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INABILITY OF CHEMICALLY GENERATED SINGLET OXYGEN TO BREAK THE DNA BACKBONE

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The capacity of a photodynamic and a chemical source of singlet molecular oxygen to cause DNA strand breakage at pH 7.8 was compared in the following systems: (1) dissolved rose bengal plus light (400 – 660 nm), (2) a novel water-soluble naphthalene-derived endoperoxide showing temperature-dependent singlet oxygen release, in the absence of light. Covalently closed circular DNA was efficiently converted to the open (relaxed) form upon exposure to dissolved rose bengal plus light in a time-dependent reaction, showing that this system was capable of causing DNA strand breakage at pH 7.8. The reaction was greatly reduced under hypoxic conditions (< 5 p.p.m. O_2), was stimulated when using D_2O instead of H_2O as a solvent and was not inhibitable by superoxide dismutase, indicating that singlet oxygen was a critical intermediate. However, comparatively large fluxes of singlet oxygen generated by the endoperoxide completely failed to produce DNA strand breaks. We conclude that, although singlet oxygen seems to play a role in DNA strand breakage by rose bengal plus light, singlet oxygen *per se* is very inefficient if not completely incapable of causing DNA strand breakage.

Key words: DNA strand breakage; oxidative DNA damage; photosensitization; rose bengal; singlet oxygen

INTRODUCTION

The participation of activated oxygen species, i.e. superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), in the production of genetic damage by ionizing radiation has been known for a long time[8,11]. The possibility that activated oxygen species produced by normal aerobic metabolism[9,13] may contribute to "spontaneous" genetic damage has become increasingly recognized[1]. Indeed, the majority of human cancers has been suggested to result from failing cellular defenses against the genotoxic action of oxygen becoming activated during normal or disturbed metabolic processes[18,47]. Brawn and Fridovich[4] have recently demonstrated

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single strand breakage of covalently closed circular DNA by an enzymatic source of $O_2^{\mp}$, a reaction that was shown to be inhibitable by superoxide dismutase, catalase and a Fe^{2+}-chelating agent, implicating the $OH^{\cdot}$ radical as the ultimate DNA-damaging agent.

Recent work[6,21,23,49] has provided evidence that singlet ($^1\Delta g$) molecular oxygen ($^1O_2$) should also be considered as a product of normal enzymatic reactions. Because of its relatively long life time, which has been estimated to be 2–4 μsec in H_2O and 20–55 μsec in D_2O,[22,25,39] $^1O_2$ can travel several μm in aqueous solutions before being quenched[14,29]; therefore $^1O_2$ generated in cytoplasm, endoplasmic reticulum or nuclear envelope of a eukaryotic cell might be able to interact with DNA in the nucleus or in mitochondria. Several observations are in line with the hypothesis of singlet oxygen being a potential carcinogen: (i) singlet oxygen quenchers such as the carotenoids and urate are supposed to be anticarcinogens[1,5]; (ii) cells from patients with Fanconi anaemia, a cancer-prone hereditary disorder, exhibit an increased level of "spontaneous" chromosomal breakage and an enhanced sensitivity to the chromosome breaking effect of O_2[19] and D_2O[20], suggesting the involvement of metabolically produced singlet oxygen in the generation of "spontaneous" genetic damage.

Another potentially important source of singlet oxygen in animal cells is presented by photodynamic sensitizer dyes, which generate $^1O_2$ upon exposure to visible light[42,46]. A substantial amount of work has been devoted to the characterization of photodynamic sensitizers acting upon linear molecules of DNA, which studies have shown that deoxyguanosine is specifically attacked in such a way that alkali-labile sites are produced which become manifest as strand breaks upon exposure to alkaline (pH > 12) conditions[3,7,16,37,41,45,46]. These lesions seem to be rapidly repaired in human cells.[27,28,38] Fiel *et al.*[12], using covalently closed circular DNA molecules as a probe, have shown that several photodynamic porphyrins can cause single strand DNA breakage directly at pH 8.2 (*without* alkaline treatment) when exposed to light of 360–390 nm; this reaction was inhibited by the singlet oxygen quencher sodium azide. It has recently been suggested that a significant part of the mutagenicity of 8-methoxypsoralen plus light in *Escherichia coli* is due to the $^1O_2$-generating capacity of this dye[34]. Indeed, 8-methoxypsoralen covalently bound to DNA was even more effective in generating $^1O_2$ than free dye[35]. Gruener and Lockwood[15] found that immobilized rose bengal plus visible light induced mutations in mammalian tissue culture cells, while visible light alone was also somewhat mutagenic; both effects were amplified by using the $^1O_2$ life time prolonging solvent deuterium oxide (D_2O) instead of H_2O. All of these observations underscore the potential importance of $^1O_2$ as an ultimate genotoxicant. However, in photodynamic studies the precise role of singlet oxygen in the destruction of DNA is difficult to assess, because (1) in addition to $^1O_2$ other activated oxygen species such as O_2^- may be produced, and (2) activated oxygen species including $^1O_2$ may react with the sensitizer, generating (a) product(s) that may inflict the damage[17,24,46].

In the present experiments we have used the assay conditions of Brawn and Fridovich[4] and Fiel *et al.*[12] to compare the efficiencies of photodynamically and chemically generated $^1O_2$ to cause strand breakage of DNA at physiological pH.

RESULTS

Two major DNA entities were observed in the untreated plasmid DNA preparations

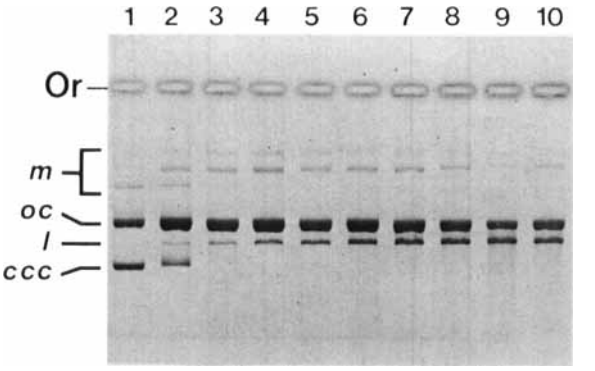

FIGURE 1 Gelelectrophoresis of pEV42 DNA after exposure to rose bengal plus light, in air-equilibrated Tris buffer containing 96.5% D_2O. Slot 1: no light; slot 2–10: light for 10, 20, 30 etc. . . . 90 minutes. *ccc*: covalently closed circular DNA molecules; *l*: full-length linear DNA molecules; *oc*: open circular (relaxed) DNA molecules; *m*: multimers of *ccc* and *oc* molecules. Or: origin.

used in this study. As illustrated in Figure 1, these were covalently closed (*ccc*) and open circular or "nicked" (*oc*) molecules, in approximately equal proportions; virtually no linear (*l*) molecules were present in the untreated samples. Figure 1 also shows the time-dependent conversion of *ccc* into *oc* and subsequently into *l* molecules upon exposure to rose bengal plus light at pH 7.8, illustrating that this photodynamic system is capable of producing single strand breaks in DNA without alkali treatment.

To assess the possible involvement of activated oxygen species in the observed DNA strand breakage reaction the effects of D_2O (which, as a solvent, supports a ca. 10 times longer life time of $^1O_2$ than H_2O[32]) and of O_2 withdrawal were studied. As shown in Figure 2, replacing the H_2O in the reaction mixture by D_2O greatly stimulated DNA breakage; stimulation was estimated to be at least 5-fold (*c.f.* ref. 32). Moreover, at an oxygen level as low as 5 p.p.m. or less (Figure 3) the DNA was much less susceptible to breakage. Furthermore, addition of superoxide dismutase and/or catalase to the reaction mixture did not affect the result, excluding $O_2^{\cdot-}$ as a critical intermediate[4,26,33]. These data strongly suggest that $^1O_2$ is a critical intermediate in the production of DNA strand breaks by rose bengal plus visible light. The low level of residual DNA breakage observed under hypoxia (Figure 3) is supposedly due to residual O_2 molecules still present under these conditions; however, the possibility of an O_2-independent DNA breakage reaction in the rose bengal plus light system (*c.f.* Peak *et al.*[36]) cannot be excluded.

Experiments to test the effect of $^1O_2$ generated chemically by the thermodissociable endoperoxide $NDPO_2$ (endoperoxide of the disodium 3,3′-(1,4-naphthylidene) diproprionate; see Figure 4) are summarized in Table I. An endoperoxide-generated $^1O_2$ flux equivalent to 10^{-4}M rose bengal illuminated for 7 mins, or even a tenfold excess, failed to produce DNA strand breaks in D_2O. Two positive controls for $^1O_2$ generation were included (results not shown). First, the amount of $^1O_2$ generated was measured in parallel incubations by using a water-soluble rubrene derivative (RTC: tetrapotassium rubrene–2,3,8,9—tetracarboxylate) as a specific $^1O_2$ trap (see Materials and Methods and Aubry *et al.*[2]). Second, when DNA was treated with the

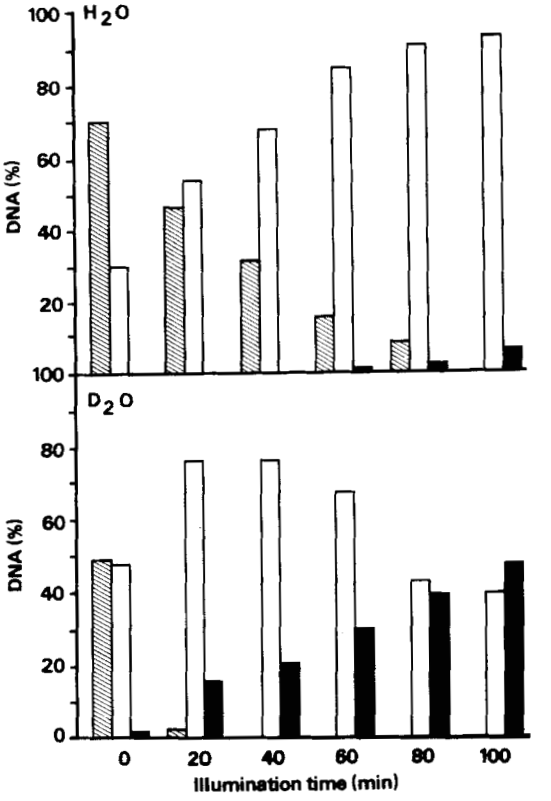

FIGURE 2 D_2O solvent effect on DNA strand breakage by dissolved rose bengal plus light. Hatched bars: covalently closed circular DNA; open bars: open circular DNA; black bars: full-length linear DNA.

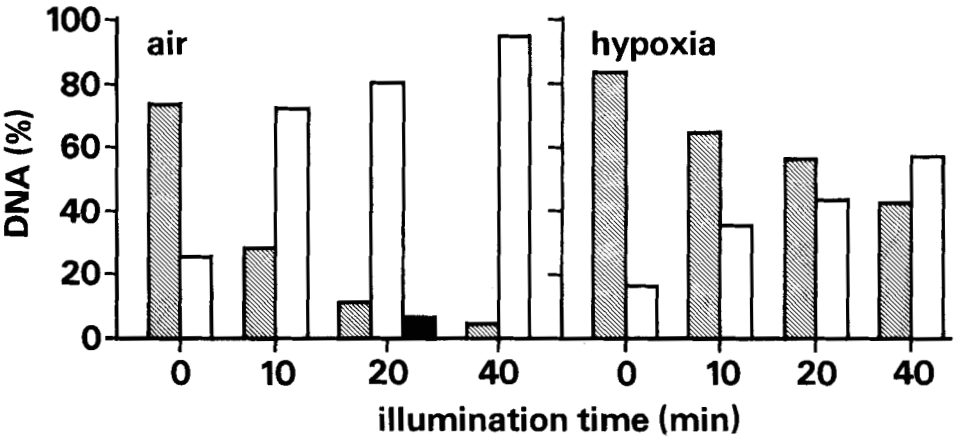

FIGURE 3 Effect of hypoxia on DNA strand breakage by rose bengal plus light; reaction mixtures, which contained 96.5% D_2O, were equilibrated with an atmosphere of air or 95% N_2/5% H_2 (less than 5 p.p.m. O_2) before being irradiated. Bars: as in figure 2.

TABLE I
DNA breakage by various sources of $^1O_2$

$^1O_2$-generating system	Reaction conditions: light (7 min)	O_2	D_2O	SOD/catalase[a]	DNA strand breakage[b]
rose bengal, 10^{-4} M[c]	+	+	+	−	++
rose bengal, 10^{-4} M[c]	+	+	+	+	++
rose bengal, 10^{-4} M[c]	+	+	−	−	+
rose bengal, 10^{-4} M[c]	+	−	+	+	−
rose bengal, 10^{-4} M[c]	−	+	+	−	−
no sensitizer	+	+	+	−	−
$NDPO_2$, 7 mg/ml[d]	−	+	+	−	−
−, + NaCl, 1M	−	+	+	−	−
−, + rose bengal, 10^{-4} M	−	+	+	−	−
−, 70 mg/ml	−	+	+	−	−

[a] SOD concentration: 10 μg/ml; catalase concentration: 20 μg/ml.
[b] no strand breakage observed; + and ++ indicate relative efficiency of strand break production.
[c] cumulative $^1O_2$ concentration: 10 mM per 7 min illumination.
[d] cumulative $^1O_2$ concentration produced with 7 mg/ml of $NDPO_2$: 10 mM.

endoperoxide $NDPO_2$ (7 mg/ml in D_2O) and subsequently exposed to alkaline conditions (see Materials and Methods) all DNA bands shown in Figure 1 had disappeared, indicating that alkali-labile lesions had been introduced by the endoperoxide treatment under conditions that failed to produce direct strand breaks.

To check the possibility of $^1O_2$ reacting with rose bengal to give a product ultimately responsible for DNA strand scission, DNA was exposed to endoperoxide plus rose bengal in the absence of light (Table I); no strand breakage was observed, however. In a final attempt to demonstrate direct $^1O_2$-mediated DNA breakage in the absence of light, DNA was exposed to endoperoxide in the presence of a high concentration of NaCl to shield off the negative charge of the DNA backbone and allow the negatively charged endoperoxide molecules (Figure 4) to come closer to the DNA. However, breakage was still not detectable.

DISCUSSION

It is clear from our experiments that rose bengal plus light has the potency to induce DNA strand breakage at physiological pH. This reaction required the presence of light and was greatly stimulated by using D_2O instead of H_2O as a solvent. Hypoxia greatly reduced the breakage reaction. These observations are consistent with $^1O_2$ being a critical intermediate in the reaction[22,32,39]. Moreover, superoxide dismutase and catalase were unable to inhibit the reaction, which excluded a contribution from O_2^- (or H_2O_2 and $OH^\cdot$ derived from it) to the breakage phenomenon[4]. These data strongly suggest that $^1O_2$ was the only activated oxygen species involved. Peak *et al.*[36] recently reported that treatment of linear DNA molecules (molecular weight ca. 10^8 daltons) with immobilized rose bengal plus 545 nm light resulted in single strand breaks as detected by alkaline sucrose gradient centrifugation, which technique cannot distinguish between strand breaks and alkali-labile lesions; the reaction was oxygen-independent and not susceptible to stimulation by D_2O. The authors concluded that DNA breakage was mainly due to "type I reactions", i.e. resulting from

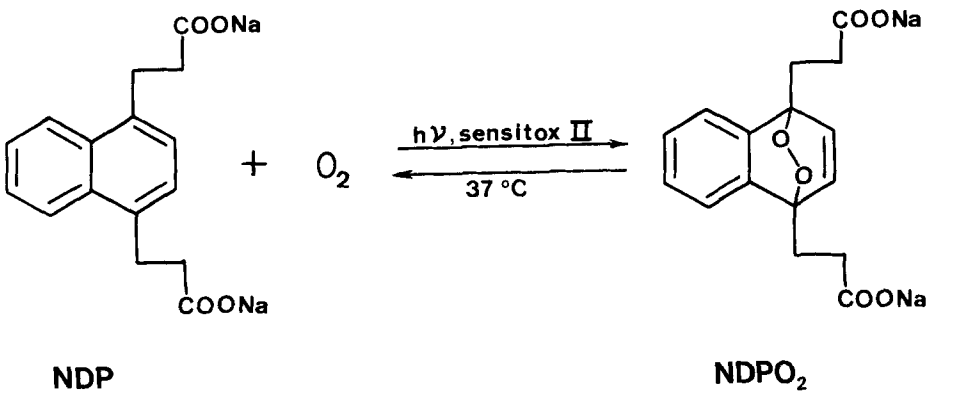

FIGURE 4 Water-soluble singlet oxygen generator, $NDPO_2$, used in the present study. About half of the O_2 released at 37°C was found to be in the singlet state $^1\Delta g$ (see Materials and Methods).

the direct interaction between excited dye and the DNA. This conclusion seems at variance with the present results. However, the discrepancy would be resolved by assuming that the majority of strand breaks observed by Peak *et al.*[36] were derived from alkali-labile lesions (produced by a type I reaction) which might be produced much more efficiently than strand break lesions at physiological pH.

We were unable to detect DNA strand breakage with comparatively large fluxes of $^1O_2$ generated chemically with the $^1O_2$ carrier $NDPO_2$ in the absence of light, implying that in addition to $^1O_2$ the presence of light plus dye was also essential for the induction of DNA strand breaks in the photodynamic system. Our data on photodynamic DNA breakage might be explained e.g. by assuming that oxygen and sensitizer molecules interact with the DNA in such a way as to result in a highly site-specific generation of $^1O_2$. The conclusion emerging from our work is that $^1O_2$ is by itself inefficient in causing direct DNA strand breakage at physiological pH. The DNA-damaging potency of $^1O_2$ *per se* must therefore reside mainly, if not exclusively, in its base-damaging properties.

MATERIALS AND METHODS

Starting materials

Rose bengal (tetraiodotetrachlorofluorescein sodium salt, Sigma Chemical Corp.) and immobilized rose bengal (Sensitox II, Chemical Dynamics Corp., South Plainfield, New Jersey) were used as received.

Plasmid DNAs (pBR 322, pEV 26 and pEV42 from Escherichia coli (mol. weights 2.6, 2.7 and 2.9 million daltons, respectively) were isolated according to the "cleared lysate" method[10,48]. The DNA preparations typically contained 50–70% covalently closed circular (*ccc*) molecules, 30–50% "open" (relaxed) circles (*oc*) and virtually no linear (*l*) molecules.

1,4-Naphtalenedipropionic acid sodium salt (NDP) was prepared from 1,4-dimethylnaphtalene (Aldrich) by bromination with N-bromosuccinimide[40]

followed by malonic synthesis[30,31], neutralisation of the diacid by sodium methylate in methanol and precipitation with ether.

Water-soluble $^1O_2$ generator, $NDPO_2$

Sensitox II (100 mg) was added to a solution of NDP (1 g) in water (0.5 ml) and methanol (9.5 ml). This mixture was irradiated, 3 hours at 5°C under stirring, with a mercury high pressure lamp (Philips SP 500) using a filter GG 515 (Schott) and maintaining a continuous bubbling of oxygen. The sensitizer was filtered off by suction and washed with methanol (20 ml) at 0°C, the resulting solution was dried by stirring at O°C with Na_2SO_4 (2 g) during 15 min.

After filtration, 60 ml ether was added to the solution at 0°C and stirred 10 min to induce precipitation of the endoperoxide $NDPO_2$. The precipitate was collected and dried 2 hours in vacuo (0.1 torr) at 0°C yielding $NDPO_2$ (80%) as white powder. HPLC analysis of this compound (RP 18 column, eluent: ethanol, 270/water, 330/H_3PO_4,1) showed that 95% of the product was photooxygenated, 4% had remained unchanged and about 1% was converted into a secondary product.

$NDPO_2$ is stable at −20°C but regenerates NDP and oxygen in aqueous solution at 37°C by a first order process ($t_{1/2} = 23$ min). By trapping $^1O_2$ with the water soluble potassium salt of rubrene-2,3,8,9-tetracarboxylic acid (RTC)[2], it appeared that 48% of the oxygen evolved by decomposition of $NDPO_2$ was in the singlet state $^1\Delta g$, while 52% was in the triplet ground state. The above reactions are summarized in Figure 4.

Irradiation conditions

Samples (50–100 μl) containing DNA (7–10 μg/ml) in 50 mM Tris HCl buffer pH 7.8, were stirred magnetically in polystyrene vials and illuminated at a distance of 5 cm, in the center of a 20 W circular (internal diameter 15 cm) white fluorescent Osram Universal lamp. Ca. 90% of the emitted light was between 400 and 600 nm with peaks at 405.8, 436.5, 547.5 and 579.8 nm. Light intensity at the site of the reaction vessel was ca. 2.8 mW/cm^2 in the range 400–600 nm, with ca. 60 μW/cm^2 at 543 nm, as measured through a Schott AL 543 interference filter (λ_{max} = 543 nm, maximal transmission 56%, half-width 19 nm).

The concentration of rose bengal was 10^{-4}M. Cumulative concentrations of $^1O_2$ generated photochemically were measured in parallel samples (without DNA) by trapping with the weakly sensitizing water-soluble $^1O_2$ trap DPATC (9,10-diphenylanthracene-2,3,6,7-tetracarboxylic acid tetrapotassium salt;[44] and appeared to be approx 1.4 mM/min.

Exposure of DNA to chemically generated $^1O_2$

Singlet oxygen generator $NDPO_2$ was added at 7 mg/ml to 50 mM Tris buffer pH 7.8 in either D_2O or H_2O and incubated at 37°C for 3 hours in the dark unless stated otherwise. These conditions caused the release of 10 mM of $^1O_2$ (cumulative concentration). Some experiments were carried out at ten-fold higher concentrations of $NDPO_2$ (70 mg/ml; 100 mM of $^1O_2$). Cumulative concentrations of $^1O_2$ were determined in parallel incubations by trapping with the water-soluble potassium salt of rubrene-2,3,8,9-tetracarboxylic acid (RTC)[2].

Gelelectrophoresis of DNA

DNA samples (1 μg in 40–60 μl) were mixed with one fifth volume of a solution containing 0.025% bromophenol blue and 3% Ficoll in 10 mM EDTA, 50 mM Tris HCl, pH 7.8, and electrophoresed on a horizontal 0.7% agarose slab gel made up in a buffer containing 89 mM boric acid, 89 mM Tris HCl, 2.5 mM EDTA, pH 7.8, using the same buffer as electrophoresis buffer.

Thirty V (30 mA) for 15 min was applied to allow the DNA to enter the gel. Electrophoresis was at 80 V, 5 mA per cm, for 4 hours.

DNA bands were visualized by soaking in a solution of ethidium bromide (5 mg in 1 liter tap water) during 30–60 min, and illuminating the gel with long wave UV light. Photographs were taken with a polaroid camera (film type 55). Negatives were scanned with a Quick Scan integrating scanning apparatus (Helena Laboratories, Beaumont, Texas). Figure 1 is a contact print made with direct positive camera paper, showing the DNA as dark bands in a light background.

Alkali treatment of DNA

DNA was tested for alkali-labile sites by addition of NaOH (0.1 M; final pH > 12) and incubation at room temperature for 15–30 min. The solution was neutralized by addition of 36 mM sodium phosphate buffer (pH 8.4) followed by addition of 0.1 M HCl.

D_2O solvent effect

The D_2O solvent effect was assessed by using reaction mixtures of which the components had been made up with D_2O (99.75% deuterium oxide, Merck) instead of H_2O. Because the DNA solutions used were generally in H_2O-containing buffers the final D_2O concentrations varied; most experiments contained 96.5% D_2O, as indicated; D_2O concentration was in no case less than 85% (v/v).

Based on the values of 55 μsec for the intrinsic lifetime of $^1O_2$ in pure D_2O and of 4.2 μsec in pure H_2O, the expected lifetimes for $^1O_2$ in H_2O/D_2O mixtures containing 96.5% D_2O and 85% D_2O can be calculated to be 38 and 21 μsec, respectively[39].

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