Oxidation of DNA bases, deoxyribonucleosides and homopolymers by peroxy radicals

Tiberiu SIMANDAN, Jing SUN and Thomas A. DIX¹

Department of Pharmaceutical Sciences, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425-2303, U.S.A.

DNA base oxidation is considered to be a key event associated with disease initiation and progression in humans. Peroxy radicals (ROO[•]) are important oxidants found in cells whose ability to react with the DNA bases has not been characterized extensively. In this paper, the products resulting from ROO[•] oxidation of the DNA bases are determined by gas chromatography/MS in comparison with authentic standards. ROO[•] radicals oxidize adenine and guanine to their 8-hydroxy derivatives, which are considered biomarkers of hydroxyl radical (HO[•]) oxidations in cells. ROO[•] radicals also oxidize adenine to its hydroxylamine, a previously unidentified product. ROO[•] radicals oxidize cytosine and thymine to the monohydroxy and

dihydroxy derivatives that are formed by oxidative damage in cells. Identical ROO[•] oxidation profiles are observed for each base when exposed as deoxyribonucleosides, monohomopolymers and base-paired dihomopolymers. These results have significance for the development, utilization and interpretation of DNA base-derived biomarkers of oxidative damage associated with disease initiation and propagation, and support the idea that the mutagenic potential of N-oxidized bases, when generated in cellular DNA, will require careful evaluation. Adenine hydroxylamine is proposed as a specific molecular probe for the activity of ROO[•] in cellular systems.

INTRODUCTION

The generation of oxidizing species from the metabolic or adventitious reduction of O₂ is associated with the aetiology of a multitude of human disease states [1–4]. Important O₂-derived oxidants formed in cells include superoxide (O₂^{•−}) and its conjugate acid, perhydroxyl (hydrodioxyl) radical (HOO[•]), hydroxyl (HO[•]) and peroxy (ROO[•]) radicals, as well as H₂O₂, all of which can react with transition metals to interconvert and generate other metal-associated oxidizing species [5–7]. Of these species, HO[•] has received the most attention owing to its established high reactivity with most organic compounds and its probable intermediacy in various metal-dependent oxidant-generating systems. In contrast, the reactivity of ROO[•] radicals is less understood, which is unfortunate because ROO[•] radicals are present in normal cells at high steady-state concentrations. Notably, ROO[•] radicals are formed in a variety of physiological and pathological processes (such as lipid and fatty acid autoxidation/peroxidation [8,9]) and are the oxidants produced after abstractions of hydrogen atoms by HO[•] followed by the addition of O₂ to the resulting carbon-centred radicals. The long biological half-lives of ROO[•] radicals (in comparison with the other O₂-derived oxidants) also favour diffusion from their site of generation and subsequent reaction in other cellular compartments. Characterizing the relative reactivities of each of the O₂-derived oxidants is of inherent chemical interest; it is also a necessary prerequisite for developing biomarkers to use to monitor the oxidants' reactivities in cells. This is a key step in the effort to link oxidant formation to biomolecular damage and disease initiation and progression in a causative fashion.

Initial efforts in our laboratory along these lines involved determining the reactivity of each of the individual O₂-derived oxidants with lipids and lipid hydroperoxides [9–13]. Both ROO[•] and O₂^{•−}/HOO[•] exhibited distinctly different patterns of re-

activity with these biologically important molecules in comparison with the established HO[•] oxidation chemistry. Thermodynamic control with the attendant selectivity was exhibited by ROO[•] and O₂^{•−}/HOO[•], whereas HO[•] is established to react under kinetic control in a less discriminate fashion. These results provided support for the contention that the individual oxidants might have different roles in pathology, but did not define unique molecular biomarkers for each oxidant that could be isolated intact from human tissue.

DNA is a main locus of attack of cellular oxidants and is a molecule whose damage can lead to disease initiation and progression. In comparison with lipids, the more complex structure of the DNA polymer provides greater opportunity to differentiate the reactivity of the individual oxidants. Thus the relative reactivity and selectivity of HO[•], ROO[•] and O₂^{•−}/HOO[•] at 'nicking' DNA (cleaving the deoxyribose-phosphodiester backbone) were compared [14,15]. Each oxidant nicked DNA by a different, characteristic, mechanism: as with lipid oxidations, greater selectivity was exhibited by ROO[•] and especially O₂^{•−}/HOO[•] than HO[•]. Mechanisms of nicking by ROO[•] have also been studied recently by Burcham and colleagues [16,17]. Oxidative damage to the individual DNA bases is a second mode of reactivity; numerous products attributable to attack on the bases by HO[•] have been characterized, primarily in pulse-radiolysis experiments (reviewed in [18–21]). Oxidation of purine bases by HO[•]-generating systems results initially in hydroxylation at the 8-position; further transformation/rearrangement can lead to the formation of ring-opened derivatives. Oxidation of the pyrimidine bases by HO[•] elicits a more complex product mixture involving primarily initial addition to the 5,6-double bond. Both purine and pyrimidine products have been used as biomarkers to monitor oxidative stress *in vivo* [22,23]. Gas chromatography/MS (GC/MS) has been a particularly useful tool in elucidating the products of oxidations of DNA bases and

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; GC/MS, gas chromatography/MS; TMS, trimethylsilyl.

¹ To whom correspondence should be addressed (e-mail dixta@muscc.edu).

monitoring their formation as biomarkers after isolation from cells and tissues [24,25].

Biological processes in which ROO[•] radicals are generated, such as oxidizing lipids, are known to result in the oxidation of DNA bases [26–29]. The oxidation of thymidine by ROO[•] has been studied recently [30]. In the present study, products of ROO[•] attack on the purine bases adenine and guanine and the pyrimidine bases cytosine and thymine were determined by GC/MS with authentic standards to support product identifications. In addition, ROO[•] oxidations were performed on each of the deoxyribonucleosides and both single-stranded and double-stranded DNA homopolymers [poly(dA), poly(dG), poly(dC), poly(dT), poly(dA)·poly(dT) and poly(dG)·poly(dC)] to evaluate whether changes in the chemical and physical forms of the bases influence the ROO[•] oxidations. The results further emphasize the unique reactivity characteristics of the individual O₂-derived oxidants, and have significance for the development and interpretation of oxidant-specific DNA-derived biomarkers of oxidative stress.

EXPERIMENTAL

Materials

2,2'-Azobis-(2-amidinopropane) dihydrochloride (AAPH, 2HCl) was purchased from Wako Chemicals (Richmond, VA, U.S.A.). Chromatographic standards of 2-amino-6,8-dihydropurine (8-hydroxyguanine) and 5-(hydroxymethyl)uracil were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Single-stranded and base-paired double-stranded homopolynucleotides were purchased from Pharmacia Biotechnology (Piscataway, NJ, U.S.A.). All other chemicals were purchased from standard supply houses. Buffers used in all incubations were prepared from distilled, deionized water.

General methods

GC/MS analysis was performed on a Finnigan Mat Series 5100 GC/MS system equipped with a 15 m DB-5 capillary column (internal diam. 0.32 mm). The temperature programme was initiated at 100 °C for 3 min followed by heating to 250 °C at a rate of 15 °C/min; the temperature was then maintained at 250 °C for 10 min. The total ion current was recorded at 70 eV at *m/z* values from 50 to 650. The files containing the mass spectral data of interest were imported into MassGraphix Plus software, which was used to plot mass spectra. A mass cut-off of 5% of the base peak was chosen to remove the baseline level of impurities from each spectrum. Specific ion monitoring was performed to confirm the presence of all significant peaks in both standard and oxidation product spectra.

Oxidations of bases with ROO[•]

Reactions contained A (25 mM), G (25 mM), C (30 mM) or T (26 mM) together with 25 mM AAPH, 2HCl in 10 ml of 0.01 M phosphate buffer, pH 7.0. Each reaction mixture was kept at 25 °C under stirring and O₂ bubbling for 4 h. G is insoluble in water at the concentrations desired for the experiments, so the reactions were heated to approx. 65–70 °C to enable the base to dissolve. Control experiments either included 10 mM 2(3)-hydroxyanisole or omitted the ROO[•] generator AAPH.

Oxidations of deoxyribonucleosides and homopolymers

Oxidations were done at identical molar concentrations and temperatures to those for the individual bases, but in much

smaller volumes (10–100 µl). All components were completely soluble; all oxidation experiments were therefore performed at 25 °C.

Work-up and analysis

The crude reaction products were worked up by the following procedure, modified from that of Dizdaroglu [31]: the solvent was evaporated under vacuum and the solid residue was stored in a vacuum desiccator over P₂O₅ for 24 h. The residues were redissolved in acetonitrile and silylated under N₂ for 30 min at 25 °C with 200 µl bis(trimethylsilyl) trifluoroacetamide. Approx. 1 µl of this solution was injected into a GC/MS instrument. For analysis of deoxyribonucleotides and homopolymers, the sugar residues were cleaved before treatment with bis(trimethylsilyl) trifluoroacetamide by dissolving approx. 1 mg of the residue in 0.5 ml of formic acid under N₂ in a small (2 ml) Teflon-lined vial followed by heating to 90 °C for 2 h; solvent was removed under a flow of N₂.

Preparation of chromatographic standards

Authentic standards of 8-hydroxyadenine [32], 6-hydroxylaminopurine [33,34], 5,6-dihydroxyuracil [35] and thymine glycol [36] were prepared and characterized in accordance with published procedures. Cytosine glycol and 5-hydroxycytosine were isolated and characterized after γ-irradiation of cytosine and revealed mass spectra of their trimethylsilyl (TMS) derivatives identical with published spectra [37].

RESULTS

Decomposition of AAPH, a standard method used to generate ROO[•] in aqueous solution, produces ROO[•] at a rate of 1.36 µmol of AAPH per litre per s [38]. ROO[•] was reacted initially with each of the free DNA bases, followed by analysis for TMS derivatives of products by GC/MS. To search for products, mass spectra of all new peaks in the total ion profile of reaction mixtures (in comparison with standards of derivatized bases) were evaluated initially. In addition, the presence of known and predicted oxidation products (as TMS derivatives) were evaluated with specific ion monitoring of the predicted *M*⁺ and (*M*–15)⁺ peaks in their mass spectra. When a tentative identification was made, a standard of the product was prepared, derivatized and run under identical GC conditions to demonstrate co-chromatography of standard and product, and to obtain a representative mass spectrum of the standard for comparison under identical chromatographic conditions.

Known or possible products of base oxidations (as seen previously in HO[•] and/or chemical oxidation experiments), as well as the *M*⁺/(*M*–15)⁺ peaks expected for the products' major TMS derivatives, are shown in Figures 1 and 2. Identified products of the ROO[•] oxidation of the free bases, and retention times of products in the standard GC analysis in comparison with authentic standards, are compiled in Table 1. In general, the mass spectra of all products exhibited strong *M*⁺ and (*M*–15)⁺ peaks and similar breakdown ions to those observed in the spectra of the authentic standards and as reported in the literature. For all experiments presented below, controls demonstrated the absence of products in reactions that were quenched immediately after initiation; therefore the derivitization procedures were not inducing product generation, and low levels of the oxidized bases were not present in the starting materials [39,40].

Figure 1 Purines and their oxidation products

(A) Adenine (A) and products of organic and free radical oxidations; (B) guanine (G) and products. The formation of each product was evaluated for all experiments after trimethylsilylation, with the numbers of TMS groups and the expected $M^+/(M-15)^+$ values (unless indicated otherwise) as follows: A (2 TMS; 279/264), **1**, **6** (3 TMS; 367/352), **2** (3 TMS, 369/354), **3–5** [2 TMS, 295/280, with 296 ($M+1$)⁺ and 279 ($M-16$)⁺ also significant], G (3 TMS; 367/352), **8** (4 TMS; 455/440), **9** (4 TMS, 457/442) and **10–12** [3 TMS, 383/368, with 388 ($M+1$)⁺ and 367 ($M-16$)⁺ also significant].

The products of oxidation of A by ROO[•] were 8-hydroxyadenine (**1**, Figure 1A), and 6-hydroxylaminopurine (**6**, Figure 1A). The product yields of **6** and **1** were about 2% and 1% of the total ROO[•] generated. [Detailed quantification of yields for all products in comparison with products formed in HO[•]-generating systems is in progress (T. Simandan, J. Sun and T. A. Dix, unpublished work).] No products were detected in reactions that omitted the ROO[•] generator, or in which an antioxidant, 2(3)-hydroxyanisole, was included; this was true of all other ROO[•] oxidations reported here. The identification of **1** is based on co-chromatography and comparison of its mass spectrum with an authentic standard (Figure 3A) and with published spectra [31,41,42] (all as TMS derivatives). The identification of **6** is based on co-chromatography of its TMS derivative with the TMS derivative of an authentic standard and the exhibition of similar mass spectra (Figure 3B). Although products **1** and **6** have identical molecular ions, the derivatized compounds are eluted over 1 min apart under the GC separation conditions (Table 1); reproducibility of peak elution under identical separation conditions was ± 0.1 min.

Oxidations of G were inherently difficult owing to its poor solubility in aqueous solution. Consequently it was necessary to run all reactions at higher temperatures to produce detectable levels of products, which resulted in a greater net flux of ROO[•] production. ROO[•] oxidized G to 8-hydroxyguanine (**8**, Figure

Figure 2 Pyrimidines and their oxidation products

(A) Cytosine (C) and products of organic and free radical oxidations; (B) thymine (T) and products. Products **14**, **17** and **24** exist as pairs of diastereomers that will give identical mass spectra. The numbers of TMS groups and the expected $M^+/(M-15)^+$ values are as follows: C (2 TMS; 255/240), **13** (3 TMS; 343/328), **14** (4 TMS; 433/418), **15** (4 TMS; 431/416), **16** (3 TMS; 344/325), **17** (4 TMS; 434/419), **18** (4 TMS; 432/417), **19** [2 TMS; 271/256 with 272 ($M+1$)⁺ and 255 ($M-16$)⁺ also significant], **20** (2 TMS; 255/240), **21** (3 TMS; 331/316), **22** (3 TMS; 316/301) and **23** (4 TMS; 449/434), T (2 TMS; 270/255), **24** (4 TMS; 448/433), **25** (3 TMS; 358/343), **26** (3 TMS; 346/331) and **27** (2 TMS; 286/271).

Table 1 Products of free base oxidation by ROO[•], with observed retention times in comparison with standards

Base	Retention time (min)		Product identity (see Figures 1–4)
	Product	Standard	
A	7.93	7.94	1
	9.07	9.15	6
G	9.92	9.88	8
C	5.75	5.70	13
	6.27	6.30	14
	6.83	6.85	18
T	4.12	4.11	26
	5.73	5.76	25
	6.12	6.15	24

1B), which was identified as its TMS derivative by co-chromatography with a TMS-derivatized authentic standard of this compound (Table 1). In addition, the ROO[•] product and

Figure 3 Mass spectra of adenine oxidation products and standards

(A, B) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of A (A), compared with the spectrum of a co-chromatographing, authentic standard of **1** (B). (C, D) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of A (C), compared with a co-chromatographing, authentic standard of **6** (D). m/e, *m/z*.

authentic **8** exhibited similar mass spectra (Figure 4). The yield of this product was about 1 % of the total ROO[•] generated.

Other known products of purine radical oxidations, specifically **2** and **7** (Figure 1A) and **9** (Figure 1B), were not detected in any of the ROO[•] oxidations. Adenine 1-*N*-oxide (**3**, Figure 1A) and guanine-3-*N*-oxide (**11**, Figure 1B) are established as the major *N*-oxide regioisomers formed in the oxidations of A and G respectively by H₂O₂/acetic acid [43], magnesium monoperoxyphthalate hexahydrate [44] and *m*-chloroperoxybenzoic acid [45,46]; neither compound was detected in any incubations. (*N*-Oxide isomers **4**, **5** and **10** would have exhibited similar mass spectra to the established isomer.) Product formation levels to 0.01 % of starting bases were detectable in controls by using specific ion monitoring (results not shown); therefore, if formed by ROO[•] attack, any of the above would be very minor products in comparison with the identified products.

Turning to the pyrimidines, the products of C oxidation by ROO[•] (Table 1) were 5-hydroxycytosine, cytosine glycol and 5,6-dihydroxyuracil (**13**, **14** and **18**, Figure 2A). The mass spectrum of each product, in comparison with its authentic standard, is shown in Figures 5(A)–5(C). The identifications of **13**, **14** and **18** are based on the comparison of their full mass spectra with authentic standards and with published spectra [22,31,42,47–49] and by co-chromatography with the authentic standards. The yield of product **13** was about 5 % of the total ROO[•] generated, whereas the yields of **14** and **18** were about 1 % each.

The ROO[•] oxidation of T resulted in the formation of thymine glycol, 5-(hydroxymethyl)uracil and 5-hydroxy-5-methylhydantoin (**24**, **25** and **26**, Figure 2B). The mass spectrum of each product is shown in Figures 6(A)–6(C). The identification of **24**

and **25** was based on full mass spectral comparison and co-chromatography with authentic standards. In the same way as for the products of C, the mass spectra of products and standards were virtually identical. The yield of both products was about 5 % of the total ROO[•] generated, whereas the yield of **26** was about 1 %. An authentic standard of **26** was not prepared; the identification is therefore considered tentative. The mass spectrum of this compound is similar to those reported previously [22,31,47–49]. Product **25** has been characterized previously as a product of ROO[•] oxidation of dT [30].

The *N*-oxide and hydroxylamine derivatives of C (**19** and **20**, Figure 2B) have been described [50], whereas the *N*-oxide of T (**27**, Figure 3B) seems to be a chemically reasonable structure. The predicted di- or tri-TMS derivatives of these compounds were not detected as products of any of the oxidizing systems; however, the stability of these derivatives is unknown. Known oxidation products of C, **15**–**17** and **21**–**23** (Figure 2A) were assayed for, but not detected, in any of the ROO[•] reaction analyses; again, their threshold of detection was 0.01 % of the ROO[•] generated. In general, although it appears as though the pyrimidines were somewhat more oxidizable than the purines by ROO[•], because products **13**, **24** and **25** formed to more than 5 % of the ROO[•] generated, whereas **6** and **8** formed only to approx. 1–2 %, this will need to be confirmed.

To evaluate the potential role of the chemical and physical structure of the DNA bases in controlling oxidations by ROO[•], ROO[•] was reacted with each of the deoxyribonucleosides (dA, dG, dC and dT) and both single-stranded and double-stranded DNA homopolymers [poly(dA), poly(dG), poly(dC), poly(dT), poly(dA)·poly(dT) and poly(dG)·poly(dC)] and products

Figure 4 Mass spectra of guanine oxidation product and standard

Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of G (A), compared with the spectrum of a co-chromatographing, authentic standard of **8** (B). m/e, m/z.

were determined. The results are reported in Table 2. All products of base oxidations by ROO[•] were observed in the different forms of DNA that were oxidized; mass spectra of products and standards were identical with those reported above for oxidation of the free bases (results not shown). Product **8** was observed in dG and poly(dG) oxidations that were performed at 25 °C; the observation of **8** as a product in oxidations of G is therefore not an artifact of the higher reaction temperature necessary to solubilize the free base. Detailed searches for other products, identical with those performed for the DNA free bases as described above, failed to indicate the formation of unique products. However, the ratios of monohydroxylated to dihydroxylated pyrimidine products were significantly different (T. Simandan and T. A. Dix, unpublished work), indicating that a detailed determination of product yields as a function of structure should be undertaken.

DISCUSSION

The results presented here demonstrate that ROO[•], an oxidant that exists in significant concentrations in cells, can oxidize the purine and pyrimidine bases to many of the products that are formed *in vivo* on DNA. Oxidations occurred whether the bases were presented as free bases, deoxyribonucleosides or homopolymers; the physical state of the DNA is therefore not qualitatively influencing product formation. (All products formed are from reaction in the major groove of the bases, so steric hindrance as a result of Watson–Crick base-pairing in the DNA duplexes is not a factor.) With regard to particular products, the oxidation of both A and G to their 8-hydroxy

derivatives, compounds that, when formed in the DNA bases, can promote base-miscoding and for which specific cellular repair processes exist [51,52], supports the contention that ROO[•] radicals might be toxicologically important oxidants. Lipid peroxidation in the presence of DNA results in the formation of 8-hydroxyguanine [28,29]; ROO[•] radicals are the main chain-propagating species of peroxidative processes and, accordingly, the putative oxidants in these systems. Furthermore 8-hydroxyguanine (**8**, Figure 1B) is the biomarker that has been used most often to monitor oxidative stress; oxidants demonstrated to produce this compound now include both HO[•] and ROO[•]. ROO[•] also oxidizes C and T to monohydroxylated and dihydroxylated products (**13**, **14**, **18**, Figure 2A; **24–26**, Figure 2B) that previously were attributed solely to oxidations by HO[•]. Oxidations of T are a major type of DNA damage *in vivo* [51] and it is possible that both ROO[•] and HO[•] are active in this damage.

ROO[•] is generally considered to be a weaker, more specific, oxidant than HO[•], as manifested by the fact that fewer products form in the oxidations by ROO[•]. For the purines, products **2**, **7** and **9** (Figures 1 and 2) were not detected in any ROO[•] oxidations. This was not surprising with **2** and **9**, because these are products arising from attack at the N7–C8 position under reductive conditions; oxidative conditions resulted in the observed **1** and **8** respectively [18–21]. In contrast, ring-contracted products of C (**21–23**, Figure 3) and T (**26**, Figure 4) were not observed as ROO[•] products. These products are believed to be secondary metabolites of pyrimidine hydroxy-hydroperoxides (Scheme 1, as shown for C, based on [18–21] and references therein), which decompose to form both dihydroxylated (e.g. **14**, formed in both ROO[•] and HO[•] oxidations) and ring-contracted (e.g. **22** and **23**, formed only in HO[•] oxidations) products. For C, products **15** and **17** (Figure 2A) were not detected in any of the ROO[•] product analyses. Observed product **18**, however, is deaminated **15**, whereas observed **17** is deaminated **14**; apparently, different products have different relative stabilities under the reaction or derivitization conditions. Deamination of C products can occur under fairly mild conditions [18–21]. The mechanism for formation of T product **25** is uncertain, but it is an established product of attack by both ROO[•] and HO[•]. Taken together, however, these results imply that the mechanisms of oxidation of purines and pyrimidines by ROO[•] and HO[•] are likely to be different; if this is correct, it could be exploited in the development of mechanistic probes for each oxidant (see below).

Of considerable interest is the demonstration that ROO[•] oxidizes A to its hydroxylamine (**6**, Figure 1A), a hitherto uncharacterized product. This is the first demonstration that an oxygen radical species can convert an amine to a hydroxylamine. Mouret et al. [53] have demonstrated that adenine 1-*N*-oxide (**3**, Figure 1A) is a product of H₂O₂ attack on dA; Yamamoto [54] tentatively identified adenine 7-*N*-oxide (**4**, Figure 1A) as a product of HO[•] attack on A. These results indicate that the biological ramifications of generating N-oxidized products in the DNA bases should be evaluated, especially because these products were first synthesized and tested for their cellular toxicity [33,34]. In the present study, particular attention was paid to the possibility of the formation of N-oxidation products by ROO[•], but only hydroxylamine **6** was a product.

N-oxidized products resulting from attack by HO[•] on the DNA bases have not been characterized. The inability of HO[•]-generating systems to generate the N-oxidized products supports the contention that these could be developed as biomarkers for ROO[•] and H₂O₂ formation and activity in cells. Mouret et al. [53] proposed using **3** as a biomarker for the presence of H₂O₂ in the vicinity of DNA; in parallel, **6** could be developed as a biomarker for ROO[•] reactivity in cells and tissues. However, certain criteria

Figure 5 Mass spectra of cytosine oxidation products and standards

(A, C) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of C (A), compared with the spectrum of a co-chromatographing, authentic standard of **13** (B). (C, D) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of C (C), compared with the spectrum of a co-chromatographing, authentic standard of **14** (D). (E, F) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of C (E), compared with the spectrum of a co-chromatographing, authentic standard of **18** (F). m/e, *m/z*.

must first be evaluated. As noted above, *N*-oxides are common products of ionic oxidations, and peracid-like species could conceivably be generated *in vivo* under conditions of severe oxidative stress. In addition, the reactivity of O₂^{•-}/HOO[•] towards the DNA bases has not been evaluated; in previous studies [10,14,15] the active oxidizing species of this acid-base pair, HOO[•], was demonstrated to perform ROO[•]-like reactions but with a higher degree of selectivity. Finally, the oxidation of intact DNA by H₂O₂ to form *N*-oxides might disfavour the formation of **3** because the oxidized nitrogen is protected in the Watson-Crick base-pairing motif. However, a key goal of defining purine-derived oxidant-specific markers does seem to be attainable.

The potential use of C and/or T products as biomarkers for the activity of individual oxidants is less certain than that for the

purine products. C and its products are regarded as too labile towards deamination to serve as stable, isolatable biomarkers. Further, with both pyrimidines, unique final products were not observed from attack by HO[•] as opposed to ROO[•], which suggests that common mechanisms of oxidation are occurring. However, the GC/MS analysis used in this study had a major flaw in that, owing to the harsh conditions of the derivatizations (especially the treatments with formic acid to cleave the sugar residues), different metastable intermediates resulting from HO[•] as opposed to ROO[•] addition could be decomposed to common final products. [This problem has been noted in studies of the oxidation of DNA components by benzoyl peroxide [55], but apparently ignored in other DNA oxidation studies, especially those using GC/MS. Co-chromatography of all underivatized,

Figure 6 Mass spectra of thymine oxidation products and standards

(A, B) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of C (A), compared with the spectrum of a co-chromatographing, authentic standard of **24** (B). (C, D) Mass spectrum of a TMS-derivatized product of ROO[•] oxidation of C (C), compared with the spectrum of a co-chromatographing, authentic standard of **25** (D). (E) Mass spectrum of a compound formed from ROO[•] oxidation of T. The compound is believed to be **26**, on the basis of comparison with published data from pulse radiolysis of thymine [31,46–50]. In addition to the appropriate *M*⁺ and (*M*–15)⁺ peaks, the spectrum reveals diagnostic ions at *m/z* 255 (*M*⁺ – TMS), and 170 (C₆H₅N₂O₂Si, diagnostic of the five-membered ring system). *m/e*, *m/z*.

UV-active, products with their authentic standards on HPLC has been achieved (including products **1**, **6**, **8**, **13**, **18** and **25**; Figures 1 and 2), which demonstrates that these are primary products of ROO[•] attack (J. Sun and T. A. Dix, unpublished work).] This would obscure mechanistic differences in adduct formation that might be exploitable as mechanistic probes for the presence of each oxidant. For example, established ROO[•] chemistry [56] indicates that a viable mechanism for the formation of product **24** is by epoxidation of the activated double bond of T followed by the addition of water to open the ring. This would contrast with the established mechanism for HO[•], which involves its addition to the double bond followed by scavenging of solution O₂ to form hydroxy-hydroperoxides (see Scheme 1) [16–18]. As noted above, the range of products characteristic of

hydroxy-hydroperoxide breakdown were not found in the ROO[•] oxidations described here. ROO[•] radicals also abstract activated allylic hydrogen atoms [57], which could lead to the formation of **25** [30]; the mechanism for formation of this product from HO[•] attack is not known.

Studies comparing the mechanisms and ratios of formation of DNA base-derived products by ROO[•] and HO[•], and the possible biological ramifications of N-oxidized bases in DNA, are in progress in our laboratory.

We thank Jack Walker, Amy Ledbetter, Trina Clark and Thomas L. L. Webb for participating in the product identification and GC/MS analyses. This work was supported by the National Institutes of Health (GM-40338, to T. A. D.) and with a postdoctoral fellowship from the Medical University of South Carolina (to T. S.).

Table 2 Products of the oxidation of deoxyribonucleosides, homopolymers and homopolymer duplexes by $\text{ROO}^\bullet$

Base	Retention time (min)		Product identity (see Figures 1–4)
	Product	Standard	
dA	7.82	7.85	1
	8.99	9.05	6
dG	10.04	9.98	8
dC	5.75	5.75	13
	6.25	6.22	14
	6.83	6.85	18
	4.11	4.12	26
dT	5.80	5.77	25
	6.10	6.10	24
poly(dA)	7.80	7.85	1
	9.03	8.95	6
poly(dG)	9.94	9.95	8
poly(dC)	5.70	5.71	13
	6.22	6.20	14
	6.84	6.85	18
	4.06	4.10	26
poly(dT)	5.89	5.88	25
	6.02	6.08	24
poly(dA) · 3poly(dT)	7.82	7.85	1
	8.99	9.05	6
	4.11	4.12	26
	5.80	5.77	25
poly(dG) · 3poly(dC)	6.10	6.10	24
	9.99	9.94	8
	5.70	5.70	13
	6.33	6.30	14
	6.91	6.86	18

Scheme 1 Cytosine hydroxy-hydroperoxides as intermediates in the formation of cytosine oxidation products (based on [6])

Only one set of isomers of the hydroxy-hydroperoxide intermediate is indicated.

REFERENCES

- Halliwell, B. M. and Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford
- Poli, G., Albano, E. and Dianzani, M. U. (eds.) (1993) *Free Radicals: From Basic Science to Medicine*, Birkhauser, Basel
- Rice-Evans, C., Halliwell, B. and Lunt, G. G. (eds.) (1995) *Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives*, Portland Press, London
- Ozben, T. (ed.) (1998) *Free Radicals, Oxidative Stress and Antioxidants: Pathological and Physiological Significance*, Plenum Press, New York
- Pryor, W. A. (1986) *Annu. Rev. Physiol.* **48**, 657–667
- von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor and Francis, London
- Sawyer, D. T. (1991) *Oxygen Chemistry*, Oxford Press, New York
- Marnett, L. J. (1986) *Carcinogenesis* **8**, 1365–1373
- Dix, T. A. and Aikens, J. (1993) *Chem. Res. Toxicol.* **6**, 2–18
- Aikens, J. and Dix, T. A. (1991) *J. Biol. Chem.* **266**, 15091–15098
- Aikens, J. and Dix, T. A. (1992) *Chem. Res. Toxicol.* **5**, 263–267
- Dix, T. A., Aikens, J. and Hess, K. M. (1992) in *Biological Free Radical Oxidations and Antioxidants* (Ursini, F. and Cadenas, E., eds.), pp. 3–8, Cleup, University Publishers, Padova
- Aikens, J. and Dix, T. A. (1993) *Arch. Biochem. Biophys.* **305**, 516–525
- Dix, T. A., Hess, K. M., Medina, M. A., Tilly, S. and Sullivan, R. W. (1994) in *Biological Oxidants and Antioxidants* (Packer, L. and Cadenas, E., eds.), pp. 13–23, Hippokrates Verlag, Stuttgart
- Dix, T. A., Hess, K. M., Medina, M. A., Tilly, S., Sullivan, R. W. and Webb, T. T. (1996) *Biochemistry* **35**, 4578–4583
- Harkin, L. A., Butler, L. M. and Burcham, P. C. (1997) *Chem. Res. Toxicol.* **10**, 575–581
- Harkin, L. A. and Burcham, P. C. (1997) *Biochem. Biophys. Res. Commun.* **237**, 1–5
- Hutchinson, F. (1985) *Prog. Nucleic Acid Res.* **32**, 115–154
- Teoule, R. (1987) *J. Radiat. Biol.* **51**, 573–589
- Kuwabara, M. (1991) *Radiat. Phys. Chem.* **37**, 691–704
- Breen, A. P. and Murphy, J. A. (1995) *Free Radical Biol. Med.* **18**, 1033–1077
- Dizdaroglu, M. (1991) *Free Radical Biol. Med.* **10**, 225–242
- Crane, P. F. (1990) *Mass Spectrometry Rev.* **9**, 505–554
- Shigenaga, M. K. and Ames, B. N. (1991) *Free Radical Biol. Med.* **10**, 211–216
- Floyd, R. A., Watson, J. A., Wong, P. K., Altmiller, D. H. and Rickard, J. (1985) *Free Radical Res. Commun.* **1**, 163–172
- Inouye, S. (1984) *FEBS Lett.* **172**, 231–234
- Ueda, K., Kobayashi, S., Morita, J. and Komano, T. (1985) *Biochim. Biophys. Acta* **824**, 341–348
- Hruszkewycz, A. M. and Bergtold, D. S. (1990) *Mutat. Res.* **244**, 123–128
- Park, J.-W. and Floyd, R. A. (1992) *Free Radical Biol. Med.* **12**, 245–250
- Martini, M. and Termini, J. (1997) *Chem. Res. Toxicol.* **10**, 234–241
- Dizdaroglu, M. (1990) *Methods Enzymol.* **193**, pp. 842–857
- Dizdaroglu, M. and Bergtold, D. S. (1986) *Anal. Biochem.* **156**, 182–188
- Giner-Sorolla, A., Medrek, L. and Bendich, A. (1966) *J. Med. Chem.* **88**, 143–144
- Chang, P. K. (1965) *J. Med. Chem.* **87**, 884–888
- Behrend, R. and Roosen, O. (1889) *Liebig's Ann. Chem.* **251**, 235–238
- Lustig, M. J., Cadet, J., Boorstein, R. J. and Teebor, G. W. (1992) *Nucleic Acids Res.* **20**, 18, 4839–4845
- Dizdaroglu, M. (1984) *J. Chromatogr.* **295**, 103–121
- Niki, E. (1990) *Methods Enzymol.* **186**, 100–108
- Ravanat, J.-L., Turesky, R. J., Gremaud, E., Trudel, L. J. and Stadler, R. H. (1995) *Chem. Res. Toxicol.* **8**, 1039–1045
- Collins, A., Cadet, J., Epe, B. and Gedik, C. (1997) *Carcinogenesis* **18**, 1833–1836
- Aruoma, O. I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1989) *J. Biol. Chem.* **264**, 20509–20512
- Blakely, W. F., Fuciere, A. F., Wegher, B. J. and Dizdaroglu, M. (1990) *Radiat. Res.* **121**, 338–343
- Stevens, M. A., Smith, H. W. and Brown, G. B. (1959) *J. Am. Chem. Soc.* **81**, 1734–1738
- Cramer, F., Randerath, K. and Schafer, E. A. (1963) *Biochim. Biophys. Acta* **7**, 150–156
- Fujii, T., Saito, T., Kizu, K., Hayashibara, H., Kumazawa, Y., Nakajima, S. and Fujisawa, T. (1991) *Chem. Pharm. Bull.* **39**, 301–308
- Subbaraman, L. R., Subbaraman, J. and Behrman, E. J. (1969) *Biochemistry* **8**, 3059–3066
- Dizdaroglu, M. (1986) *Bio Techniques* **4**, 536–546
- Dizdaroglu, M. (1985) *Anal. Biochem.* **144**, 593–603
- Schulte-Frohlinde, D. and von Sonntag, C. (1985) in *Oxidative Stress* (Sies, H., ed.), pp. 11–40, Academic Press, London
- Delia, T. J., Olsen, M. J. and Brown, G. B. (1965) *J. Org. Chem.* **30**, 2766–2768
- Marnett, L. J. and Burcham, P. C. (1993) *Chem. Res. Toxicol.* **6**, 771–785
- Teebor, G. W., Boorstein, R. J. and Cadet, J. (1988) *Int. J. Radiat. Biol.* **54**, 131–150
- Mouret, J.-F., Polverelli, M., Sarrazini, F. and Cadet, J. (1991) *Chem. Biol. Interact.* **77**, 187–201
- Yamamoto, O. (1980) *Radiat. Res.* **21**, 239–247
- Hazlewood, C. and Davies, M. J. (1996) *Arch. Biochem. Biophys.* **332**, 79–91
- Ingold, K. U. (1969) *Acc. Chem. Res.* **2**, 1–9
- Porter, N. A. (1986) *Acc. Chem. Res.* **19**, 262–268