Peroxy Radical Oxidation of Thymidine

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The peroxy radical (ROO•) is unique among reactive oxygen species implicated in the production of DNA damage in that it possesses an extremely long half-life (order of seconds) and is predicted to have a relatively greater chemical selectivity in its reactions relative to other radical intermediates. Yet no product studies of the reactions of ROO• with bases, nucleosides, or DNA have appeared, and thus no meaningful predictions can be made regarding its potential involvement in the production of DNA base damage and the mutagenic process. We report here on the reaction products formed by peroxy radical with thymidine, a major target of oxidative base damage. ROO• reacts with thymine to yield predominantly 5-Me oxidation products. The highly mutagenic 5-(hydroperoxymethyl)-2′-deoxyuridine, 5-formyl-2′-deoxyuridine, and 5-(hydroxymethyl)-2′-deoxyuridine are produced by peroxy radical oxidation. In contrast, 5-Me oxidation products are minor products of thymidine oxidation by •OH, which yields predominantly saturated derivatives via addition to the 5,6 double bond. A plausible mechanistic scheme for the formation of the base oxidation products of thymidine by peroxy radicals is presented. Attack at the deoxyribose moiety resulting in oxidative depyrimidination is also found to occur, as indicated by free base release. Phosphodiester backbone cleavage resulting in single and double strand breaks is also catalyzed by peroxy radical, as demonstrated using a plasmid nicking assay.

Introduction

The reaction processes which bring about oxidative damage of the nucleic acids are of great interest owing to the important contribution they make to mutagenesis. The various classes of reactive oxygen radicals within cells likely vary in their capacity to produce specific kinds of DNA damage. The nucleophilic/electrophilic character of the individual radical species involved, as well as their lifetimes and abundance, will influence the relative distribution of oxidative base damage that is observed. Little is known about the relative abundance of various radical species produced under physiological conditions as a consequence of metabolic or adventitious oxidation, or the relative contributions of different radicals to the production of individual base lesions. Among reactive oxygen radicals, the greatest attention has been focused on the reactions of the hydroxy radical (•OH) with DNA substrates. Radiolytic reactions occurring in water as a result of γ irradiation (*1*), metal ion promoted Fenton-type reactions (*2*), and decomposition reactions of H_2O_2 (*3*) result in the production of hydroxy radicals. This extremely reactive intermediate yields a wide variety of base oxidation products with DNA arising predominantly from initial addition to double bonds. In pyrimidines, this typically leads to products characterized by saturation of the 5,6 double bond (*3*−*6*). In contrast to the short-lived, highly reactive hydroxy radical, other biologically important radical species which possess greater chemical stability may also play an important role in the formation of oxidative base lesions in DNA, since they are not readily quenched by random molecular encounters and may traverse large intracellular distances prior to reacting. Moreover, greater radical stability implies greater selectivity in the kinds of chemical modifications produced. Hence one might expect to find a limited set of DNA damage products characteristic of this exposure, *i.e.*, a mutagenic signature distinct from that produced by hydroxy or other nonselective radicals.

Good candidates for such reactive intermediates would be organic peroxy radicals (ROO•), such as those formed by reaction of molecular oxygen with carbon centered radicals. Carbon radicals may be readily formed by at least two pathways, one involving H• abstraction by other radicals, or by the addition of •OH to double bonds. Addition of molecular oxygen to provide peroxy radicals (autoxidation) is believed to be a common biological event (*7*). As Marnett has pointed out, peroxy radicals may contribute to oxidative DNA damage and thus play an important role in carcinogenesis (*8*), but suprisingly little is known about the types of products that result from reaction with DNA.

Hydroperoxides (ROOH), potential precursors of peroxy radicals, have been shown to produce DNA damage in the presence of low valent transition metal ions. Hydroperoxides of lipids have been shown to cause alkali labile modification of double stranded DNA fragments in the presence of metal ions such as copper. Thymines and guanines were found to be the most frequently modified bases, although no oxidation products were identified (*9*, *10*). Autoxidized lipids have been shown to promote the formation of 8-hydroxyguanine in calf thymus DNA, an effect potentiated by transition metal ions such as Cu(II) and Fe(III) (*11*). It has been shown that enhanced lipid peroxidation in isolated mitochondria correlates with a marked increase in the 8-hydroxyguanine content of mtDNA (*12*). Reactions of the peroxy radical of dimethylarsine with DNA have been identified as a possible cause of the mutagenicity of arsenates, and *in vitro* DNA double strand breaking activity has been demonstrated (*13*). Peroxy radicals are believed to be important intermediates in these reactions, although direct evidence for their participation is lacking.

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In order to assess the potential for peroxy radicals to mediate oxidative DNA damage, we have explored the reactions of the water soluble radical initiator ABAP[1,2] [2,2′-azobis(2-methylpropionamidine) dihydrochloride] with nucleosides and plasmid DNA in O_2 saturated buffer solutions. This free radical initiator has been shown to provide peroxy radicals in a linear, kinetically reproducible fashion upon thermolysis in the presence of oxygen (*14*). This method has been used to model biologically relevant oxidation reactions by peroxy radicals (*15*, *16*).

Because of reports identifying thymine as a major target of oxidation by metal ion promoted decomposition of organic hydroperoxides, as well as the fact that oxidation products of thymine constitute the majority of DNA damage resulting from ionizing radiation (*17*), we have examined the reaction of thymidine with peroxy radicals. We have identified the major base oxidation products by GC-MS, ^{1}H-NMR, and chemical correlation with authentic compounds when feasible. The structure of a novel compound, 3,3′,4,4′-tetramethylsuccinimidine, derived from ABAP thermolysis is also reported.

Materials and Methods

Reagents and Standards. Thymidine and thymine were purchased from Aldrich Chemical Co. (Milwaukee, WI). ABAP was purchased from ACROS organics (Geel, Belgium).

Caution: *ABAP (AAPH) is toxic and is an irritant. Use care in handling.*

5-HmdU (5-(hydroxymethyl)-2′-deoxyuridine) was purchased from Sigma (St. Louis, MO); purity was checked by FPLC using the conditions described below for reaction analysis. 5-HPMdU (5-(hydroperoxymethyl)-2′-deoxyuridine) was synthesized from 5-HmdU by a modification of the procedure of Hahn and Wang (*18*), omitting recrystallization from 10% MeOH. FPLC purification of the reaction products provided the desired hydroperoxide in 35−40% yield. This compound has also been isolated from menadione photosensitization of thymidine in oxygenated aqueous solution (*21*). 5-FodU (5-formyl-2′-deoxyuridine) was prepared by Dr. Lawrence C. Sowers using a modification of the procedure of Cline *et al.* (*19*). Thymine glycol was also a gift of Dr. Sowers. *cis*-5-Hydroxy-6-hydroperoxy-5,6-dihydrothymine was prepared by H_2O_2 oxidation of *cis*-thymine glycol under acidic conditions as described previously (*20*). This material, although isolable using the chromatographic conditions described below, was found to be unstable at room temperature, consistent with a reported half-life of ∼3 days at 22 °C (*21*).

Oxidation Reactions. All reagents and buffers were prepared using doubly distilled deionized water (Barnstead Nanopure system). Typically, 40 μmol of thymidine was dissolved in 4 mL of buffer (KH_2PO_4, pH = 4.5 or 7.0). O_2 was then bubbled into the solution for ∼15 min to achieve saturation, prior to the addition of 50 μmol (13.6 mg) of ABAP. The reaction was maintained at 40 °C for 25 h. Reactions were terminated by quick freezing in dry ice followed by lyophilization. Samples were then resuspended in 1 mL of water or buffer prior to analysis by FPLC.

Chromatographic Analyses. Reactions were chromatographed using a Pharmacia FPLC system equipped with a PepRPC 5 (C_2/C_{18}) HR 5/5 or HR 10/10 column. Separation was achieved using isocratic elution in a manner essentially identical to that described by Tofigh and Frenkel (*22*) for purification of thymine hydroperoxides: 0.5 M NH_4HCO_2, pH = 3.5. Flow rates of 0.5 or 2.5 mL/min for analytical (HR 5/5) and preparative (HR 10/10) runs, respectively, were used; analyte detection was by UV monitoring at 254 nm. Desalting of fractions was achieved by rechromatography over a MonoQ HR 5/5 strong anion exchange column eluted with doubly distilled water. TLC was performed on silica gel 60 F_{254} plates (Merck) using ethyl acetate/2-propanol 2.5:1.

Spectroscopic Methods. All UV spectra were recorded using a Perkin-Elmer Lambda 220 spectrometer. Analytical tests for organic peroxides, monitoring the formation of I_3^- via oxidation of KI, were conducted spectophotometrically using a molar extinction value of 15 400 M^{-1} cm^{-1} at 361 nm (*23*).

Mass Spectral Analyses. Gas chromatography/mass spectrometry (GC-MS) was performed on a Hewlett Packard 5970 mass selective ion detector interfaced to a Hewlett-Packard 5890A gas chromatograph. A fused-silica capillary column coated with cross-linked 5% phenylmethyl silicone gum was used for GC separation with helium as the carrier gas. Samples were injected in the split mode with the injector port at 250 °C. Mass spectra were obtained at 70 eV (*24*). Derivatization of nucleosides was carried out in dry acetonitrile (distilled from activated 4 Å molecular sieves). BSTFA [bis(trimethylsilyl)-trifluoroacetamide] was obtained from Aldrich Chemical Co. Approximately 100 nmol of analyte (estimated spectrophotometrically by assuming a molar extinction coefficient of ∼10 000 M^{-1} cm^{-1}) was dried in a Pierce hypovial and capped with a Teflon lined septum under argon. Aliquots of 100 μL each of acetonitrile and BSTFA were added via gas-tight syringe, and the samples were heated to 130 °C for 30 min.

Electrospray mass spectral data for exact mass determinations were collected on a Finnegan MAT 900 magnetic sector instrument. This was equipped with a microscale electrospray interface (*25*). Samples were introduced in 20% CH_3CN/0.1% TFA (trifluoroacetic acid). Lysine and phenylalanine were used as internal standards.

NMR. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Unity 300 MHz NMR instrument. Samples were dissolved in D_2O or d_6-DMSO containing 0.75% 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (Aldrich Chemical Co.) as an internal reference standard.

Plasmid Assay. An aliquot of 1 μg of DNA plasmid pGEM9Zf(−) (Promega, Madison, WI) was dissolved in 500 μL of O_2 saturated phosphate buffer, pH = 7.0 maintained at 4 °C, followed by 500 μL of the appropriate 2× ABAP solutions to yield final concentrations of 1 μM, 100 μM, or 1 mM. The reactions were then maintained at 40 °C for a period of 1 h, whereupon the DNA was split into two equal aliquots and precipitated by the addition of sodium acetate (10 mM final concentration) and 2 volumes of absolute ethanol. The pellets were dried on a Speed-Vac and redissolved in 10 μL of TBE containing loading buffer. Electrophoresis was carried out on a 8 cm × 6 cm 1% agarose gel run at 90 V for 3 h on a horizontal submarine gel apparatus. Bands were visualized by ethidium staining of the gels and exposure to 254 nm light. Gel documentation was performed using a UVP Gel Documentation system (San Gabriel, CA).

Results

Analysis of the Reaction Products of Peroxy Radical with Thymidine. A typical chromatogram of the reaction products obtained after 25 h at 23 °C is shown in Figure 1. Physical data for the reaction products are provided in Table 1. Approximately 30% of thymidine was converted to products during the course of this reaction. Reaction of 5-methyluridine under identical conditions gave rise to a nearly identical reaction profile, with some additional minor (<5% of total products) early eluting components, possibly arising from sugar modification (data not shown). These products were not analyzed further. The major products of the

[1] Abbreviations: 5-HPMdU, 5-(hydroperoxymethyl)-2′-deoxyuridine; 5-FodU, 5-formyl-2′-deoxyuridine; 5-HMdU, 5-(hydroxymethyl)2′-deoxyuridine; ABAP, 2,2′-azo-bis(2-methylpropionamidine) dihydrochloride; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TFA, trifluoroacetic acid; TBE, Tris/borate/EDTA; TMS, trimethylsilyl; 5-HPMU, 5-(hydroperoxymethyl)uracil.

[2] The abbreviation AAPH [2,2′-azobis(amidinopropane) dihydrochloride] is also used.

Table 1. Physical Data for the Reaction Products[a]

compd	yield (%)[b]	UV λ_{max} (nm)	R_f[c]	MS (m/z)[d]	NMR[e]	t_R (min)[f]
1a	18.3	264	0.81	270 (M^+), 255	CH_3, 1.9; H_6, 7.65	5.95 (2.3)
2c	12.25	264	0.63	358 (M^+), 343, 269, 255	CH_2, 4.4; H_6, 7.90; $H_{1'}$ 6.3; $H_{2'}$, 2.4; $H_{5'}$, 3.8	8.5 (5.3)
3c	6.0	235, 280	0.88	284 (M^+), 195, 256, 241	CH 9.1; H_6, 8.8; $H_{1'}$, 2.30; $H_{2'}$, 2.4: $H_{5''}$, 3.8	16.1 (4.2)
4a	trace		nd	370 (M^+), 355, 281, 270, 266, 255	nd	(12.6)
5c	17.2	264	0.76	nd*	CH_2, 4.72; H_6, 8.10 $H_{1'}$ 6.3; $H_{2'}$, 2.4; $H_{5'}$, 3.8	12.2
6a	46.2	240	0.10	154.1344 ($M^+ + 1$)‡	CH_3, 1.35; NH, 9.65, [CH_3, 24.3, q; C, 53.1, s; C=X, 197.25, s]§	13.5

[a] Compound designations refer to products shown in Figure 3. [b] Expressed as relative yields of isolated oxidation products, average of three determinations. [c] R_f values calculated on silica gel TLC plates as described in Materials and Methods. [d] Ion fragments from GC-MS of TMS derivatives. [e] Chemical shifts in D_2O, except for **6a** (DMSO), given in ppm upfield from TMSPA-d_4. [f] Retention times in parentheses correspond to GC retention times of TMS derivatives; additional values are FPLC retention times using elution conditions described in the text.
‡ Electrospray MS data, positive ion mode. § ^{13}C-NMR data in D_2O. *nd = not determined.

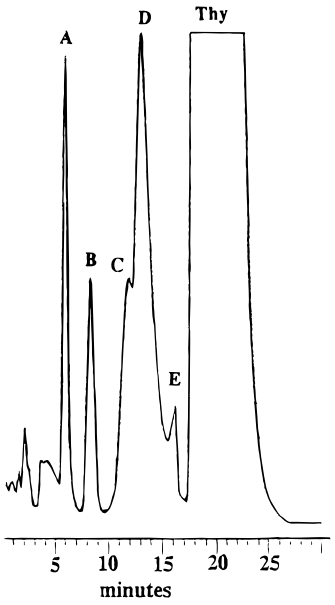

Figure 1. FPLC chromatogram of the reaction products obtained from the oxidation of thymidine (Thy) by peroxy radical generated from the thermolysis of ABAP in the presence of saturating O_2. Peak identities are as follows: A, thymine; B, 5-HMdU; C, 5-HPMdU; D, 3,3′,4,4′-tetramethylsuccinimidine; and E, 5-FodU.

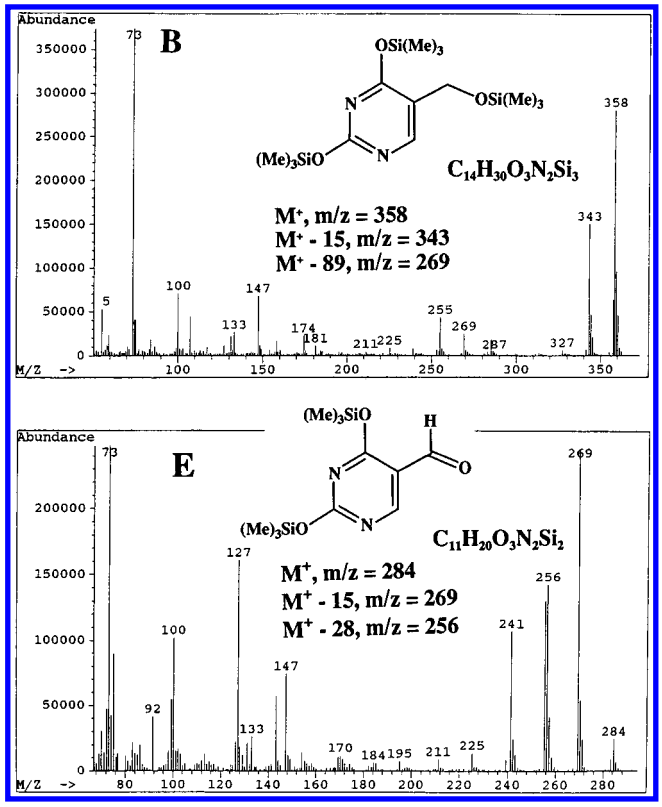

Figure 2. GC-MS spectra of fractions corresponding to the trimethylsilyl derivatives of peak B and peak E in Figure 1. The GC retention times are provided in Table 1.

5-methyluridine reaction were shown by UV analysis to have the same spectral properties as the corresponding products obtained in the thymidine reaction. These observations with 5-methyluridine indicated that the major base oxidation products were the same for DNA and RNA nucleosides. Analyses were therefore confined to the deoxynucleoside reaction, owing to the availability of reference compounds for oxidative base damage.

The peak labeled A in Figure 1, eluting at 5.95 min, possessed a UV λ_{max} of 264 nm. TMS derivatization and GC-MS analysis of this fraction yielded an $M^{\bullet+} = 270$, an intense ion fragment at m/z 255, and other ions consistent with the fragmentation of thymine. Confirmation of this peak as the free base was achieved by coinjection on FPLC and co-spotting on TLC with authentic sample. Examination of the starting material by FPLC indicated no contamination of nucleoside with free base; therefore, depyrimidination occurred as a result of the reaction with peroxy radical.

The fraction corresponding to peak B, with a retention time of 8.5 min, similarly displayed a UV consistent with an unsaturated pyrimidine (264 nm). GC-MS analysis of the trimethylsilyl derivative of peak B, shown in Figure 2, top panel, revealed a base ion at m/z 358. Supporting this assignment were the expected ion fragments corresponding to loss of CH_3 (m/z 343) and OTMS (*O*-(trimethylsilyl), m/z 269). The electron ionization mass spectrum of this compound is virtually identical to that previously reported for 5-(hydroxymethyl)uracil (*26*). The ion fragment corresponding to the intact nucleoside and other ions derived from the sugar series are in general of such low abundance as to be of little diagnostic value for the deoxypyrimidines (*27*). Thus the question of whether peak B corresponds to the free base or the nucleoside of (hydroxymethyl)uracil could not be determined by GC-MS analysis. The identity of peak B as the nucleoside was confirmed by NMR analysis (Table 1), coinjection on FPLC, and coelution on silica TLC plates with authentic 5-HMdU.

The partially resolved peak C in Figure 1 was characterized by a UV λ_{max} of 264 nm, while the fraction corresponding to peak D absorbed maximally at 240 nm. The KI test for peroxides yielded a positive result for the longer wavelength-absorbing component, but D was

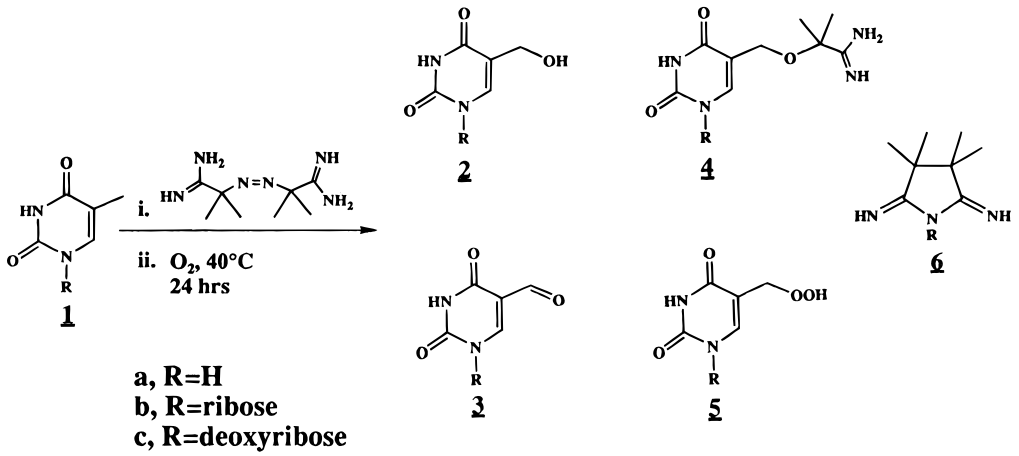

Figure 3. Reaction scheme and structures of the reaction products characterized in the ABAP initiated peroxy radical oxidation of thymidine.

judged not to contain any peroxide moiety. Attempts to obtain trimethylsilyl derivatives of peak C for GC-MS analysis using a variety of conditions resulted in decomposition. Since 5-HPMdU appeared to be a likely candidate for an unsaturated hydroperoxide derived from thymidine, this compound was synthesized using a modification of the procedure of Hahn and Wang (*18*), using the deoxynucleoside rather than the free base. Comparison of the retention times and coinjection of this material with the reaction products of the thymidine/ABAP/O_2 reaction and purified peak C demonstrated that peak C was indeed HPMdU. NMR comparison of peak C with material prepared as described above as well as the data reported for the nucleoside prepared photochemically (*21*) substantiated this assignment. NMR data for the nucleoside in D_2O are reported in Table 1.

The fraction corresponding to peak D was derivatized and examined by GC-MS. The major GC peak obtained following derivatization was broad and not well resolved. Sampling the mass spectra at several points along this peak revealed many ion fragments of low intensity, which rendered it impossible to clearly assign parent ions (data not shown). A relatively late eluting (12.6 min) minor component, in contrast, gave a sharp peak in the GC chromatogram. Discrete ion fragments were observed for this species, although the ion abundances were found to be lower than those derived from 5-HMdU. The assignment of the $M^{\bullet+}$ ion to an $m/z = 370$ was supported by the presence of M − 15, M − 89, and M − 104 ions at $m/z = 355$, 281, and 266, corresponding to loss of CH_3, OTMS, and CH_3 + OTMS, respectively (*27*, *28*). This compound was assigned the structure **4a** shown in Figure 3. Since only GC-MS data on this trace component could be obtained, we could not assign the nucleoside.

Further support for this assignment is provided by the observation of an ion fragment corresponding to loss of the unusual side chain ($C_5H_{11}N_2O$, $m/z = 115$). In general, ion fragments arising from side chain loss from the parent molecular ion of modified nucleosides and bases provide good evidence for their structural assignment. This observation has been of value in the identification of base modified tRNA components (*28*). We also observed side chain loss of $TMSO^+{=}CH_2$ (m/z 103) from the 5-(hydroxymethyl)uracil base ion (m/z 358) (Figure 2**,** top panel). Heteroatom containing side chains such as those in **4a** generally result in lowered abundances of the basic series of characteristic ions, consistent with what is found in the present case (data not shown) (*28*). Silylation of the guanidine group of the side chain was not observed. Similar behavior has also been noted during attempts to prepare volatile derivatives of arginine by BFTSA for GC-MS analysis.[3]

NMR analysis of peak D in D_2O revealed a lack of 2′-deoxyribose proton resonances. Only two proton resonances were observed, one assigned as CH_3 (1.35 ppm) and an absorbance due to HOD (4.8 ppm). The CH_3 resonance was shifted upfield relative to the 5-Me group of thymine (Table 1, entry **6a**) and was consistent with the chemical shift assignment of Me groups of other saturated thymidine derivatives such as thymine glycol (*20*). This initially suggested that peak D was possibly a saturated pyrimidine derivative, consistent with a UV λ_{max} of 240 nm. We considered the possibility that this compound might be thymine glycol or perhaps 5(6)-hydroperoxy hydroxy thymine, but FPLC coinjection with authentic standards prepared as described above failed to correlate peak D with these known compounds.

A single broad, D_2O exchangeable NH signal was assigned at 9.65 ppm in DMSO-d_6. This ruled out the possibility of an intact pyrimidine derivative, owing to the absence of more than one imino resonance. ^{13}C-NMR data acquired with and without proton decoupling revealed only three types of carbons (Table 1), two of which were quaternary, while the other signal derived from a methyl group. Since we were unable to obtain informative GC-MS data on the main component of peak D, electrospray mass spectral analysis was undertaken. Peak matching was used to determine the exact molecular weight to millimass unit accuracy. Consideration of the various molecular formulas consistent with an exact mass of 153.1344 ($C_{10}H_{18}O$, $C_8H_{15}N_3$, H_4N_{10}) yielded only one possible candidate consistent with all spectroscopic data: $C_8H_{15}N_3$. Peak D was therefore identified as succinimidine derivative **6a** in Figure 3. That **6a** was not an oxidation product of thymidine, but rather was derived from ABAP oxidation, was verified by running the reaction in the absence of nucleoside. FPLC analysis of the ABAP oxidation products revealed a major component possessing the same retention time, UV, and NMR as peak D in Figure 1.

UV spectrophotometric analysis of peak E revealed a double maxima at 235 and 280 nm. This suggested the possibility that E possessed a formyluracil chromophore (*29*). NMR in D_2O confirmed the presence of an aldehyde

[3] John E. Shively, personal communication.

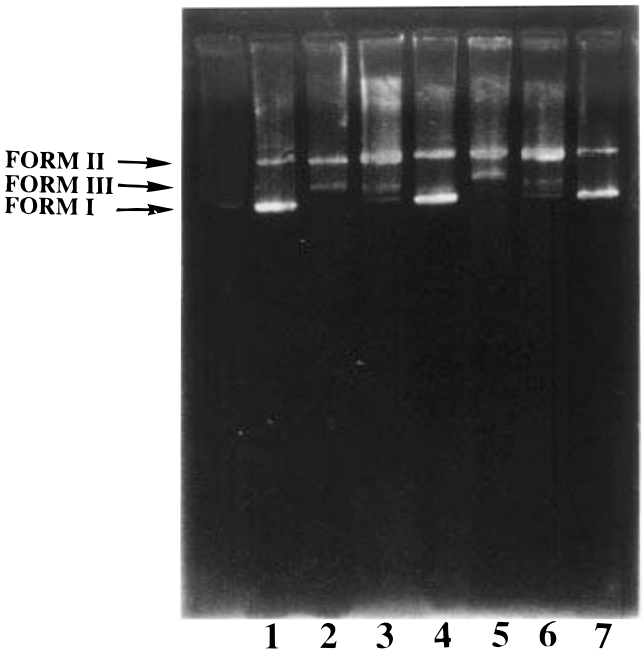

Figure 4. Agarose gel electrophoresis of pGEM9Zf(−) plasmid exposed to varying concentrations of peroxy radical for 60 min. Lane 1, untreated control plasmid; lanes 2 and 5, 1 mM ABAP; lanes 3 and 6, 100 μM; lanes 4 and 7, 1 μM.

proton at 9.2 ppm (s, 1H), supporting this assignment. GC-MS analysis was entirely consistent with the bis-TMS derivative of formyluracil, with a parent ion at m/z = 284 and a characteristic M − CO^+ ion at m/z 256 (Figure 2, bottom panel). Final proof of the assignment of peak E was obtained by coinjection with authentic compound on FPLC.

Peroxy Radical Produces DNA Strand Breaks. In order to examine the ability of peroxy radical to effect chain breaks in DNA, the conversion of supercoiled pGEM9Zf(−) plasmid into Form II (closed circular) and Form III (linear) DNA was examined (Figure 4). Agarose gel electrophoretic analyses of plasmid reactions revealed that 1 mM ABAP in O_2 saturated buffer is sufficient to deplete all detectable supercoiled (Form I) DNA from the reaction, resulting primarily in the formation of linear DNA (lanes 2 and 5). Predominantly double strand breaks, resulting in Form III DNA, with little or no increase in Form II, can be observed at this concentration. At 10-fold lower ABAP concentrations, some supercoiled DNA is still present, but the increase in circular DNA appears greater than formation of linear DNA (lanes 3 and 6).

Discussion

The results described above provide the first direct evidence that peroxy radicals can react with nucleosides to form oxidatively damaged bases and lend support to a proposed role for peroxy radicals in the production of DNA damage *in vivo*. It is significant that the major products resulting from base oxidation of thymidine by peroxy radical (**2c**, **3c** and **5c,** Figure 3) have been previously identified as only minor products resulting from ionizing radiation exposure of either free bases, nucleosides, or DNA (*30*). The major products produced by γ radiolysis of thymine in aqueous solution are saturated pyrimidines formed by the addition of $^{\bullet}OH$ to the 5,6 double bond (*3*−*6*). Although it is possible that small amounts of saturated thymine derivatives are

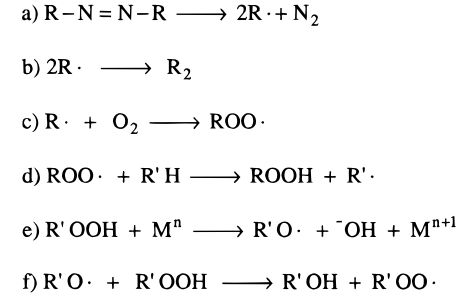

Figure 5. (a−d) Free radical reactions occurring upon the thermolysis of ABAP in the presence of O_2. R is the *sec*-amidinopropane moiety of ABAP. (e and f) Metal ion promoted reduction of hydroperoxides yielding alkoxy and peroxy radicals. R′ is any alkyl group of an organic hydroperoxide.

produced in the ABAP/O_2 reaction, our results clearly show that 5-Me oxidation products are formed predominantly, in contrast to hydroxy radical oxidation.

The available evidence indicates that thymine derived products resulting from oxidation of the 5-Me group are likely of greater importance in mutagenesis than those arising from 5,6 double bond addition. Saturated pyrimidines such as thymine glycol are not mutagenic *in vivo* (*31*), and biochemical experiments have shown that several DNA polymerases either are quantitatively arrested at these lesions, or insert adenine opposite these sites (*32*). In contrast, 5-HMdU has been shown to be mutagenic in several strains of *Salmonella typhimurium* (*33, 34*) and in mammalian cells (*35*). 5-FodU has also been shown to be mutagenic in bacteria (*29*), while its mutation inducing potential in mammalian cells has not been determined. The structural basis for the mutagenicity of products resulting from 5-Me oxidation of thymidine is unclear at the present time, but one possibility is that the normal base pairing arrangements are disrupted by chemical modification, leading to base substitution errors during replication. A possible mechanism for mutagenicity based upon mispairing has recently been proposed in light of the unusual acidity of the N3 proton of 5-formyluracil, although additional biochemical experiments are required to validate this hypothesis (*36*). The mutagenicity of 5-hydroperoxyuracil (5-HPMU) and 5-HPMdU has been recognized for some time, and this effect appears to be greatly enhanced by low valent transition metal ions, particularly Cu^{2+} (*37, 38*). This metal ion promoted mutagenicity can be most readily explained by the formation of reactive radical species formed from reduction of the hydroperoxy moiety (see below). Thus reactions mediated by peroxy radical which favor the production of 5-Me thymine oxidation products may comprise an important route to mutagenic base damage *in vivo*. Moreover, the oxidative depyrimidination observed in the present study may contribute to the formation of mutagenic abasic sites at dT residues in DNA.

Figure 5a−d depicts several reactions of ABAP which occur upon thermal dissociation in the presence of oxygen. Thermolysis of ABAP, depicted in reaction a, produces a tertiary carbon centered radical, the *tert*-amidinopropyl radical. Although dimerization of carbon radicals within the solvent cage has been commonly observed with diazo initiators (reaction b), we were suprised to discover that an intermolecular cyclization to yield a succinimidine derivative **6a** (Figure 3) takes place readily in a pH independent fashion (data not shown). In light of this and additional mechanistic

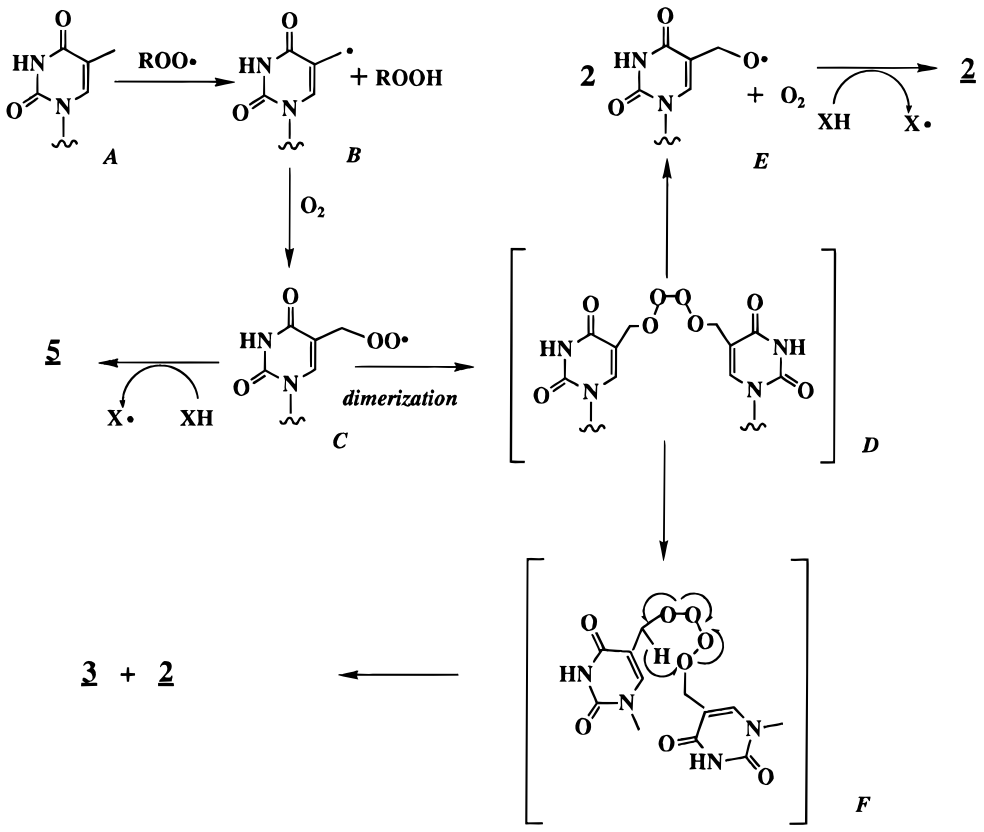

Figure 6. Mechanistic scheme for the formation of base oxidation products produced by reaction with peroxy radical reaction of thymidine. Product numbers correspond to those of Figure 3.

information, it is highly probable that the cyclization proceeds via a free radical rather than an ionic mechanism.[4] To the best of our knowledge, this major side product resulting from the thermolysis of ABAP has not been previously reported. Its characterization should therefore be of interest to those using this water soluble radical initiator, particularly in view of the relatively large amounts formed (Figure 1, peak D).

Radicals which diffuse out of the solvent cage react rapidly with dissolved oxygen to generate the tertiary peroxy radical shown in reaction c. Tertiary peroxy radicals terminate chains several orders of magnitiude more slowly than primary or secondary peroxy radicals (*39*); thus they readily participate in chain propagation reactions such as that shown in d of Figure 5. The position of H abstraction for a given substrate will depend upon the ease of oxidizability by peroxy radical, dictated in large part by the stability of the resulting radical R′•. Peroxy radicals are sufficiently good oxidants ($E° \sim 1000$ mV at pH = 7) to abstract allylic and bis-allylic hydrogens (*40*) and play an important role in the autoxidation of polyunsaturated fatty acids (*41*, *42*).

An additional important route to peroxy radical formation, likely important in biological systems, is the one electron reduction of hydroperoxides by low valent transition metal ions (Figure 5e,f). This reaction intitally provides alkoxy radicals (e). Because they are stronger oxidants (*40*), and hydroperoxides are present in excess, these intermediates may initiate peroxy radical chains via H abstraction from hydroperoxides as depicted in (f). Metal ion promoted reduction of HPMdU is not an important route for the production of alkoxy or peroxy radicals in the present studies, since extreme care was taken to exclude even adventitious metal ions through the use of glass distilled, deionized water and purification of buffer solutions through Chelex-100.

Figure 6 provides a mechanistic rationale for the observed base oxidation products identified in the reaction of peroxy radicals with thymidine. Hydrogen abstraction at 5-Me by peroxy radical yields the 5-methyl-2′-deoxyuridylyl radical (**B**). This is likely the initial reactive thymine intermediate formed by peroxy radical, and its formation is consistent with the ability of peroxy radical to abstract allylic hydrogens. Exposure of thymine to hydroxy radical produces **B** as a minor intermediate, as determined by electron spin resonance at acidic/neutral pH; but at high pH (≥13), this species is formed predominantly (*4*). Consistent with this, the product distribution is observed to shift from saturated thymine products to 5-Me oxidation products with increasing pH (*43*). This is likely the result of increased aromatic character of the thymine dianion resulting from deprotonation at N3 and N1 (*44*), decreasing the susceptibility to nucleophilic attack and favoring a radical with increased benzylic character. In our experiments, however, the reactions of thymidine with ROO• were conducted at slightly acidic or neutral pH, and the use of the nucleoside precludes the possibility for N1 deprotonation. Hence the predominant reaction pathway involving 5-Me H abstraction reflects the selectivity of ROO• rather than any pH induced changes in the ionization state of thymine.

Radical **B** has been shown to be the most stable of several radical species generated from 1-methylthymine in the solid state, much more stable in fact than radical intermediates which result from H^+/e^- addition to the C5/C6 double bond (*45*). The stability of these radicals likely derives in part from the resonance stabilization

[4] M. Martini and J. Termini, manuscript in preparation.

common to allylic radicals. That the 5-methyl-2′-deoxyuridylyl radical can form in DNA has been demonstrated by tritium release from [5-*Me*-^{3}H]thymine containing DNA (*46*). This is not suprising owing to the disposition of the 5-Me group within the major groove of B-DNA, where it would be expected to be accessible to free radical species.

Reaction of **B** with O_2 yields the 5-peroxy radical of thymidine (**C**), which can abstract a suitably activated hydrogen from XH (*e.g.*, ROOH or **A)** to provide **5**, the hydroperoxide of thymidine. **C** may also dimerize in solution to yield tetroxide intermediate **D** (*47*). This may decompose in a manner which terminates the chain (nonradical pathway) or allows for propagation (radical pathway). Disproportionation of this intermediate can provide O_2 and 2 equiv of alkoxy radical **E**, which ultimately yields 5-HMdU in the chain propagating pathway. **E** may react with the *tert*-amidinopropyl radical to provide **4** (Figure 3), which was only detected as the base in trace amounts by GC-MS. An alternative pathway for the formation of **4** involves reaction of the *tert*-amidinopropoxy radical with **B**. Although we cannot discriminate between these two possibilities, the detection of **4** provides direct evidence for some of the mechanistic intermediates proposed in the oxidation of thymidine by peroxy radical.

Alternatively, **D** can decompose via the internal transfer of an α hydrogen to produce 5-HMdU (**2c**), 5-FodU (**3c**), and O_2 via a concerted pathway called the Russell rearrangement (*48*) depicted in **F**. This mechanism predicts the formation of equimolar amounts of carbonyl and hydroxyl compounds in a chain terminating reaction. The occurrence of this 1:1 product stoichiometry upon tetroxide decomposition has been used as evidence in support of this mechanism (*48*, *49*). In the present case, however, nonequivalent amounts of **2c** and **3c** are produced from peroxy radical oxidation of thymidine. If **D** decomposed nonpreferentially through both pathways, a 3:1 ratio of 5-HMdU to 5-FodU would be predicted. Our observed product ratio is closer to 2:1 (Table 1). Some fraction of **E** may decompose via β scission of hydrogen to yield 5-FodU. β scission of the alkoxy radical to yield formaldehyde and the 5-vinyl radical of uracil is unlikely given the unfavorable energy of activation for the latter intermediate (*50*), as well as the lack of detectable formation of 5-oxouracil products.

The oxygen dependent release of thymine from the nucleoside by peroxy radical observed in the present work is consistent with C1′ or C4′ H abstraction. A 1:1 ratio of thymine:ribonolactone would imply the former pathway (*51*), but since we have not analyzed the carbohydrate fraction, we cannot discriminate between these possibilities. However, since abstraction of C1′ H by peroxyl radical would lead to the more stable radical (*52*), we feel this pathway is more likely in light of the propensity of ROO$^\bullet$ to favor the thermodynamic product. It is unclear whether results of studies on the peroxy radical oxidation of nucleosides will bear directly on the mechanism(s) of strand cleavage in double-helical DNA. For example, a thermodynamically favorable C1′ H abstraction in nucleosides by peroxy radicals in solution might be kinetically unfavorable in DNA due to the constraints of B-helix geometry. Additional studies at the polymer level are required to address this point.

The observation of strand breakage by peroxy radical is consistent with its proposed involvement in oxygen dependent backbone cleavage by diverse DNA damaging agents such as bleomycin (*53*) and hydroxy radical (*54*). Peroxy radicals centered on pyrimidine bases can similarly initiate strand breaks in an intramolecular fashion, by mediating H atom abstraction from an adjacent sugar moiety within DNA (*55*). Peroxy base radicals are readily formed during radiolysis of aerobic solutions of DNA by diffusion controlled addition of oxygen to carbon radicals resulting from hydroxy radical addition (*56*). The reactivity of $^\bullet$OH with the carbohydrate moiety of poly(U) is too low to account for the observed yield of strand breaks, with >90% of hydroxy radical adding to the 5,6 double bond of uracil to produce base radicals (*57*). Time resolved measurements show that the half-life for strand breaks in poly(U) initiated by hydroxy radicals under aerobic conditions is coincident with the rate of decay of base peroxy radicals (*54*, *55*), *i.e.*, several seconds (*58*). Base peroxy radicals generated *in situ* at a unique thymine residue (at C5) within a polydeoxynucleotide have been shown to give rise to strand breaks, providing direct support for this hypothesis (*59*). Whether 5-Me thymine peroxy radicals such as those implicated in this study play a role in strand breakage will be the subject of future study.

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