Mutagenesis by Peroxy Radical Is Dominated by Transversions at Deoxyguanosine: Evidence for the Lack of Involvement of 8-oxo-dG[1] and/or Abasic Site Formation[†]

Michael R. Valentine,[‡] Henry Rodriguez,[§] and John Termini*,[‡]

Departments of Molecular Biology and Biology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010

Received December 22, 1997; Revised Manuscript Received March 17, 1998

ABSTRACT: Oxidative damage of DNA by endogenously generated oxygen radicals contributes to the mutagenic process. Hydroxy, alkoxy, and peroxy radicals all have the potential to react with DNA, giving rise to strand breaks and potentially mutagenic oxidative base damage. Although reactions of the hydroxy radical with DNA have been well studied, far less is known about the reactivities of these other radicals with DNA and their mutation-inducing potential. Frequencies of DNA base modifications and strand break densities caused by peroxy radical (ROO•) oxidation were measured by glyoxal gel electrophoretic analysis. We report the spectrum of mutations induced in *Escherichia coli* upon transfection with peroxy radical treated DNA carrying the *lacZ*α gene as a reporter. Transfection of DNA exposed to micromolar amounts of peroxy radical resulted in a 30-fold increase in mutation frequency in non-SOS-inducible cells. Sequencing analysis of DNA isolated from mutants showed that among base substitution mutants 88% consisted of transversions at G, with a nearly equal number of G → C and G → T mutants. Transition mutations were rarely detected, in contrast to control experiments. Electrophoretic analysis of peroxy radical treated DNA exposed to NaOH, Nth, and Fpg proteins demonstrated that abasic sites are not formed to any detectable degree. The oxidative G lesions are sensitive to digestion by the Fpg protein. We were unable to detect the formation of 8-oxo-dG by HPLC/electrochemical analysis of peroxy radical oxidation of dG, suggesting that the G → T transversions were not caused by this base lesion.

The role of catabolic and metabolic processes in the production of endogenous oxidants in vivo and their involvement in the degradation of cellular and nuclear membranes and proteins are now widely apppreciated. These processes are also thought to be involved in the production of DNA damage, in theory contributing to the mutagenic process independently of any environmental mutagen exposure. The production of hydrogen peroxide by various organelles and enzymes and the presence of O_2 within cells provide the substrates for the generation of free radical intermediates of oxygen. This can occur via sequential one-electron reduction by adventitious low-valent transition metal ions or "leakage" of electrons and/or reduction of radical species from biological redox pathways. Thus the $1e^-$ reduction of hydrogen peroxide yields the hydroxy radical (•OH, the Fenton reaction), while reduction of O_2 yields the superoxide ion ($O_2^{\bullet -}$). The hydroxy radical, also produced during the radiolysis of water, is a powerful mutagen owing to its high reactivity as a DNA oxidant, and its mutation-inducing potential has been well-studied (*1*, *2*). Superoxide demonstrates little or no reactivity toward the DNA bases, since it is a poor oxidant at physiological pH (*3*); however, it may be involved in strand-nicking of DNA (*4*). More importantly for its role in mutagenesis, however, the superoxide ion may undergo one-electron reduction by either transition metal ions or superoxide dismutase to produce H_2O_2.

The extreme reactivity of the hydroxy radical ($\tau_{1/2} \sim 10^{-9}$ s) requires that it be generated in close proximity to an oxidizable substrate to generate damage; i.e., its reactivity is essentially diffusion controlled. However, it is apparent that •OH may also be important in the generation of secondary radical species with the capacity for DNA damage. These secondary radicals would likely possess greater chemical stability than •OH and would therefore be expected to display greater chemical selectivity in their reaction chemistry (*5*). Thus, in contrast to relatively nonselective oxidants such as hydroxy radical which produce many products upon direct reaction with DNA (*6*), these secondary radicals may produce a set of characteristic products which defines the exposure, i.e., a chemical/mutagenic signature distinct from that produced by hydroxy or other less selective radicals. One such radical species of great importance in biology is the peroxy radical (ROO•). Peroxy radicals have unusual stability, with approximate half-lives on the order of seconds (*7*), and play a prominent role in chain initiation and propagation reactions in the autoxidation of lipids (*8*).

Peroxy radicals are formed from the addition of molecular oxygen to carbon radicals. Carbon radicals may be readily formed by at least two pathways, one involving H• abstraction by other radicals and one by the addition of radicals such as •OH to double bonds, as depicted in Scheme 1. Marnett has

[†] This work was supported by PHS Grant GM 53962. H.R. was supported by PHS Grant CA 69449 to Drs. G. P. Holmquist and S. A. Akman.

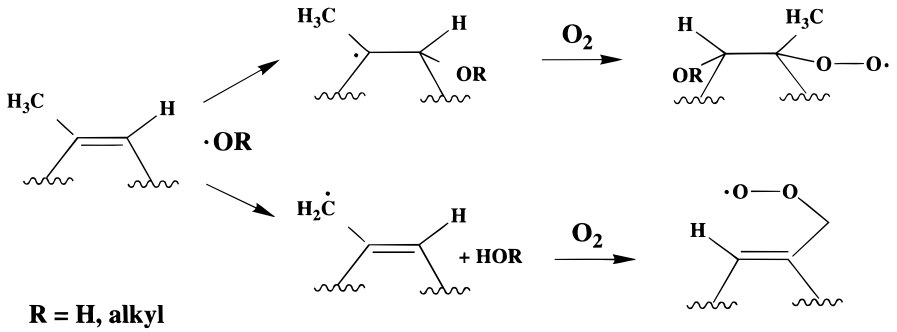
* To whom correspondence should be addressed.

[‡] Department of Molecular Biology.

[§] Department of Biology. Present address: Biotechnology Division, NIST Bldg. 222, Gaithersburg, MD 20899.

S0006-2960(97)03132-2 CCC: $15.00
Published on Web 04/22/1998

Scheme 1



pointed out that not only may peroxy radicals contribute to the induction of mutations by direct reaction with DNA, but they are also likely involved in the activation by oxidation of a variety of procarcinogens (*9*). Thus peroxy radicals may play an important role in carcinogenesis. Despite this, virtually nothing is known about DNA damage resulting from reaction with peroxy radicals and its mutagenic potential.

Some work has appeared exploring the mutagenic potential of lipid hydroperoxides. Hydroperoxides (ROOH), potential precursors of peroxy radicals, have been shown to promote the formation of 8-hydroxyguanine in calf thymus DNA, an effect potentiated by transition metal ions (*10*). Increased levels of lipid peroxidation in isolated mitochondria correlate with an increased level of 8-hydroxyguanine in mtDNA (*11*). Lipid hydroperoxides have also been shown to produce piperidine-sensitive lesions at G and T in a metal ion dependent reaction, although no oxidation products were identified (*12*, *13*). Hydroperoxides of sterols have been shown to be mutagenic in the *Salmonella* reversion assay (*14*), and lipid hydroperoxides produced by rat liver microsomes have mutation-inducing activity in *Escherichia coli* (*15*).

It is difficult to determine in these experiments the extent of involvement of peroxy radicals, since they are produced as secondary radicals in the transition metal ion catalyzed decomposition of hydroperoxides. The $1e^-$ reduction of hydroperoxides initially provides alkoxy radicals. These may abstract H• from other hydroperoxides to yield peroxy radicals, but alkoxy radicals would also be expected to function as DNA oxidants. The chemistry of alkoxy radicals with DNA remains to be explored. Moreover, breakdown products from lipid alkylperoxides such as malondialdehyde and other autoxidation byproducts can also react with DNA and have mutagenic consequences. This experimental difficulty may be circumvented through the use of a reagent which generates peroxy radicals directly in a manner which precludes or minimizes self-reaction of the radical and formation of side products. The water-soluble radical initiator ABAP[1] [2,2′-azobis(2-methylpropionamidine) dihydrochloride] produces tertiary carbon radicals upon thermolysis which in the presence of O_2 provides peroxy radicals in a kinetically reproducible fashion (*16*). This reagent has been used to model biologically relevant oxidation reactions by peroxy radical (*17*, *18*).

We have recently characterized the reaction products of peroxy radical generated by this method with deoxythymidine, and we have found that in contrast to the reaction with •OH, which produces at least 10 base damage products via initial addition to the 5,6 double bond, only three major products are produced by peroxy radical oxidation (*19*). All involve oxidation of the C-5 Me group. Interestingly, these products, formed in minor amounts in the •OH reaction, are among the most mutagenic oxidation products of thymidine (*20*−*22*). Here we report on some biochemical and biological aspects of peroxy radical oxidation of DNA. Using a *lacZ*α forward mutation assay, we show that transversions at G dominate the mutation spectrum and that peroxy radical is a moderately strong mutagen in this system. DNA transfection efficiency and modified base and strand break densities are shown to be dependent on peroxy radical concentration in a dose-dependent manner. Data on the nature of the predominant G lesion is also presented, which together suggest that 8-oxo-dG and/or abasic sites play little, if any, role in the mutational outcome.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* XL1-Blue strains (Stratagene, La Jolla, CA), *RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacZ*α [F′ *proAB lacZ*α*I*^{q}*Z*Δ*M15* Tn*10* (Tet^r)]^c), and JM 103, *endA1 supE sbcBC thi-1 strA*Δ*(lac-pro)[F′traD36 lac*^{q}Δ*M15 proAB*] (P1 lysogen), were used as hosts in the forward mutation assays. Bacteriophage M13mp18 containing *lacZ*α was used to restore β-galactosidase activity via intracistronic α complementation.

Reaction of Phage DNA with Peroxy Radical. All solutions were prepared with doubly distilled deionized water (Barnstead Nanopure system). ABAP [2,2′-azobis(2-methylpropionamidine) dihydrochloride] was obtained from ACROS Organics (Geel, Belgium). Ten micrograms of M13mp18 DNA was dissolved in 250 μL of water which had been previously saturated with oxygen by sparging with 99.99% purified O_2 for 20 min at 0 °C. This yields a solution approximately 1 mM in O_2. Aliquots of freshly made ABAP stock solutions were immediately added to give final concentrations of 10 μM to 4 mM initiator in 500 μL of total reaction volume. The reactions were allowed to proceed for 1 h at 40 °C. DNA was isolated by precipitation with 0.1 vol of 3 M sodium acetate and 2 vol of 100% ethanol.

Transfection Efficiency. Competent XL1-Blue cells were transfected according to published methods (*23*), using 1 μg

[1] Abbreviations: 8-oxo-dG, 8-hydroxy-2′-deoxyguanosine; ABAP, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; IPTG, isopropyl β-D-thiogalactopyranoside; AP, apurinic/apyrimidinic; Nth, endonuclease III; Fpg, formamidopyrimidine DNA glycosylase; ddd, doubly distilled deionized; PGK, phosphoglycerate kinase; ssb, single-strand break.

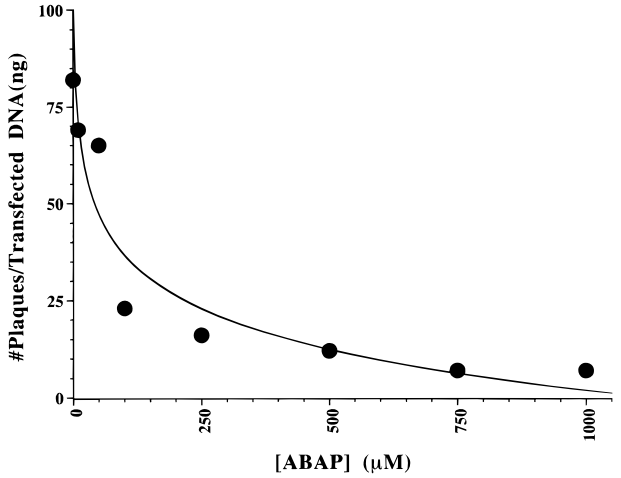


FIGURE 1: Inhibition of plaque-forming ability by peroxy radical. Phage DNA was treated for 1 h at 40 °C with varying concentrations of radical initiator under saturating O_2 to generate peroxy radicals. Units of intiator concentration are micromolar. Each point represents the average of at least two determinations.

of peroxy radical treated single-stranded M13mp18 phage DNA. XL1-Blue cells were also transfected via electroporation with a Bio-Rad Gene Pulser according to the manufacturer's directions. Peroxy radical mediated inhibition of transfection was determined by peroxy radical oxidation over a range of intiatior concentrations from 0.01 to 1.0 mM under saturating O_2 conditions. These samples were transfected by electroporation into XL1-Blue cells and plated to give 30−300 plaques/plate. The decrease in transfection efficiency as a function of concentration is shown in Figure 1. Transfection efficiency is defined as the number of plaques obtained per nanogram of transfected DNA. Approximately 4−10 μL of transfected cells was added to 3 mL of top agar (1% bactotryptone, 1% NaCl, 0.5% yeast extract, and 0.7% agar) with 40 μL of 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 4 μL of 200 mg/mL IPTG (isopropyl β-D-thiogalactopyranoside), and 200 μL of XL1-Blue cells grown in L broth (1% bactotryptone, 1% NaCl, and 0.5% yeast extract) at 37 °C for 6−8 h. This mixture was poured onto L plates (1% bactotryptone, 1% NaCl, 0.5% yeast extract, and 1.5% agar) and incubated at 37 °C for 8−12 h.

Scoring of Mutant Plaques. Plaques with inactive β-galactosidase were scored as white or light blue plaques. Mutant plaques were picked using sterile pasteur pipets, placed in 1 mL of L broth, incubated at room temperature for 1−2 h, and then stored at 4 °C. Verification of mutant plaques was obtained by replating the mutant phage together with wild-type phage.

Isolation of Mutant DNA. Single-stranded DNA from verifiable mutants was isolated using QiaPrep Spin M13 kits. Approximately 1−5 μg of DNA was obtained from each mutant for sequencing analysis.

DNA Sequencing. Dideoxy DNA sequencing using flourescent nucleotides was accomplished with an ABI Prism 377XL DNA sequencer (PE/Applied Biosystems, Foster City, CA). The following primers were designed to cover the entire coding region of the structural gene: 5′GGCGAAAGGGGGATGTG3′ and 5′AACCAATAGGAACGCCA3′. The primers were synthesized by the DNA Synthesis Core Facility of the City of Hope. These were complementary to positions +181−197 and +527−543, respectively, of the *lacZ*α structural gene.

Quantitation of Strand Break and Base Modification Density. Purified human male fibroblast DNA (2.5 μg) was reacted with 0.03−5.0 mM ABAP initiator as described above for the peroxy radical oxidation of phage DNA. After ethanol precipitation, samples were resuspended in 4.5 μL of ddd H_2O, 2 μL of 100 mM sodium phosphate, pH 7.0, 10 μL of DMSO, and 3.5 μL of 6 M glyoxal and incubated at 50 °C for 1 h. Three microliters of loading dye (50% glycerol, 10 mM sodium phosphate, and 0.25% each bromphenol blue and xylene cyanol) was added, and the samples were immediately subjected to electrophoresis in 0.6% agarose and 10 mM sodium phosphate, pH 7, at 3−4 V/cm. A mixture of *Hae*III-restricted ΦX-174 DNA and *Hin*dIII-restricted λ DNA was used as a marker lane for molecular weight calibration. Gels were stained with 1 μg/mL acridine orange for 45 min and destained overnight in water. Gels were visualized with a Vista Fluorescence/Fluorimager Si (Molecular Dynamics Inc., Sunnyvale, CA) using the Molecular Dynamics ImageQuant software. From the digitized image data, the single-stranded DNA break distribution was determined by comparison to the size−mobility calibration curve obtained using the marker lane. The DNA break density (1/Mn) was calculated as described previously (*24*). To estimate the extent of base modifications, breaks were introduced at the site of base lesions using a combination of base excision repair proteins which would excise modified bases and create breaks at abasic sites using terminal digestion conditions (see below). Integration of these data provides the sum of direct strand breaks and enzyme-induced breaks. Correction for the former provides an estimate of the base modification frequency.

Reactions of Peroxy Radical Treated DNA with Alkali and the DNA Glycosylase/AP Lyase Proteins Nth and Fpg. The 5′ regulatory region and first exon (−436 to +371) of the human phosphoglycerate kinase 1 gene (PGK1, ref *25*) was subcloned in Bluescript SK+ (Stratagene, San Diego, CA). Ten micrograms of plasmid DNA was sequentially digested with 10 units each of *Bsp*EI and *Bsm*I. The two fragments produced (3.3 kb and 12 bp) were 3′-end-labeled using the Klenow fragment of DNA polymerase (10 units) and [^{32}P]-dCTP. Following phenol/chloroform extraction, the 3.3-kb fragment was isolated on a Sephadex G-50 microspin column and ethanol precipitated. The labeled DNA was reacted with peroxy radicals and ethanol precipitated as described above using 100 μM ABAP initiator. Samples were resuspended in deionized H_2O to a final concentration of 26 ng/μL. DNA was titrated with increasing amounts of *E. coli* derived Fpg or Nth proteins (10−300 ng) in 100 μL of the respective buffers, incubated for 1 h at 37 °C, and subsequently analyzed by autoradiography to determine the minimal amount of endonuclease required for "terminal digestion" (data not shown). This is defined as the minimal amount of enzyme required to maximize band intensities at enzyme-reactive sites in DNA (*24*), and in the present case it was determined to be ∼150 ng each for Nth and Fpg proteins. Control reactions were incubated with buffer alone. Reactions were quenched by the addition of 50 μL of 10 M NH_4-OAc, 1 μL of 20 mg/mL glycogen, and 2.5 vol of cold ethanol. Precipitated, dried DNA was resuspended in 7 μL of formamide loading buffer containing 0.05% each of xylene

cyanol and bromophenol blue and was subjected to electrophoresis on a 60-cm 6% polyacrylamide/7 M urea gel. Alkaline hydrolysis reactions were carried out by the addition of 100 μL of 0.4 M NaOH to 100-μL solutions of 1.3 μg of peroxy radical treated DNA and incubation at 40 °C for 1 h. Reactions were quenched as described above for the enzyme digestions.

Nucleoside Oxidation Reactions and Chromatographic Analysis. Nucleosides dT, dC, dA (Aldrich Chemical Co., Milwaukee WI), and dG (Chem-Impex, Intl., Wood Dale, IL) were each dissolved in 50 mL of 0.1 M KH_2PO_4 buffer (pH 5.5) to create a solution that was 250 μM in deoxynucleoside. All phosphate buffer stock solutions were filtered through 500 g of Chelex 100 Na to remove metal ions. Chromatogaphic standards of 8-hydroxy-2′-deoxyguanosine (Sigma, St. Louis, MO) were prepared in this buffer. Each solution was then saturated with O_2 at room temperature by sparging with 99.99% oxygen for 20 min. ABAP was added to give a 10-fold molar excess of initiator, and the reaction mixtures were sealed and placed in a shaking incubator at 40 °C. Aliquots (500 μL) were withdrawn at 24 h and analyzed by HPLC using a Supelco LS−C18 reverse-phase analytical column. Detection was either by optical absorbance (Kratos SF773 diode array) or by electrochemical methods (Coulochem 2, Chelmsford, MA). The column was initially equilibrated for 5 min at a flow rate of 1.5 mL/min with 90% A:10% B: 0% C followed by a ternary gradient which reached 40% A:10% B:50% C over a period of 15 min. Solvents/buffers: A, water; B, 0.2 M KH_2PO_4, pH = 4.5; C, MeOH.

RESULTS

Peroxy Radical Induced Inhibition of Bacteriophage M13mp18 Transfection. The ability of peroxy radical to induce DNA damage was investigated by studying phage reproduction following exposure to peroxy radical. Single-stranded phage DNA was incubated with various concentrations of peroxy radical prior to transfection. The ability to replicate was then determined by monitoring the plaque forming efficiency, expressed as number of plaques/nanogram of transfected DNA. The viability of the single-stranded phage DNA would be reduced as a consequence of peroxy radical induced strand breaks. In this regard, the viability curve can be considered to be primarily an indicator of the extent of breaks introduced into single-stranded DNA as a function of peroxy radical concentration. The contribution of base modifications which would block replication of transfected phage DNA is considered to be small. These results are summarized in Figure 1. In the absence of peroxy radical exposure, transfection yielded ~80 plaques/ng, defined as 100% survival. Relatively small amounts of radical initiator in the presence of oxygen had a significant impact on transfection efficiency. Concentrations in the 40−50 μM range reduce phage survival by ~50%. Since ABAP thermolyzes slowly at 37 °C (τ1/2 = 175 h), the estimated amount of peroxy radical formed during the 1-h incubation period is ~100-fold less (see Discussion). At 100 μM initiator, ~30% of oxidized phage DNA yields transfectants. This dosage was used in the forward mutation assay.

Relative Distribution of Peroxy Radical Induced Base Modifications and Strand Breaks. A determination of the base modification frequency following oxidation with peroxy radical requires the use of double-stranded DNA. This is because the assay employs DNA glycosylases/AP lyases to create strand breaks at modified bases, and these enzymes require double-stranded substrates for activity. The data in Figure 2, derived from integration of glyoxal gel electrophoretic analyses of peroxy radical treated human fibroblast DNA, indicate that the number of strand breaks induced by peroxy radical lag behind the yield of base modifications at every concentration examined. The extent of base modification increases more rapidly than strand breaks with increasing concentration, an effect most pronounced at [ABAP] ≤ 1 mM. The increase in yield of both base modifications and strand breaks is logarithmic. At 100 μM the ratio of base modifications to strand breaks reaches ~4.4. (1.3 base modifications vs 0.33 dsb per kilobase).

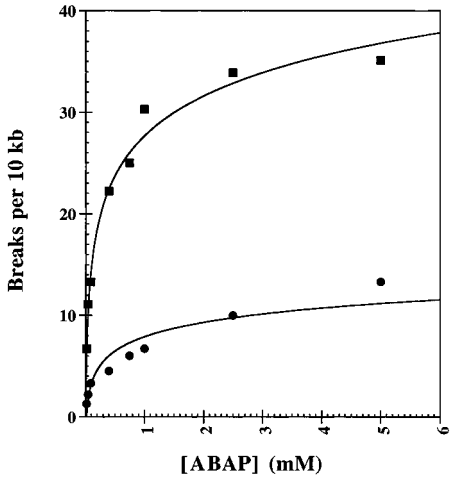


FIGURE 2: Frequency of strand breaks (●) and base modifications (■) as a function of increasing concentration of peroxy radical. Purified male fibroblast DNA was oxidized by peroxy radicals at different initiator concentrations for 1 h at 40 °C. DNA was then analyzed for single-strand break distribution (M_n) by electrophoresis on neutral glyoxal gels. Fibroblast DNA subjected to the same isolation procedure without exposure to peroxy radical was used to determine the endogenous break density and to correct all subsequent measurements (~0.2 break/10 kb). Break densities were calculated as previously described (*24*). To analyze the base modification frequency, oxidized DNA was reacted with a combination of Fpg and Nth proteins using saturating conditions to induce breaks at modified purines and pyrimidines. Correction for breaks resulting from direct attack on the carbohydrate backbone yielded the values for base modifications recognizable by Fpg and Nth.

Mutation Frequency. The spontaneous (control) mutation frequency, determined by counting light blue/white plaques produced upon transfection of untreated phage DNA carrying the *lacZ*α gene into XL1-Blue hosts, was found to be 3×10^{-4} /transfectant (Figure 3). This is the mutation frequency observed in the absence of activation of the "error prone repair" pathway, the so-called SOS response. The XL1-Blue cells are $RecA^-$, and are thus by definition non-SOS-inducible. Transfection of phage DNA which had been exposed to peroxy radical (100 μM radical initiator and 1 mM O_2 at 40 °C) resulted in a greater than 30-fold increase in mutation frequency (1×10^{-2}/transfectant) when expressed in XL1-Blue cells. Transfection of identically treated DNA into a related yet $RecA^+$ strain (JM103) yielded a lower level of induced mutations as compared to the XL1-Blue cells. The backround or spontaneous mutation frequency

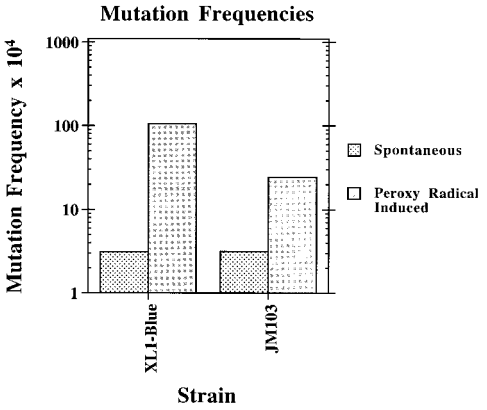


FIGURE 3: Spontaneous and induced mutation frequencies for *E. coli* strains XL1-Blue (RecA$^-$) and JM 103 (RecA$^+$) transfected with M13mp19 containing the *lacZ*α gene. Spontaneous frequencies were measured following transfection with untreated plasmid DNA, while induced mutants resulted from transfection with peroxy radical oxidized plasmids.

Table 1: Peroxy Radical Induced and Spontaneous Mutations

	transversions	transitions	frameshifts	duplications
induced	G → T 20	C → T 2	−G 3	0
	G → C 28		−C 1	
	C → G 1			
spontaneous	G → T 3	C → T 9	−G 2	1
	G → C 2	G → A 1	+C 2	
	A → T 1	A → G 1		

observed in RecA$^+$ JM 103 cells was essentially the same as that observed for the RecA$^-$ XL1-Blue strain (3.16×10^{-4}/transfectant).

The use of either single- or double-stranded DNA as a substrate for peroxy radical oxidation did not affect the frequency of mutations observed following transfection. Peroxy radical treatment of double-stranded M13mp18 (rf) followed by transfection into XL1-Blue cells gave rise to a mutation frequency which was within 10% of that found for the single-stranded vector. The use of single-stranded DNA to "fix" the mutagenic lesions did not appear to bias the mutation frequency.

Peroxy Radical Mutation Spectrum. A summary of the spontaneous and peroxy radical induced mutations in the *lacZ*α gene is provided in Table 1. The complete map of induced and spontaneous mutations is shown in Figure 4. The entire *lacZ*α sequence, including the promoter region, was examined for mutations recovered in the XL1-Blue strain. The most striking result from the induced mutant sequencing data was that ∼87% of peroxy radical induced mutants (48/55) consisted of transversions at G. The extent of G → C and G → T transversions was roughly the same, with a slight preponderance of the former. In contrast, spontaneous transversions at G occurred with a frequency of ∼25%. Among spontaneous mutants, transitions at C predominated, accounting for nearly half the mutants. Conversely, these appeared rarely in the induced mutations, where they occurred with a frequency of <5%.

There also appear to be a number of "hotspots" at which transversions tend to cluster. Most of these appear in the coding region of the gene. Prominent examples of this can be seen in Figure 4 at positions 199, 202, and 324. In some cases, the peroxy radical induced hotspots coincide in position with the spontaneous mutants, although at an elevated frequency. Thus, for example, at position 324 a relatively rare spontaneous G → C transversion is observed, while seven transversions of this type are induced by peroxy radical at this position.

The only types of frameshift mutants which appear to be induced by peroxy radical are deletions. No insertions are observed in the induced spectra, although two appear among the spontaneous mutants; in both cases at short runs of C (−42, 87). A total of four deletions are observed in the induced mutants. At position 73, two spontaneous and two induced deletions at G are observed. An unusual C → G transversion appears among the induced mutants at position −56 in the promoter region. It is unclear whether it is peroxy radical induced or a rare spontaneous change.

Lability to Alkali and Enzymatic Recognition of the Peroxy Radical G Lesion in DNA. The nature of the predominant lesion at G sites produced in DNA by peroxy radical was probed using alkaline hydrolysis and enzymatic digestion. The production of chain breaks by alkali at G sites would be consistent with the presence of abasic sites (*26*). Treatment with alkali should result in strand breaks at abasic sites through an anti-β-elimination reaction involving the C-2′ hydrogen (*27*). In addition to the hemiacetal abasic sites formed by hydrolytic depurination/depyrimidination, the C-1′, C-2′, and C-4′ oxidized AP (apurinic/apyrimidinic) sites should also react with alkali to generate strand breaks owing to the presence of acidic α-carbonyl hydrogens. Chain breaks may also be induced via alkali-labile base modifications which give rise to AP sites indirectly. The Nth and Fpg proteins were also used to probe the nature of the peroxy radical lesions. These proteins possess both DNA glycosylase and AP lyase activities. The Fpg protein removes the ring-opened imidazole derivatives (resulting from rupture of the C-8/N-9 bond) of guanine bases (*28*). It also appears to be one of the major repair enzymes involved in the removal of 8-oxo-2′-deoxyguanosine (*29*). Nth protein shares the AP endonuclease activities of the Fpg protein in addition to excising a wide variety of saturated, fragmented, and ring-contracted bases derived from pyrimidines (*30*, *31*). Pyrimidine oxidation products which do not result in saturation of the 5,6 double bond, e.g., 5-(hydroxymethyl)-2′-deoxyuridine, are not substrates for this enzyme (*32*, *33*). The AP lyase activities of the Fpg and Nth proteins create strand breaks at abasic sites produced by oxidation or hydrolytic depurination/depyrimidination (*34*).

The autoradiogram of Figure 5 reveals the strand break susceptibility of peroxy radical treated DNA following exposure to either alkali, Fpg, or Nth proteins. The DNA substrate is a linearized ^{32}P-end-labeled double-stranded plasmid containing the human PGK1 gene (*25*). Oxidized DNA was prepared by exposing plasmids to peroxy radicals using the same conditions described for the mutagenesis assay immediately prior to digestion with alkali or Nth/Fpg proteins. Lanes 1−5 show the backround breaks obtained for unoxidized plasmid DNA (control) using various digestion conditions. Lanes 6−8 duplicate these conditions with DNA that has been exposed to peroxy radical. Control DNA treated with alkali (lane 5) does not seem to differ significantly from untreated control (lanes 1 and 2). Peroxy radical oxidation of DNA does not result in an increase in the number of alkali-labile sites relative to unoxidized control

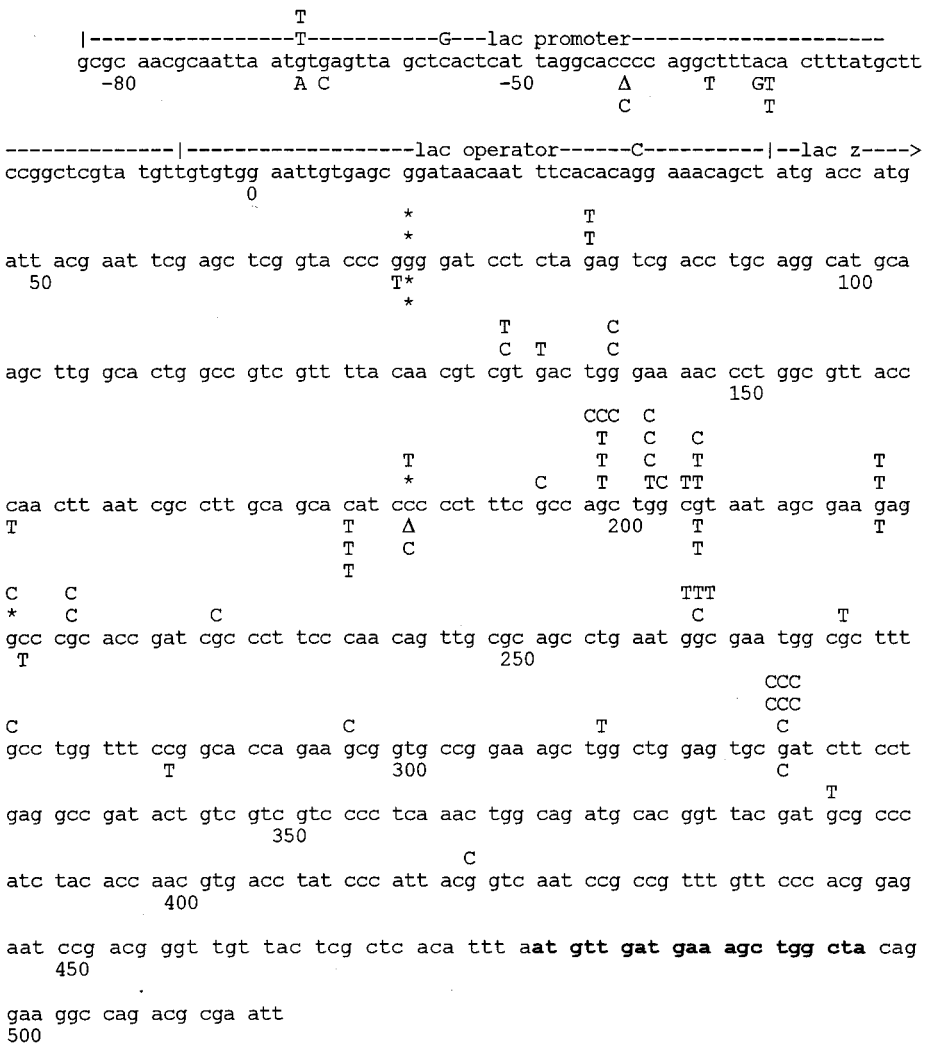


FIGURE 4: Map of peroxy radical induced and spontaneous mutants for the entire M13mp19 *lacZ*α gene. Induced changes are indicated above the coding sequence, while spontaneous changes are noted below. Frameshift mutants are denoted as △ for insertions, while * indicates deletions. The sequence outlined in bold corresponding to positions 478−497 represents a spontaneous duplication. Base substitution mutations are tabulated in Table 1.

(compare lanes 5 and 8). Similarly, treatment of peroxy radial treated DNA with Nth protein does not seem to produce any sensitive lesions significantly above backround (lane 6 vs 3). In contrast to this result, lesions produced by peroxy radical appear to be sensitive to the Fpg protein (lane 7 vs 4), producing breaks at predominantly G sites. It appears unlikely that these sites are abasic in nature since they are neither substrates for the AP endonuclease activity of the Nth protein at high concentrations nor susceptible to cleavage by alkali. The lack of Nth-sensitive sites is consistent with prior studies of peroxy radical oxidation of thymidine which demonstrated that saturated pyrimidines were not formed to any detectable extent (*19*).

Peroxy Radical Oxidation of Deoxyguanosine. The oxidation of dG by peroxy radical was examined as a model reaction to provide information on the transversion inducing G lesions. The HPLC trace of a typical reaction of dG with peroxy radical is shown in Figure 6A. The diode array isogram data obtained from this chromatogram is shown in the top panel. In addition to recovered starting material, eluting at 4.9 min, two new products are formed, eluting at 5.8 and 7.8 min, respectively. The early eluting components at ≤1.5 min are low wavelength absorbing components that are produced as side products from the non-oxygen-dependent decomposition of ABAP (*19*), and do not represent dG oxidation products. In contrast to the reaction of dT with peroxy radical under identical conditions, more than half of the starting material is consumed in the reaction. The isogram reveals a long-wavelength absorbance at 278 nm for the major product, while the latest eluting component absorbs maximally at 296 nm. No free guanine is detected in the chromatogram. The presence of 8-oxo-dG appeared possible since its λ_{max} at pH 4.5 is close to that of the latest eluting component. However; authentic 8-oxo-dG possesses a retention time of 5.32 min under these chromatographic conditions (Figure 6B). The possibility of formation of very minor amounts of this oxidation product, undetectable by optical absorbance, was investigated by the use of electrochemical detection. Analysis of the reaction failed to detect the presence of 8-oxo-dG, within a sensitivity range of ≥1 ng/mL (data not shown). The structural elucidation of the two major oxidation products is in progress.

DISCUSSION

The majority of research on oxidative DNA damage has focused on the reaction products and mutagenicity of the

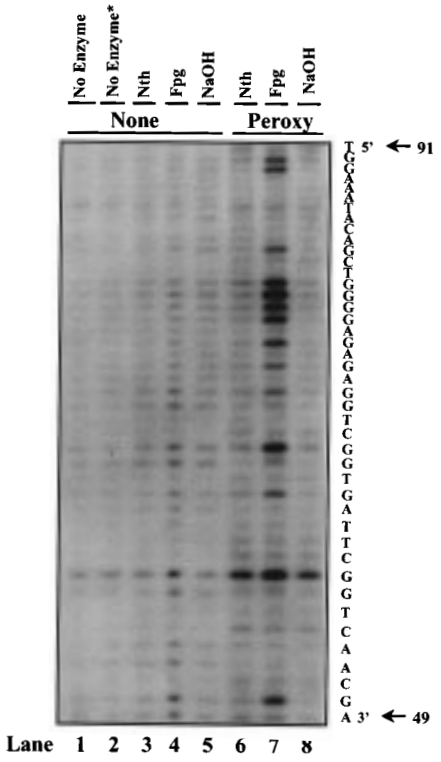


FIGURE 5: Electrophoretic analysis of the reactions of peroxy radical treated DNA with alkali and the DNA glycosylase/AP lyase proteins Fpg and Nth. ^{32}P-end-labeled, linearized PGK1 plasmid DNA was treated with peroxy radical and then reacted with either 750 ng of Nth protein (lane 6) or 500 ng of fpg protein (lane 7). Control lanes 3 and 4 reveal the backround breaks elicited by Nth and Fpg proteins, respectively, on unoxidized DNA. Reactions with NaOH were designed to test for the presence of alkali-labile sites.

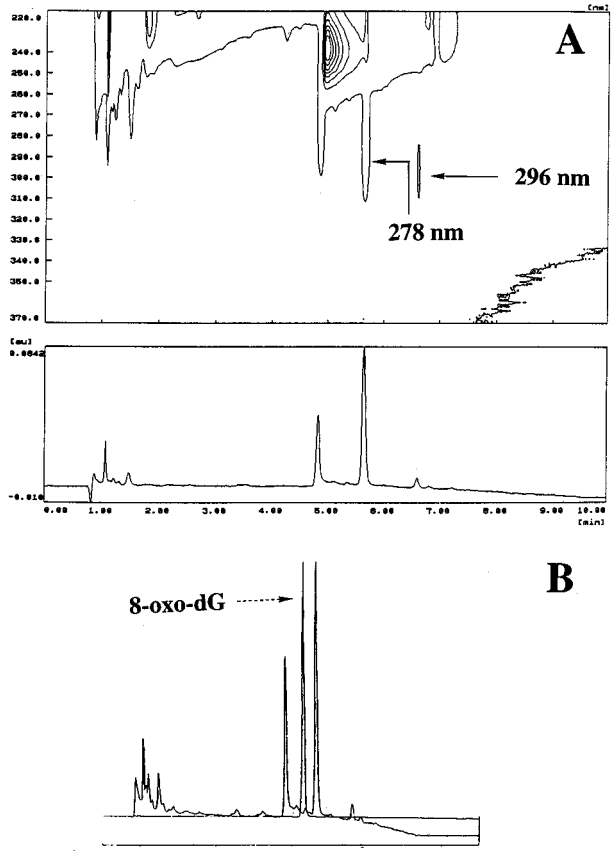


FIGURE 6: HPLC chromatographic analysis of the reaction of dG with peroxy radicals. (A) Top figure shows the isogram obtained from the HPLC trace shown directly below. (B) Superposition of the reaction chromatogram with that of an 8-oxo-2′-deoxyguanosine standard.

hydroxy radical, formed either from the radiolysis of water or the 1e$^-$ reduction of endogenous hydrogen peroxide. The study of other biologically relevant radical species which participate in DNA damage and mutagenesis was inspired in part by evidence which presented a role for pyrimidine base peroxy radicals in the formation of strand breaks upon aerobic pulse radiolysis (*35*−*37*). The notion that a highly reactive radical species such as •OH may react with DNA in the presence of oxygen to generate more stable radical intermediates with their own characteristic pattern of reactivity suggests that "secondary" radicals such as peroxy or alkoxy may play important roles in production of endogenous DNA damage.

Although these studies were informative about the involvement of peroxy radicals in strand breaks, it was apparent that little information was available concerning peroxy radical oxidation reactions of the nucleic acid bases and the resulting potential for mutagenesis. The data of Figure 2 demonstrate that base oxidation by peroxy radical occurs more readily than strand breaks in DNA. This would suggest that peroxy radicals have a greater potential for mutagenesis via mechanisms involving translesional synthesis than through those involving DNA breakage. It can be seen from the data in Figure 2 that the yield of modified bases increases more rapidly than strand breaks at low initiator concentrations. Whether there is a prior dependence on the formation of modified bases for the generation of strand breaks, as is the case for pyrimidine base peroxy radicals generated by •OH addition, is unclear from the present work. Optical pulse radiolysis in conjunction with time-resolved light scattering methods might provide insight into this question (*36*). One possibility is that only a small subset of base damage products or radical precursors are competent at effecting strand breaks; thus a number of base modifications would accumulate prior to the appearance of strand breaks.

The fact that base modification occurs at least 4 times more readily than strand breaks at low initiator concentrations reflects the greater oxidizability of the bases relative to the ribophosphate moiety. The estimates of base modification presented in Figure 2 are on the conservative side since it is likely that not all base lesions were excised by the combination of DNA glycosylases/AP lyases used in this study. This is not the case with ssb's, since they are detected directly in the glyoxal gel analysis. The ratio of base modifications to ssb's is higher than that reported for the Cu^{2+}/H_2O_2/ascorbate system, which utilized an identical procedure to calibrate breaks and modifications (*24*). The greater degree of strand breaks in this case may reflect the greater reactivity of the radical intermediate(s) produced in the Cu^{2+}/H_2O_2/ascorbate reaction. Whether the principal oxidant in this case is •OH remains to be demonstrated, since it has been shown that the extent of reaction of hydroxy radicals with ribose is too slow to account for the observed yield of strand breaks (*35*). The preferential reaction of peroxy radical with the bases, and the overwhelming preference for reaction at guanine, likely reflects the greater chemical selectivity of this species relative to that produced by the Fenton reaction with low-valent transition metal ions.

In these experiments, peroxy radicals were generated via thermolysis of the water-soluble diazo initiator ABAP in the presence of saturating concentrations of O_2. The half-life of this initiator at 37 °C is ~175 h; thus, the rate of formation of carbon-centered radicals over the first few hours is linear. Since the reaction of these radicals with oxygen is diffusion controlled (*38*), the rate of peroxy radical formation must also be linear. The slow formation of peroxy radicals limits the probability for radical quenching by self-condensation and maximizes opportunities for substrate reaction. Radical yields at 37 °C may be estimated over the linear range of initiator decomposition according to the equation of Niki, where t is in seconds (*16*):

$$[ROO^{\bullet}] = 1.36 \times 10^{-6}[ABAP] \times t$$

Applying this approximation to the ABAP concentrations of Figures 1 and 2 reveals levels of $ROO^{\bullet}$ at least 2 orders of magnitude lower than that of the initiator, implying that even submicromolar concentrations of radical may exert significant toxicity and mutagenicity.

Since the base moieties were the principal target of peroxy radical oxidation of DNA (Figure 2), replication of oxidized template DNA was expected to give rise to mutations via translesional DNA synthesis. Transfected single- or double-stranded plasmid DNA exposed to 500 nM peroxy radicals resulted in a >30-fold increase in the mutation frequency over untreated transfectants. The striking result here is that nearly all the base-substitution mutations are G transversions, with a virtually equivalent number of G → C and G → T substitutions. In contrast, base mutations arising from transfection of unoxidized DNA were primarily transitions at cytosine (Table 1). Transversions at G have been previously observed in forward mutation assays in *E. coli*, where the oxidizing radicals were generated by Fenton or Udenfriend reaction of DNA. G → C transversions was observed by Loeb and co-workers in the mutagenic spectrum of Udenfriend-oxidized (Fe^{2+}, O_2) DNA (*1*). Forty percent of the base substitutions observed in the *lacZ*α forward mutation assay were G → C transversions, while the next most abundant were C → T transitions (23%). This transition was detected rarely in our studies with peroxy radical induced mutants (<4%), yet it appears frequently among the spontaneous mutants (Table 1). The ~ 30-fold enhancement in mutation frequency obtained by McBride et al. (*1*) is similar to the level of mutation induction that we observe. However, the mutations we observe arise from translesional synthesis in the absence of RecA and the associated SOS response. The DNA lesions produced by low-valent transition metal ion oxidations require error-prone DNA repair synthesis (SOS response) to give levels of mutability comparable to those obtained by peroxy radicals. Thus, there appear to be some fundamental differences in the kinds of base damage produced by peroxy radical. Some of these are already apparent. For example, 8-hydroxyguanine is produced by the Fenton and Udenfriend reactions, where it may account in part for the G → T transversions observed, but this base lesion is not produced by peroxy radical oxidation. It will be of interest to determine whether the lesion at guanosine which gives rise to G → C transversions following treatment with peroxy radical is identical to that produced in Udenfriend/Fenton-oxidized DNA. This oxidation product must allow base pairing with dG to produce this mutant. This transversion has also been observed following treatment with alkylating agents such as 4-nitroquinoline 1-oxide (*39*). In this case, C-8 adduction at guanine forces the base into the syn conformer which can formally base pair with an opposed dG in the anti conformation. This lesion is the only known example of a G → C transversion causing a base damage product whose identity is known. Perhaps the dG oxidation product possesses a similar conformational geometry and hydrogen bonding arrangement.

The finding of an elevated level of mutations in a $RecA^-$ strain transfected with peroxy radical oxidized DNA prompted us to examine the mutation frequency in a related yet $RecA^+$ strain (JM101). Here the frequency of mutations was 10 times higher than the backround mutation level (which was identical in $RecA^+$ and $RecA^-$ strains), yet it was still about 3-fold less than that observed in the $RecA^-$ strain. It would appear that the presence of a functional RecA gene product can limit the mutagenic potential of peroxy radical induced DNA damage, although how this might occur is presently unclear.

G → T transversions are known to arise via replication of template DNA containing either 8-oxo-dG or abasic sites (*40*−*43*). Strand breaks at G sites of oxidized DNA following treatment with the Fpg protein (Figure 5) initially suggested the presence of abasic sites, imidazole ring-opened purine derivatives (fapy derivatives), or 8-oxo-dG. Resistance to cleavage by alkali, as well as resistance to digestion by a second AP lyase, the Nth protein, argued against the presence of abasic sites. Consistent with this, free guanine was not detected in model oxidations of dG. Sensitive electrochemical detection methods failed to confirm the presence of 8-oxo-dG. It is unlikely that fapy guanine is produced in the reaction to any extent, since this lesion is apparently lethal in bacteria in the absence of repair and does not appear to give rise to a characteristic mutant spectrum (*44*). The Fpg-sensitive G lesion is possibly a modified fapy derivative, or an oxidized purine hitherto unrecognized as a substrate for Fpg glycosylase/AP lyase.

While this work was nearing completion, a report appeared describing the mutations induced in *E. coli* by peroxy radicals using a similar assay (*45*). While those authors also identified G transversions as the predominant mutation, very few G → C transversions were found, in contrast to this report. Induction of the SOS response in $RecA^+$ strains was also required to detect induced mutants, yet this frequency was less than 10-fold over the spontaneous backround. Moreover, the predominant G → T transversions were proposed by those authors to arise from abasic sites. We present evidence that conflicts with this view, although the differences may reflect nonidentical genetic backrounds and/or differences in methodology. Those authors do not appear to have controlled the partial pressure of oxygen in the DNA reactions, which are described as being "incubated in open reaction vessels". We have observed variations in the product distributions of the peroxy radical reaction products of dG as a function of O_2 partial pressure; thus, it is possible that the conditions used by Harkin et al. (*45*) failed to generate the same distribution of lesions that gave rise to our observed mutant pattern. The different genetic background of the strains used in these two studies may also account for some differences.

We previously demonstrated that peroxy radical reaction with thymidine gives rise to 5-Me oxidation products (*19*). Failure to observe mutations at dT in our assay may have been due to the lack of mutagenicity of some C-5 Me oxidized thymidine lesions in bacteria (*46*) or the efficient repair of others. We have recently completed in vitro mapping studies of peroxy radical DNA damage on genomic DNA for selected exons in the PGK1 and P53 genes using the ligation-mediated PCR technique (Rodriguez et al., unpublished experiments). Eighty-seven percent of the thousands of base damaged sites detected were at G. Guanine possesses the lowest ionization potential among the standard Watson−Crick bases and is often observed to be the most damaged base when DNA is exposed to oxidizing conditions (*47*). Thus, it is not suprising that G is the preferred target for a highly selective radical oxidant like ROO•. With respect to its mutagenic profile, the peroxy radical appears similar to singlet oxygen, which also is a strong inducer for transversions at guanine (*48*, *49*). Interestingly, singlet oxygen can be produced via the decomposition of peroxy radical dimers (*50*), and it is possible that it is produced in our reaction. However, the major oxidative product of the singlet oxygen reaction with DNA is 8-hydroxy-2′-deoxyguanosine, which is not detected in the peroxy radical reaction. G → C transversions produced by singlet oxygen have been detected in a mutation assay using mamallian cells (*49*). Whether this lesion shares identity with those responsible for G → C transversions in the Fenton/Udenfriend or peroxy radical oxidations will be the subject of future investigation.

ACKNOWLEDGMENT

We wish to thank Dr. Timothy O'Connor for his generous gifts of Fpg DNA glycosylase and Nth protein and Dr. Lawrence C. Sowers for assistance with the chromatographic analyses. We also thank Dr. Adam Bailis for helpful discussions.

REFERENCES

1. McBride, T. J., Preston, B. D., and Loeb, L. A. (1991) *Biochemistry 30*, 207−213.
2. Moraes, E. C., Keyse, S. M., Pidoux, M., and Tyrell, R. M. (1989) *Nucleic Acids Res. 17*, 8301−8311.
3. Bielski, B. H. J. (1978) *Photochem. Photobiol. 28*, 645−649.
4. Dix, T. A., Hess, K. M., Medina, M. A., Sullivan, R. W., Tilly, S. L., and Webb, T. L. (1996) *Biochemistry 35*, 4578−4583.
5. Buettner, G. R. (1993) *Arch. Biochem. Biophys. 300*, 535−543.
6. Tèoule, R. (1987) *Int. J. Radiat. Biol. 51*, 573−579.
7. Pryor, W. A. (1986) *Annu. Rev. Physiol. 48*, 657−667.
8. Porter, N. A., Caldwell, S. E., and Mills, K. A. (1995) *Lipids 30*, 277−290.
9. Marnett, L. J. (1987) *Carcinogenesis 8*, 1365−1373.
10. Park, J.-W., and Floyd, R. A. (1992) *Free Radical Biol. Med. 12*, 245−250.
11. Hruszkewycz, A. M., and Bergtold, D. S. (1990) *Mutat. Res. 244*, 123−128.
12. Inouye, S. (1984) *FEBS Lett. 172*, 231−234.
13. Ueda, K., Kobayashi, S., Morita, J., and Komano, T. (1985) *Biochim. Biophys. Acta 824*, 341−348.
14. Smith, L. L., Smart, V. B., and Made Gowda, N. (1986) *Mutat. Res. 161*, 39−48.
15. Akasaka, S., and Yonei, S. (1985) *Mutat. Res. 149*, 321−326.
16. Niki, E. (1990) *Methods Enzymol. 186*, 100−108.
17. Pryor, W. A., Strickland, T., and Church, D. F. (1988) *J. Am. Chem. Soc. 110*, 2224−2229.
18. Ham, A.-J. L., and Liebler, D. C. (1995) *Biochemistry 34*, 5754−5761.
19. Martini, M., and Termini, J. (1997) *Chem. Res. Toxicol. 10*, 234−241.
20. Kasai, H., Iida, A., Yamaizumi, Z., Nishimura, S., and Tanooka, H. (1990) *Mutat. Res. 243*, 249−253.
21. Boorstein, R. J., and Teebor, G. W. (1988) *Cancer Res. 48*, 5466−5470.
22. Wang, S. Y., Hahn, B. S., Batzinger, R. P., and Bueding, E. (1979) *Biochem. Biophys. Res. Commun. 89*, 259−263.
23. Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet. 16*, 339−346.
24. Rodriguez, H., Drouin, R., Holmquist, G. P., O'Connor, T. R., Boiteux, S., Laval, J., Doroshow, J. H., and Akman, S. A. (1995) *J. Biol. Chem. 270*, 17633−17640.
25. Singer-Sam, J., Keith, D. H., Tani, K., Simmer, R. L., Shively, L., Lindsay, S., Yoshida, A., and Riggs, A. D. (1984) *Gene 32*, 409−417.
26. Jones, A. S., Mian, A. M., and Walker, R. T. (1968) *J. Chem. Soc. C*, 2042−2044.
27. Mazumder, A., Gerlt, J. A., Absalon, M. J., Stubbe, J., Cunningham, R. P., Withka, J., and Bolton, P. H. (1991) *Biochemistry 30*, 1119−1126.
28. Boiteux, S., O'Connor, T. R., Lederer, F., Gouyette, A., and Laval, J. (1990) *J. Biol. Chem. 265*, 3916−3922.
29. Tchou, J. Kasai, H., Shibutani, S., Chung, M. H., Laval, J., Grollman, A. P., and Nishimura, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4690−4694.
30. Gates, F. T., and Linn, S. (1977) *J. Biol. Chem. 252*, 2802−2807.
31. Breimer, L. H., and Lindahl, T. (1984) *J. Biol. Chem. 259*, 5543−5548.
32. Breimer, L. H., and Lindahl, T. (1985) *Mutat. Res. 150*, 85−89.
33. Dizdaroglu, M., Laval, J., and Boiteux, S. (1993) *Biochemistry 32*, 12105−12111.
34. Haring, M., Rüdinger, H., Demple, B., Boiteux, S., and Epe, B. (1994) *Nucleic Acids Res. 22*, 2010−2015.
35. Deeble, D. J., Schulz, D., and Von Sonntag, C. (1986) *Int. J. Radiat. Biol. 49*, 915−926.
36. Jones, G. D. D., and O'Neill (1990) *Int. J. Radiat. Biol. 57*, 1123−1139.
37. Barvian, M. R., and Greenberg, M. M. (1995) *J. Am. Chem. Soc. 117*, 8291−8292.
38. Michaels, H. B., and Hunt, J. W. (1977) *Radiat. Res. 72*, 18−31.
39. Fronza, G., Campomenosi, P., Iannone, R., and Abbondandolo, A. (1992) *Nucleic Acids Res. 20*, 1283−1287.
40. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. (1992) *J. Biol. Chem. 267*, 166−172.
41. Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) *Nature 349*, 431−434.
42. Kunkel, T. A., Schaaper, R. M., and Loeb, L. A. (1983) *Biochemistry 22*, 2378−2384.
43. Sagher, D., and Strauss, B. (1983) *Biochemistry 22*, 4518−4526.
44. Tudek, B., Boiteux, S., and Laval, J. (1992) *Nucleic Acids Res. 20*, 3079−3084.
45. Harkin, L. A., Butler, L. M., and Burcham, P. C. (1997) *Chem. Res. Toxicol. 10*, 575−581.
46. Levy, D. D., and Teebor, G. W. (1991) *Nucleic Acids Res. 19*, 3337−3343.
47. Jovanovic, S. V., and Simic, M. G. (1986) *J. Phys. Chem. 90*, 974−978.
48. Decuyper-Debergh, D., Piette, J., and Van de Vorst, A. (1987) *EMBO J. 6*, 3155−3161.
49. Costa de Oliveira, R., Ribeiro, D. T., Nigro, R. G., Di Mascio, P., and Menck, C. F. M. (1992) *Nucleic Acids Res. 20*, 4319−4323.
50. Nakano, M., Takayama, K., Shimizu, Y., Tsuji, Y., Inaba, H., and Migita, T. (1976) *J. Am. Chem. Soc. 98*, 1974−1975.

BI973132M