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Formation of 8-Hydroxy(deoxy)guanosine and Generation of Strand Breaks at Guanine Residues in DNA by Singlet Oxygen[†]

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ABSTRACT: Singlet molecular oxygen (¹O₂) was generated in aqueous solution (H₂O or D₂O) at 37 °C by the thermal dissociation of the endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate (NDPO₂). Guanosine and deoxyguanosine quench ¹O₂ with overall quenching rate constants of 6.2 × 10⁶ M⁻¹ s⁻¹ and 5.2 × 10⁶ M⁻¹ s⁻¹, respectively. Reaction with ¹O₂ results in the formation of 8-hydroxyguanosine (8-OH-Guo) and 8-hydroxydeoxyguanosine (8-OH-dGuo), respectively, with a yield of 1.5% at 1 mM substrate with an NDPO₂ concentration of 40 mM; a corresponding 8-hydroxy derivative is not formed from deoxyadenosine. In D₂O the yield of 8-OH-Guo is 1.5-fold that in H₂O. Sodium azide suppresses 8-OH-Guo and 8-OH-dGuo production. In contrast, the hydroxyl radical scavengers, *tert*-butanol, 2-propanol, or sodium formate, do not decrease the production of the 8-OH derivatives. The formation of 8-OH derivatives is significantly increased (2-5-fold) by thiols such as dithiothreitol, glutathione, cysteine, and cysteamine. With use of a plasmid containing a fragment of the mouse metallothionein I promoter (pMTP3') and a novel end-labeling technique, the position of 'O₂-induced single-strand breaks in DNA was examined. Strand breaks occur selectively at dGuo; no major differences (hot spots) were observed between individual guanines.

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Electronically excited molecular oxygen (singlet oxygen, ¹O₂)¹ may be generated by photochemical reactions through transfer of excitation energy to ground-state oxygen (³O₂) from suitable excited triplet-state sensitizer (photoexcitation). It can also be produced in biological systems by dark reactions (chemiexcitation), e.g., in lipid peroxidation, and by enzyme actions such as those catalysed by lactoperoxidase, lipoygenase, and chloroperoxidase (Cadenas & Sies, 1984; Anufsky, 1989). ¹O₂ is produced during photooxidation of a variety of biological compounds and xenobiotics. Since ¹O₂ is relatively long-lived, with half-times in the range of milliseconds, considerable diffusion of singlet oxygen is possible with a radius estimated to be in the range of 100 Å (Schnuriger & Bourdon, 1968; Moan, 1990).

Singlet oxygen has been shown to be capable of inducing DNA damage and to be mutagenic (for review, see Piette, 1990). The guanine moiety has been observed to become hydroxylated at C8 on photolysis of oxygenated DNA solutions in the presence of the sensitizer methylene blue (Floyd et al., 1989). It was concluded that ¹O₂ is the species responsible for this reaction, in agreement with the fact that ¹O₂ reacts preferentially with free guanine nucleotides (Piette & Moore, 1982; Cadet et al., 1983; Kawanishi et al., 1986). Hence, it is of interest to ascertain whether ¹O₂ is capable of forming

8-OH derivatives with guanines and study possible factors influencing their formation. 8-Hydroxylation of guanine has also been identified as an important process in OH[•] radical induced damage to DNA (Floyd et al., 1986, 1988; Kasai et al., 1986; Aruoma et al., 1989). 8-OH-dGuo is a reaction product that can be easily measured and that has therefore been used as an indicator of oxidative DNA damage in vivo (Floyd et al., 1986; Kasai et al., 1986).

The 'O₂-induced damage to DNA also leads to strand breaks (Wefers et al., 1987; Di Mascio et al., 1989a, 1990). Decuyper-Debergh et al., (1987) have shown that 'O₂-induced mutagenicity results from single nucleotide substitutions occurring predominantly at the guanosine residues. However, the method is indirect since it involves measuring base substitutions after repair. Using a newly developed method that allows the detection of end-labeled nicked fragments on sequencing gels, we assign here the base position at which 'O₂-induced single-strand breaks occur in plasmid DNA and check the possibility of the occurrence of "hot spots".

Recent studies (Rougee et al., 1988; Kaiser et al., 1989; Devasagayam et al., 1991a) have shown that thiols and sulfur-containing amino acids quench ¹O₂. Interestingly, if performed in the presence of DNA, this quenching is accompanied by a large increase in the number of strand breaks

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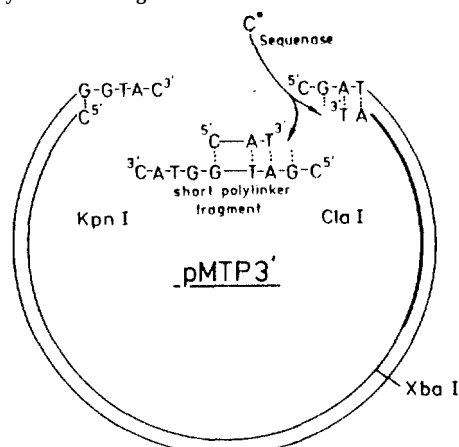
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¹ Abbreviations: ¹O₂, singlet molecular oxygen; NDP, 3,3'-(1,4-naphthylidene) dipropionate; NDPO₂, endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate; EDTA, ethylenediaminetetraacetic acid; DETAPAC, diethylenetriaminepentaacetic acid; OH[•], hydroxyl radical; 8-OH-Guo, 8-hydroxyguanosine; 8-OH-dGuo, 8-hydroxydeoxyguanosine; MTP, metallothionein promoter.

Scheme 1: Plasmid pMTP3' Used for Detection of Single-Strand Breaks by End Labeling



The fragments resulting from pMTP3' after cleavage with *Kpn*I and *Cla*I are shown. Only 3'-recessed ends can be labeled by Sequenase 2.0. C* indicates [α - 32 P]dCTP. The thick line indicates the strand of the metallothionein promoter in which single-strand breaks are detectable over a range of about 300 b (see Figure 6).

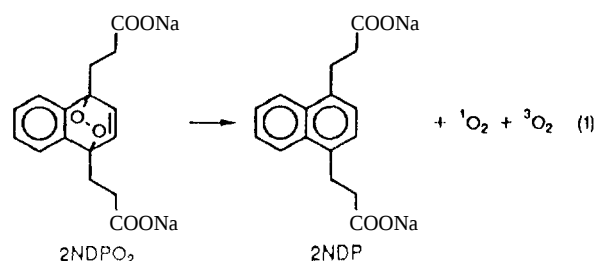
(Devasagayam et al., 1991b). On the other hand, thiols were reported to decrease oxidative damage to DNA induced by ionizing radiation (Fahey, 1988; Held, 1988) and mutagenicity induced by oxygen radicals (De Flora et al., 1989). Conversely, thiols can damage DNA in the presence of metal ions (Reed & Douglas, 1989) or can be mutagenic under certain conditions (Glatt et al., 1983; Ross et al., 1986; Glatt, 1989). Therefore, in view of this complex effect of thiols on the results of oxidation reactions it was of interest to study $^1\text{O}_2$ -induced changes in the guanine (deoxy)nucleosides.

EXPERIMENTAL PROCEDURES

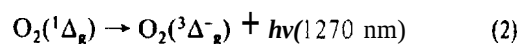
Reagents. Deuterium oxide (99.8%), DL-dithiothreitol, DL-cysteine, glutathione, deoxyguanosine, deoxyadenosine, and diethylenetriaminepentaacetic acid (DETAPAC) were from Sigma. Guanosine was from Ega-Chemie. Cysteamine hydrochloride was purchased from Schuchardt. All other chemicals were from Merck. Water used in our studies was purified by Millipore filtration. The endoperoxide of the disodium salt of 3,3'-(1,4-naphthylidene) dipropionate (NDPO₂) was prepared as described (Di Mascio & Sies, 1989). The product was identified by ^1H NMR and IR spectroscopy. Restriction enzymes were from Boehringer Mannheim and were regularly checked for endonuclease activity.

Plasmids. Plasmid pBR322 DNA was prepared by use of the Qiagen Plasmid kit, and the effect of NDPO₂ on single-strand break formation was studied as described previously (Di Mascio et al., 1989a). The 850-bp *Eco*RI-*Bgl*II fragment from pMTSV (kindly provided by Dr. R. D. Palmiter, Seattle) comprising the murine metallothionein 1 promoter (Stuart et al., 1984) was cloned into the *Eco*RI and *Bam*HI site of the polylinker of pBluescript KS+ to yield pMTP. pMTP3' was obtained from pMTP by deleting the *Eco*RI-*Bst*EII-(5') fragment and religating the fragments after filling in with Klenow polymerase. This plasmid thus contains a fragment of the mouse MT-I promoter flanked by several additional restriction sites (thick line in Scheme 1).

Generation and Quenching of $^1\text{O}_2$ by Guanosine and Deoxyguanosine. Singlet oxygen was generated by the thermal dissociation of the endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate (NDPO₂) yielding 3,3'-(1,4-naphthylidene) dipropionate (NDP), $^1\text{O}_2$, and triplet molecular oxygen (Di Mascio & Sies, 1989) (reaction 1).



The solvent used to estimate quenching constants was 50 mM sodium phosphate buffer in D₂O, pD 7.4, pD being taken as the pH measured with a glass electrode + 0.4 pH units (Salomaa et al., 1964). At 37 °C, 3 mL of the buffer was placed in a thermostated, closed glass cuvette. A total of 15–30 μL of an NDPO₂ stock solution in D₂O was added, to give a final concentration of 5 mM NDPO₂. The resulting infrared photoemission of $^1\text{O}_2$ (reaction 2) was monitored at 1270 nm



with use of a germanium diode photodetector (Di Mascio & Sies, 1989). At the maximum of the monomol emission intensity, achieved within 5–6 min, 10–50 μL of the freshly prepared quencher (guanosine or deoxyguanosine) in deaerated 50 mM sodium phosphate buffer in D₂O, pD 7.4, was added and the resulting intensity was recorded. The overall quenching constant $k_q + k_r$ was calculated from Stern–Volmer plots of the dependence on guanosine or deoxyguanosine concentrations of the emission intensities at 1270 nm (see Figure 1) and the $^1\text{O}_2$ decay constant of $1.9 \times 10^4 \text{ s}^{-1}$, as determined according to Valduga et al. (1988) using photoexcitation of methylene blue as $^1\text{O}_2$ source and time-resolved spectroscopy (courtesy of Professor S. Braslavsky and Dr. D. Mártire, Mulheim, F.R.G.).

Formation of 8-OH-Guo and 8-OH-dGuo with $^1\text{O}_2$. The reaction mixture for the assay contained 1 mM of guanosine or deoxyguanosine in 50 mM sodium phosphate buffer in 40% D₂O, pD 7.4, at 37 °C. The reaction was started by adding NDPO₂ at a final concentration of 40 mM and stopped by adding 10 mM sodium azide. The 8-OH derivatives formed were identified and quantitated by use of high-pressure liquid chromatography on a Nucleosil-5-C₁₈ (4.6 $\times$ 125 mm) column with optical and electrochemical detection. The mobile phase used for the separation of 8-OH-Guo was 2 mM KH₂PO₄/20 mM NaClO₄/4% methanol, pH 4.0, at a flow rate of 0.8 mL/min; for 8-OH-dGuo methanol was 10%. Under these conditions, the retention times for 8-OH-Guo and 8-OH-dGuo were 17 and 21 min, respectively. The absorption spectra and retention times of the 8-OH-Guo and 8-OH-dGuo produced by $^1\text{O}_2$ were compared to those obtained by reaction with radiation-chemically produced hydroxyl radicals (Dizdaroglu, 1985; Candeias and S.S., unpublished results) and found to be the same and identical, respectively. The absorption spectrum of 8-OH-dGuo produced by $^1\text{O}_2$ was also compared to that of the compound as described by Culp et al. (1989) and to that of the authentic 8-OH-dGuo kindly provided by Dr. H. Kasai, National Cancer Research Center, Tokyo, Japan. Calibration of the yield of 8-OH-dGuo was performed by measuring the absorption of 8-OH-dGuo at 293 nm and taking $\epsilon(293 \text{ nm}) = 9700 \text{ M}^{-1} \text{ cm}^{-1}$ (Culp et al., 1989), using 8-bromoguanine as an internal standard and taking $\epsilon(250 \text{ nm}) = 13940 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon(293 \text{ nm}) = 4850 \text{ M}^{-1} \text{ cm}^{-1}$. The electrochemical detector was operated at 0.7 V.

Generation of Single-Strand Breaks in Plasmid DNA. Single-strand breaks were generated by incubating 200 μg of plasmid pMTP DNA/mL with 40 mM NDPO₂ in 50 mM

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sodium phosphate buffer, pH 7.4 in 80% D₂O, at 37 °C (Di Mascio et al., 1989a). DNA samples incubated with NDP were taken as controls. Reactions were terminated by the addition of 10 mM sodium azide and by immersing the samples in ice. Single-strand breaks were detected by electrophoresis on 0.8% agarose gels. In a typical 2-h incubation, around 50% of supercoil was converted to relaxed circle as compared to 5–10% in controls.

Analysis for Position of Single-Strand Breaks. After the induction of single-strand breaks, the reaction mixture was dialyzed against STE buffer (100 mM sodium chloride, 10 mM Tris, 1 mM EDTA, pH 7.5) for 72 h with several changes. In some experiments, DNA was concentrated by precipitation with ethanol before digestion with restriction enzymes. To label the 3' end of the nicked fragment of DNA specifically, two consecutive restriction digests were performed. In the plasmid pMTP3' (Scheme 1), digestion with 3 units of *Clal*/μg of DNA produces two 5' protruding ends. Digestion with 3 units of *KpnI*/μg of DNA cleaves off a short piece of polylinker leaving behind a 3' protruding end on the remainder of the plasmid. End labeling at 3' recessed ends by incubation with 5 μCi of [α -³²P]dCTP/μg and 2 units of Sequenase 2.0 (USB, Cleveland, OH) therefore occurs on a short specific 27-base fragment from the polylinker and on fragments resulting from single-strand breaks in the metallothionein promoter insert (thick line in Scheme 1). Sequenase 2.0 lacks exonuclease activity and does not label at the strand breaks themselves. The labeling reaction was terminated by addition of EDTA (20 mM), and the reaction mixture was either used directly or further purified and concentrated by phenol extraction and ethanol precipitation before loading onto a 6% polyacrylamide/5M urea gel in TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). Total radioactivity loaded was similar for NDP control and NDPO₂-treated samples. On every gel, sequencing lanes of the promoter insert were run in parallel for the identification of the nicked sites. Sequencing reactions were performed by the Sanger method with use of Sequenase and ³⁵S-labeled deoxynucleotide triphosphates and MI3 reverse primer. Gels were dried and exposed for various times for Kodak films. Autoradiographs were scanned by employing an LKB laser densitometer with GSXL software.

RESULTS

Singlet Oxygen Quenching by Guanosine and Deoxyguanosine. The quenching of monomol photoemission by ¹O₂ by guanosine is shown in Figure 1A. Figure 1B shows the Stern–Volmer plot, the overall singlet oxygen quenching rate constant, $k_q + k_r$, being $6.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, where k_q is the physical quenching rate constant and k_r is the chemical reaction rate constant. The value for the overall quenching rate constant of deoxyguanosine is $5.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This number is almost identical with that ($5.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) measured for 2'-deoxyguanosine 5'-monophosphate by use of time-resolved ¹O₂ emission decay techniques (Lee & Rogers, 1987).

Formation of 8-OH Derivatives by Singlet Oxygen. HPLC tracings of the formation of 8-hydroxy(deoxy)guanosine as measured by electrochemical and UV detection are shown in Figure 2. 8-OH-Guo formation due to exposure to guanosine to NDPO₂-generated ¹O₂ in D₂O buffer is compared to H₂O buffer in Figure 2 and Table I, the yield being higher in D₂O by a factor of 1.5. Enhanced formation of D₂O is attributed to a larger half-life of singlet oxygen in D₂O (Monroe, 1985). ¹O₂ also induced the formation of 8-OH-dGuo from deoxyguanosine (Figure 2, right-hand traces, and Table I). However, when deoxyadenosine was exposed to ¹O₂, there was no

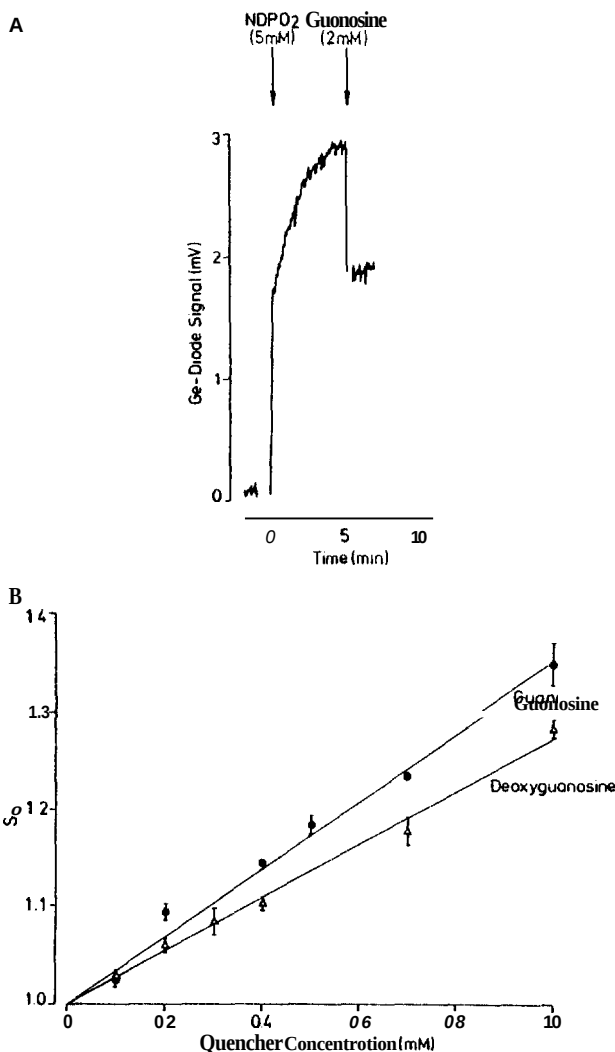


FIGURE 1: (A) Quenching of NDPO₂-generated singlet oxygen monomol photoemission at 1270 nm by guanosine. (B) Stern–Volmer plots for the quenching of singlet oxygen by guanosine (●) and deoxyguanosine (▲) in 50 mM sodium phosphate buffer in D₂O, pH 1.4.

Table I: Formation of 8-OH-Guo by NDPO₂-Generated Singlet Oxygen^a

additions	time (min)	8-OH-Guo (μM)	8-OH-dGuo (μM)
none	60	nil	nil
NDP	60	nil	ND ^b
NDPO ₂ , D ₂ O buffer	0	2.0	nil
	60	15.6	13.6
NDPO ₂ , H ₂ O buffer	0	1.6	ND
	60	10.3	ND

^a A 1 mM solution of guanosine or deoxyguanosine was exposed to 40 mM NDP or NDPO₂ in 50 mM sodium phosphate buffer in D₂O (pH 7.4) or H₂O (pH 7.4). The reaction mixture was incubated at 37 °C, and the reaction was stopped by adding 10 mM sodium azide. The 8-OH-Guo or 8-OH-dGuo formed was determined as described in Experimental Procedures. ^b Not determined.

formation of the corresponding 8-OH product, in agreement with similar results of Cadet et al. (personal communication). Production of 8-hydroxydeoxyadenosine, however, occurs readily by radiation-chemically generated OH[•] radicals (Cadet & Berger, 1985; Dizdaroglu, 1985; Dizdaroglu & Bergtold, 1986; Steenken, 1989; Dizdaroglu & Gajewsky, 1990). Table I also shows that there is no formation of 8-OH-Guo when guanosine is incubated at 37 °C in the presence of NDP.

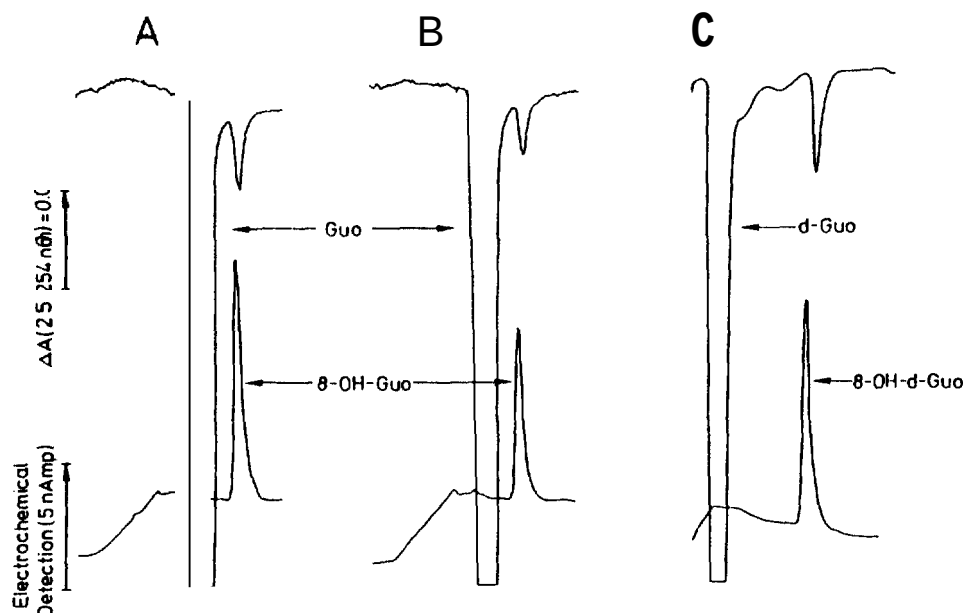


FIGURE 2: Detection by HPLC of the 8-OH-Guo (A, B) and 8-OH-dGuo (C) formation from Guo or dGuo. Singlet oxygen was generated from NDPO_2 (40 mM) in 50 mM sodium phosphate buffer, pH 7.4 in D_2O (A, C) or H_2O (B). Detection was by UV absorbance (top) and electrochemistry (bottom).

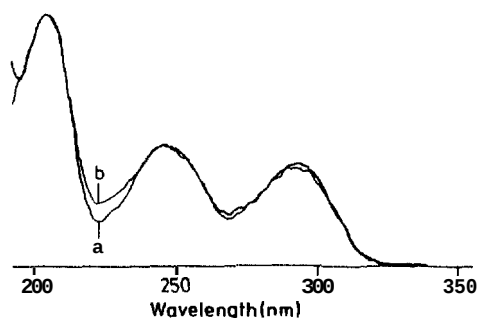


FIGURE 3: Absorption spectrum of 8-OH-dGuo (b) produced by singlet oxygen from NDPO_2 as compared to authentic 8-OH-dGuo (a).

Figure 3 shows that the UV absorption spectrum of 8-OH-dGuo produced by $^1\text{O}_2$ and that of the authentic reference compound are practically identical. The absorption spectra of 8-OH-dGuo and 8-OH-Guo produced by $^1\text{O}_2$ and $\text{OH}^\cdot$ are also similar (not shown). On the basis of 8-bromoguanine as internal standard and with use of optical detection, the concentration of 8-OH-dGuo formed from the incubation of 1 mM deoxyguanosine with 40 mM NDPO_2 was determined to be 15 μM , corresponding to a yield of 1.5% on the basis of deoxyguanosine.

Sodium azide (2 mM) significantly decreases the yield of 8-OH-Guo as well as that of 8-OH-dGuo whereas the $\text{OH}^\cdot$ scavengers *tert*-butanol (100 mM), 2-propanol (100 mM), or sodium formate (10 mM) are ineffective. The metal chelators EDTA and DETAPAC likewise have no effect, suggesting that metal ions are not involved in the formation of the 8-OH derivatives.

The time course of $^1\text{O}_2$ -induced formation of 8-OH-dGuo is similar to that of single-strand breaks in plasmid pBR322 DNA (Di Mascio et al., 1989a; Devasagayam et al., 1991b) (Figure 4). The formation of 8-OH-Guo observed decreases from a plateau at pH 6–7 to <10% at pH 9 (Figure 5).

Modulation of Singlet Oxygen Induced 8-OH-Guo Formation by Thiols. Thiols significantly increase the $^1\text{O}_2$ -induced formation of 8-OH-Guo or 8-OH-dGuo (Table II). The enhancing effect of thiols, as exemplified by dithiothreitol, was not significantly altered by the $\text{OH}^\cdot$ scavengers *tert*-butanol

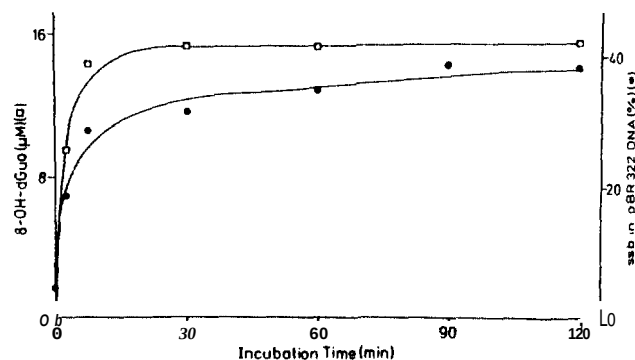


FIGURE 4: Time course of formation by singlet oxygen of 8-OH-dGuo and single-strand breaks. Deoxyguanosine (1 mM) or plasmid pBR322 DNA (2 μg) was exposed to 40 mM NDPO_2 in 50 mM sodium phosphate buffer in D_2O (pH 7.4) at 37 $^\circ\text{C}$.

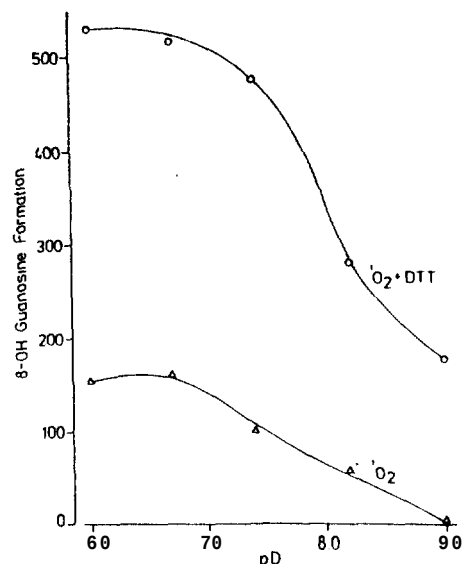


FIGURE 5: pH dependence of singlet oxygen induced (A) and singlet oxygen/thiol (O₂·DTT) induced 8-hydroxyguanine formation. Conditions are as in Figure 4; incubation was for 1 h at 37 $^\circ\text{C}$. The value obtained with $^1\text{O}_2$ at pH 7.4 (15.2 μM) was taken as 100%. Dithiothreitol (DTT) concentration was 10 mM.

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Table II: Modulating Effect of Thiols on Singlet Oxygen Induced Formation of 8-OH-(d)Guo^a

thiol	8-OH-Guo (μM)	8-OH-dGuo (μM)
no additions	14.7	15.2
dithiothreitol	69.7	64.4
glutathione	59.1	66.3
cysteine	41.3	ND
cysteamine	31.5	ND

^aThe incubation medium contained 1 mM guanosine or deoxyguanosine, 40 mM NDPO₂, and 10 mM of the respective thiols in 50 mM sodium phosphate buffer in D₂O, pD 7.4. Incubation was for 1 at 37 °C.

(100 mM) or sodium formate (10 mM) or by metal chelators EDTA (1 mM) or DETAPAC (1 mM). Evidence for non-involvement of radicals in the sensitization by thiols is the fact that ascorbate, a scavenger of thiol peroxy radicals (RSOO[•]) (Tamba et al., 1986), left the enhancing effect of dithiothreitol almost unchanged. The modulating effect of thiols on ¹O₂-induced 8-OH guanosine production is dependent on the pD of the buffer used (Figure 5).

Characterization of ¹O₂-Induced Single-Strand Breaks in Plasmid DNA. Single-strand breaks induced in plasmid DNA were further characterized by a newly developed end-labeling method described in Experimental Procedures. Since each single-strand fragment carries the same amount of label after end labeling, the intensity of the bands on the sequencing gels is proportional to their overall abundance (Figure 6). The gel resolves fragments up to 300 bases 5' to the *Cla*I site. Single-strand breaks induced by NDPO₂ can be identified by comparison to the single-strand break background in the NDP control lane. This background level proved critical for the method. All reagents employed, particularly batches of restriction enzymes, had to be carefully checked for nicking activity. For the same reason, precipitation steps were kept to a minimum. The positions where nicks have occurred can be identified by comparison to the sequencing reactions run in parallel on the gel. The sequencing products contain primer and originate outside the polylinker and are therefore 96 bases longer (when the *Cla*I site is used for labeling) than the corresponding single-strand fragments (see dotted lines in Figure 6).

The result is that ¹O₂-induced single-strand breaks occur selectively at guanines. At 120 min of treatment, all Gs were detectable on the gels and no major variation was visible. A similar pattern was already obtained after 10 min of ¹O₂ treatment (Figure 7), indicating no major differences in the reactivity of individual Gs or parts of the metallothionein promoter sequence. However, a close comparison of the densitometer scans shown in Figure 7 reveals that one individual G reacts slowly (arrow). By the present method, "hot spots" comparable to those obtained with alkylating agents (Nehls et al., 1984) were not detected within the metallothionein promoter sequence although it contains several CG-rich regions as well as parts with lower CG content.

DISCUSSION

Reactivity of ¹O₂ with dGuo and Guo. In DNA only the guanine moiety appears to have an appreciable reactivity toward singlet oxygen. The rate constants of singlet oxygen reaction with DNA and dGMP, determined from the measurements of the first-order decay constants of the infrared luminescence of ¹O₂, generated by photooxidation of Rose Bengal, were found to be $5.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $5.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ respectively (Cadet et al., 1985). With the nucleosides, we have obtained the overall quenching constants of $6.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $5.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for Guo and dGuo, respectively.

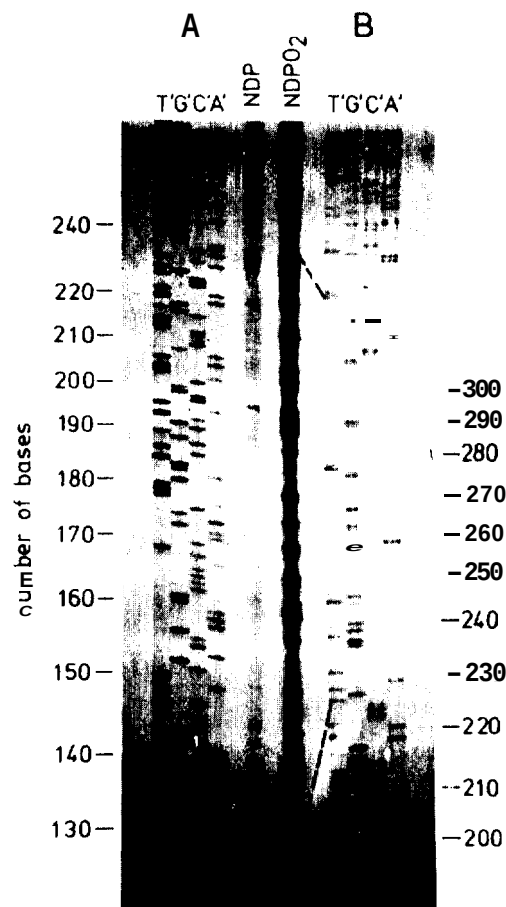


FIGURE 6: Autoradiograph of pMTP3' DNA end labeled after treatment with NDPO₂ and NDP for 120 min (central lanes) as compared to dideoxynucleotide sequencing products. The number of bases indicates the length of fragments. Since the Sanger sequencing products represent the strand opposite from the 3'-end-labeled NDPO₂-induced fragments, the complementary bases (T', G', C', A', corresponding to the end-labeled strand) are indicated. The sequencing mixtures were loaded with the NDPO₂-treated DNA (A) and 30 min earlier (B). Since the Sanger products are 96 bases longer, the fragments in B run approximately alongside the corresponding nicked fragments (matched by dotted lines). The sequence between 226 and 330 indicated in the sequencing lanes in B corresponds to bases 215 to 111 upstream of the MT-I transcriptional start site.

These values are similar to that for dGMP but are much lower than those of carotenoids or tocopherols, which are on the order of 10^{10} and $10^8 \text{ M}^{-1} \text{ s}^{-1}$, respectively (Foote et al., 1968, 1974; Yamauchi & Matsushita 1977; Di Mascio et al., 1989b; Kaiser et al., 1990).

The present study shows that guanosine and deoxyguanosine when exposed to NDPO₂-generated singlet oxygen produce significant amounts of 8-OH derivatives, indicators of oxidative DNA damage (Floyd et al., 1986, 1988). The formation of ¹O₂-induced 8-OH-Guo does not seem to involve metal ions as shown by lack of inhibition by metal chelators. A similar observation also has been made with singlet oxygen induced strand breaks in plasmid DNA (Devasagayam et al., 1991b). OH[•] radicals are also not involved, as evidenced by the lack of effect of the OH[•] scavengers tert-butanol, 2-propanol, and sodium formate. The enhancing effect of deuterated buffer as well as inhibition by azide points to ¹O₂ as the species responsible. This conclusion is in agreement with the interpretation by Floyd et al. (1989) and Ravanat et al. (1991), who have shown that methylene blue or phthalocyanine plus light induces the formation of 8-hydroxydeoxyguanosine.

Ravanat et al. (1991) also studied the specific singlet oxygen oxidation products of 3',5'-di-O-acetyl-2'-deoxyguanosine using

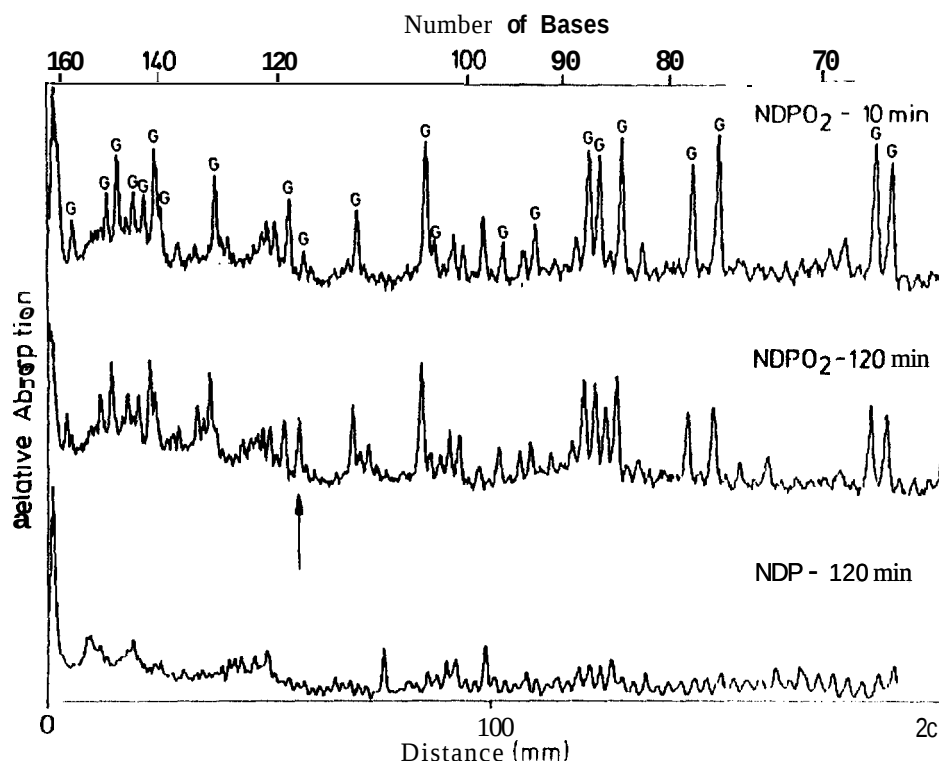
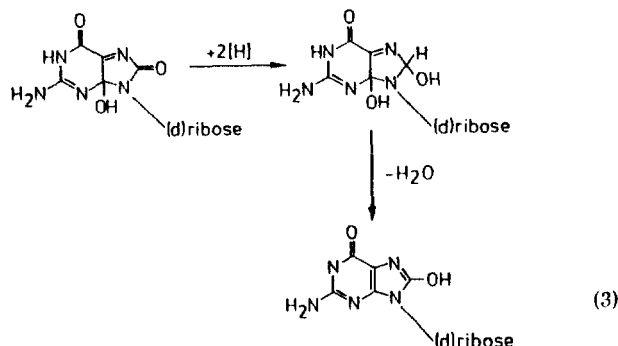


FIGURE 7: Laser densitometer scans of the autoradiograph of the gel with pMTP3' DNA treated with NDPO, for 10 and 120 min and NDP for 120 min. The G signal at position 117 differs significantly between the 10- and 120-min lanes (arrow).

$^{18}\text{O}_2$. One of the main products characterized was a 4-hydroxy-8-oxoguanosine derivative, which was postulated to be formed via an unstable endoperoxide as an intermediate. It is possible that such a type of endoperoxide is an intermediate also in the formation of 8-OH-Guo or 8-OH-dGuo. For the endoperoxide to give the 8-hydroxypurine, two reducing equivalents (2H) have to be supplied.

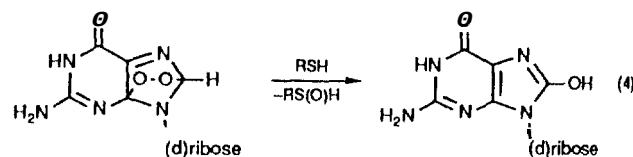


Strand Break Formation. The mechanism of $^1\text{O}_2$ -induced strand break formation is unknown. Formation of single-strand breaks and formation of 8-hydroxydeoxyguanosine initially occur with similar kinetics (Figure 4). Schneider et al. (1990) have reported that single-strand nicking occurs approximately 17-fold less frequently than does formation of 8-hydroxydeoxyguanosine. These results are inconsistent with the idea that 8-hydroxydeoxyguanosine causes strand breaks. It may, however, be possible that 8-hydroxydeoxyguanosine and strand breaks are derived from a common precursor, possibly the endoperoxide.

Thiol Effects. Thiols enhance the $^1\text{O}_2$ -induced formation of 8-OH derivatives significantly. This enhancement was seen with dithiothreitol (Figure 5) and with glutathione, cysteine, and cysteamine, which occur at up to millimolar concentrations in biological tissues. This effect is in contrast to the normally found and well-documented antioxidant and radioprotective

role of thiols (Fahey, 1988; Sies, 1989). It is interesting that thiols have also an enhancing effect on the formation of strand breaks (Devasagayam et al., 1991b).

It may be argued that the observed enhancement by thiols results from the formation of reactive radical species. However, our studies using scavengers do not support this argument. Sevilla et al. (1990), who studied the reaction of thiols with molecular oxygen, have postulated the sequential formation of the thiyl radical ($\text{RS}^\bullet$), the thiol peroxy radical ($\text{RSOO}^\bullet$), the sulfonyl radical ($\text{RSO}_2^\bullet$), and the sulfonyl peroxy radical ($\text{RSO}_2\text{OO}^\bullet$). Since these radicals are likely to be scavengable by ascorbate (Tamba et al., 1986), the observation (vide supra) that the sensitizing effect of thiols on the formation of 8-hydroxy(deoxy)guanosine cannot be inhibited by ascorbate indicates that these radicals are *not* involved in the formation of the $^1\text{O}_2$ -induced damage. This conclusion is supported by the observation (S.S., unpublished results) that on production of thiol peroxy radicals by radiation-chemical methods hydroxylation of the 8-position of (deoxy)guanosine does not occur. It is therefore tentatively suggested that the sensitization is by covalent as opposed to radical pathways. One possibility would be by interaction with the peroxide suggested by Cadet et al. (1990), as shown below.



However, regarding single-strand break formation, free radicals could be involved in the extra damage generated by thiols, because this effect was partially counteracted in pBR322 by radical scavengers (Devasagayam et al., 1991b). To summarize, it appears that of the two effects of $^1\text{O}_2$ on DNA studied here, i.e., strand break formation and hydroxylation at C8 of guanine, the latter is not the cause of the former,

although the strand breaks do occur at the guanines. Further studies are necessary in order to understand the mechanism of these and related phenomena.

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Registry No. NDP, 18071-16-2; 8-OH-Guo, 3868-31-3; 8-OH-dGuo, 88847-89-6; Guo, 18-00-3; dGuo, 961-07-9; O₂, 7782-44-7; DL-dithiothreitol, 27565-41-9; DL-cysteine, 3374-22-9; glutathione, 70-18-8; cysteamine, 60-23-1.

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