Oxidation of 2′-deoxyguanosine by H_2O_2–ascorbate: evidence against free OH• and thermodynamic support for two-electron reduction of H_2O_2

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Received (in Cambridge, UK) 4th August 2000, Accepted 6th December 2000
First published as an Advance Article on the web 11th January 2001

Of the four major nucleosides in DNA, 2′-deoxyguanosine (dG) is the most easily oxidized. The formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) by oxidation of free dG, as well as of dG in DNA, by hydrogen peroxide (H_2O_2) and the reducing agent ascorbate ($HAsc^-$) was measured using HPLC with high sensitivity electrochemical detection. High concentrations of the hydroxyl radical ($OH^•$) scavengers PBN, DMPO, pentoxifylline and imidazole had a very limited protective effect on 8-oxodG formation, which provides evidence against free $OH^•$ production as the major mechanism. A possible mechanism involves a "two-electron reduction of H_2O_2" with oxidation of $HAsc^-$ and dG, producing the guanine radical cation ($dG^{•+}$) leading to 8-oxodG. This mechanism is, based on changes in Gibbs free energy ($\Delta G_0'$) at pH 7, much more favourable than $OH^•$ production. Catalase from *Aspergillus niger* and the aminoxyl TEMPO almost completely inhibited 8-oxodG formation, whereas bovine catalase cleaved the free dG molecule. Substantial 8-oxodG formation from free dG occurred even at 0 °C.

Introduction

In vivo, hydrogen peroxide (H_2O_2) can diffuse into the nucleus and oxidize DNA, and 2′-deoxyguanosine (**1**) (dG) has been shown to be oxidized to potentially mutagenic[1–3] 8-oxo-7,8-dihydro-2′-deoxyguanosine (**4**) (8-oxodG, Scheme 1) in the presence of H_2O_2 and a reducing agent.[4,5] The mechanism of dG (**1**) oxidation under these conditions is unclear but has been suggested to involve hydroxyl radical ($OH^•$) produced by transition-metal catalysed one-electron reduction of H_2O_2[4,5] [the Fenton reaction, reaction (4b) below].[6] Exposure of water to ionizing radiation,[7] as well as one-electron reduction of H_2O_2,[8] produces the very reactive $OH^•$, which can attack dG (**1**) forming an 8-OH-8-H-dG$^•$ adduct (**3**), that upon oxidation gives 8-oxodG (**4**).[7] 8-oxodG (**4**) can also be formed by one-electron oxidation of dG (**1**) to give the guanine radical cation **2** ($dG^{•+}$), which is subsequently hydroxylated in water (Scheme 1).[9,10] Guanine repeat sequences (GGG and GG) and especially 8-oxodG (**4**) sites are efficient traps of fast cation transfer through DNA, being preferentially oxidized.[11,12] One-electron oxidation of 8-oxodG (**4**) containing oligonucleotides has been found to lead to the formation of guanidinohydantoin (**8**),[11] which could be formed by ring opening and decarboxylation of the unstable 5-hydroxy-8-oxo-7,8-dihydro-2′-deoxyguanosine (**7**) (5-OH-8-oxodG).[13] The reaction rate between $OH^•$ and guanosine (7.8×10^9 M^{-1} s^{-1})[14] is similar to that of many $OH^•$ scavengers (PBN, 8.6×10^9 M^{-1} s^{-1};[15] pentoxifylline, 7.7×10^9 M^{-1} s^{-1};[16] DMPO, 4.3×10^9 M^{-1} s^{-1};[17] and imidazole, 3.9×10^9 M^{-1} s^{-1}[18]).

To determine the effects of $OH^•$ scavengers on the inhibition of 8-oxodG (**4**) formation, DNA and free dG (**1**) were incubated with 50 μM H_2O_2 and 2 μM ascorbate ($HAsc^-$). The catalytic removal of H_2O_2 was also investigated [eqn. (1)]:

$$2H_2O_2\ (MnO_2\ (s),\ KI \text{ or catalase}) \longrightarrow 2H_2O + O_2 \quad (1)$$

The steady-state level of 8-oxodG (**4**) in DNA is used as a biomarker for oxidative stress.[19] However, the 8-oxodG (**4**) level is hard to determine, since artifactual 8-oxodG (**4**) can be formed during the work-up procedure by the oxidation of dG (**1**),[20] and also since 8-oxodG (**4**) can be lost by further oxidization. DNA is commonly hydrolysed with nuclease P_1 at pH 5.3 (37 °C, 1–2 h) during the work-up procedure, and liberated dG (**1**) is particularly sensitive to oxidation. Suitable antioxidants and work-up procedures have been investigated that could be used to obtain the true 8-oxodG (**4**) level in cellular DNA, by preventing oxidation of the nucleosides during work-up.[20]

Results and discussion

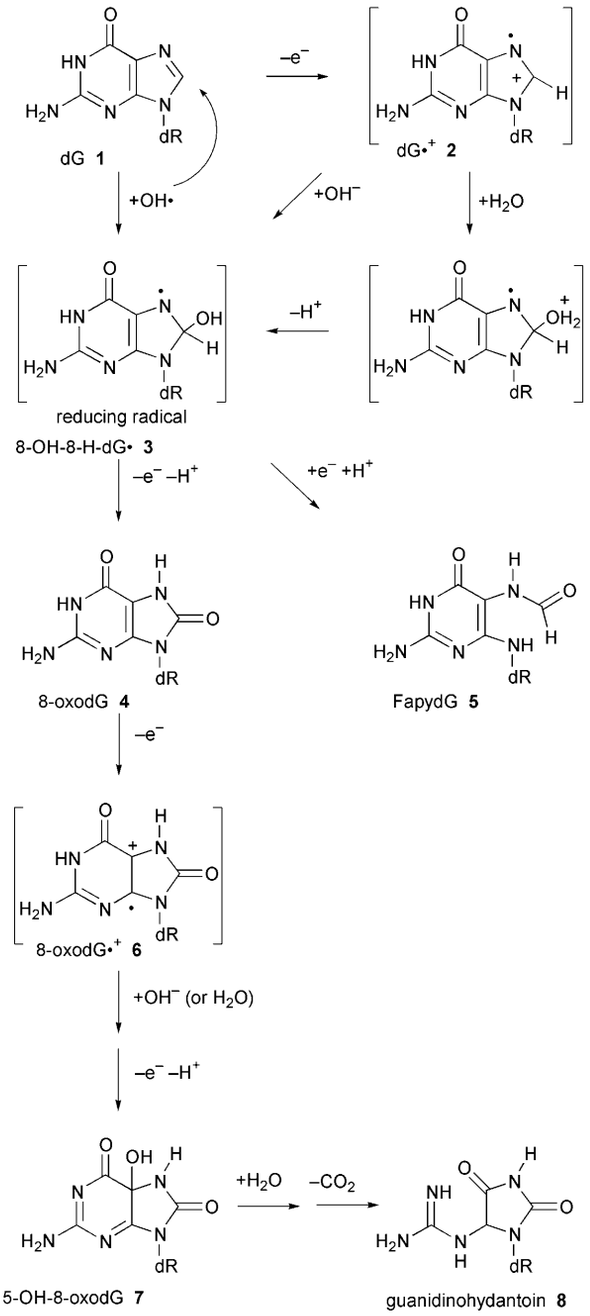
Evidence against free $OH^•$ formation as the major mechanism

When free dG (**1**) (Fig. 1) or rat liver DNA (Fig. 2) was incubated with 50 μM H_2O_2 and 2 μM ascorbate, the addition of $OH^•$ scavengers (PBN, DMPO, pentoxifylline and imidazole) at high concentrations relative to dG (**1**) had almost no effect on the inhibition of 8-oxodG (**4**) formation. This suggests that formation of free $OH^•$ is not the major mechanism responsible for 8-oxodG (**4**) formation. Melatonin (Fig. 1) has been suggested to have inhibitory effects on metal ion-catalysed oxidation processes, acting as a metal ion deactivator.[21]

Inhibition of 8-oxodG formation by H_2O_2 removal

For H_2O_2 removal alone, KI, MnO_2 and catalase (*Aspergillus niger* and bovine) were all very effective at a concentration of 30 weight-% of H_2O_2 (bubble test). Catalase was effective even at 0 °C (bubble test). However, when free dG (**1**) was incubated under the conditions used to hydrolyse DNA, together with 50 μM H_2O_2 and 2 μM ascorbate, MnO_2 had only a modest effect as an inhibitor of 8-oxodG (**4**) formation (Fig. 1), bovine catalase (50 U mL^{-1}) cleaved the dG (**1**) molecule (seen by UV detection) and KI (1 mM and 100 μM) destroyed the electrochemical chromatogram. Catalase from *Aspergillus niger* inhibited 8-oxodG (**4**) formation almost completely under these conditions (Fig. 1), showing that the O_2 present with ascorbate did not oxidize dG (**1**) at 37 °C when H_2O_2 was removed. Incubation of free dG (**1**) with H_2O_2 alone did not result in significant 8-oxodG (**4**) formation (data not shown). Removal of Zn^{2+} in the H_2O_2–ascorbate system did not affect the 8-

 DOI: 10.1039/b006394k



Scheme 1 Possible mechanisms for the $OH^{\bullet}$ addition to, and one-electron oxidation of, dG (**1**) giving 8-oxodG (**4**), and further oxidation of 8-oxodG (**4**) (dR: 2-deoxyribose).

oxodG (**4**) levels (data not shown). Removal of background H_2O_2 in the buffers using catalase can prevent oxidation of nucleosides, but it should be confirmed that catalase does not damage the biomolecules being analysed.

Effect of TEMPO as inhibitor of 8-oxodG formation

When the aminoxyl TEMPO was added to the incubation mixture (50 μM H_2O_2 and 2 μM ascorbate) of free dG (**1**) (Fig. 1) or DNA (Fig. 2), 8-oxodG (**4**) formation was almost completely blocked. The electron acceptor TEMPO,[22,23] added at an elevated concentration relative to ascorbate, can quickly oxidize ascorbate and the reduced hydroxylamine form of TEMPO did not reduce H_2O_2 significantly to give any 8-oxodG (Figs. 1 and 2). Thus, TEMPO can prevent H_2O_2 from being reduced (and thereby oxidizing the nucleosides) by oxidizing the reducing agent. Injections (100 μL) of 1 mM TEMPO were not found to disturb the electrochemical detection of 8-oxodG.

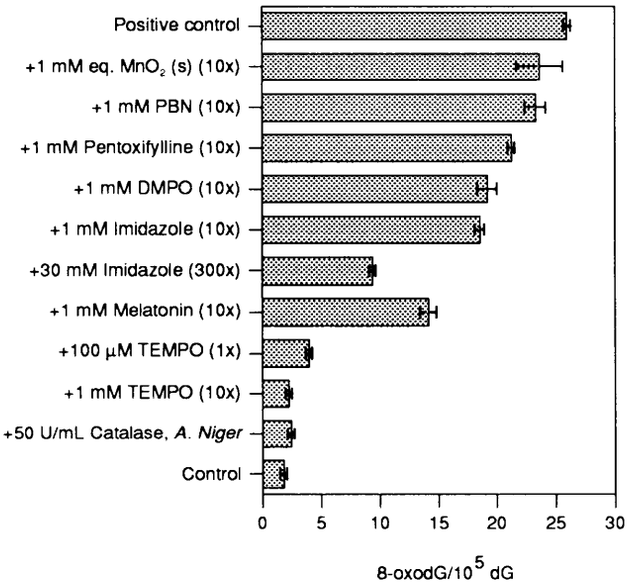


Fig. 1 Effects of antioxidants on the inhibition of 8-oxodG (**4**) formation in free dG (**1**). Positive control: incubation (37 °C, 1.5 h) of 100 μM dG (**1**) with 50 μM H_2O_2 and 2 μM ascorbic acid in DNA hydrolysis buffer (50 mM sodium acetate, 0.2 mM Zn^{2+}, pH 5.3). The control is without H_2O_2 and ascorbic acid. Each point represents three analyses and is shown as mean ± SD. In parentheses is the concentration of the antioxidant relative to dG (**1**).

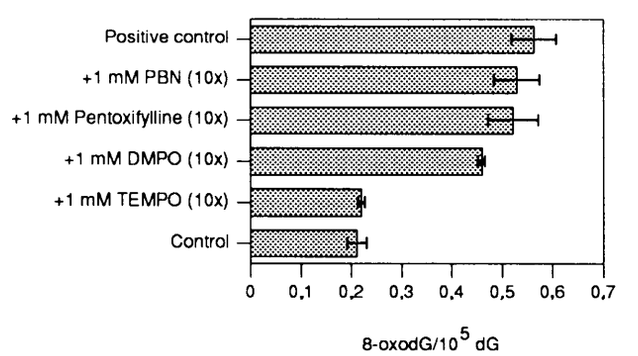


Fig. 2 Effects of antioxidants on the inhibition of 8-oxodG (**4**) formation in DNA. Positive control: incubation (37 °C, 1.5 h) of 40 μg rat liver DNA with 50 μM H_2O_2 and 2 μM ascorbic acid in distilled water. All solutions were set to approximately pH 7.4 using NaOH (use of a buffer that could scavenge $OH^{\bullet}$ was intentionally avoided). The dG (**1**) concentration was approximately 100 μM (calculated with a 22% dG (**1**) content in DNA, an average nucleotide weight of 325 g mol^{-1} and a total volume of 271 μL). The control is without H_2O_2 and ascorbic acid. The DNA was washed, hydrolysed and analysed by HPLC for 8-oxodG (**4**) content. Each point represents three analyses and is shown as mean ± SD. In parentheses is the concentration of the antioxidant relative to dG (**1**).

Temperature effect on 8-oxodG formation

When free dG (**1**) was incubated with 50 μM H_2O_2 and 2 μM ascorbate at temperatures between −15 to +52 °C for 1.5 h (Fig. 3), the 8-oxodG (**4**) formation could be fitted to a third order equation, with the least 8-oxodG (**4**) formation occurring at the lowest temperatures. However, 8-oxodG (**4**) formation occurred significantly more at all tested temperatures *versus* the control (without H_2O_2 or ascorbate). Still, temperature reduction can serve as an extra protective measure to prevent oxidation of nucleosides.

Thermodynamic support for a mechanism involving "two-electron reduction of H_2O_2"

A possible mechanism involves a transition metal-mediated non-$OH^{\bullet}$ two-electron reduction of H_2O_2 with formation of the guanine radical cation **2** ($dG^{\bullet+}$) [reactions (2)–(3)]:

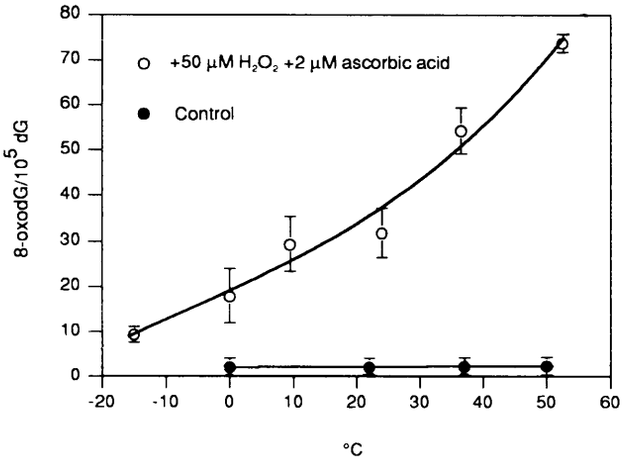


Fig. 3 Temperature dependence of 8-oxodG (**4**) formation by oxidation of dG (**1**). Incubation of 100 μM dG (**1**) in 20 mM Na_2HPO_4–NaH_2PO_4 (pH 7.4) for 1.5 h. Each point represents three analyses and is shown as mean ± SD. The increase of the 8-oxodG/10^5 dG ratio with temperature could be approximated to a third order equation.

$$HAsc^- + Fe^{3+} \longrightarrow H^+ + Asc^{\bullet -} + Fe^{2+} \quad (2a)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe(H_2O_2)^{2+} \quad (2b)$$

$$Fe(H_2O_2)^{2+} + dG \longrightarrow Fe^{3+} + 2OH^- + dG^{\bullet +} \quad (2c)$$

$$\Sigma: HAsc^- + H_2O_2 + dG\ (\text{Trans. metals}) \longrightarrow H^+ + Asc^{\bullet -} + 2OH^- + dG^{\bullet +} \quad (2)$$

$$OH^- + dG^{\bullet +} \longrightarrow \text{8-OH-8-H-dG}^{\bullet} \longrightarrow \text{8-oxodG} + H^+ + e^- \quad (3)$$

For iron and copper, the intermediates $Fe(H_2O_2)^{2+}$ and $Cu(H_2O_2)^+$ could exist,[5,24] and the reduced transition metal could be chelated to DNA. At pH 7 the reduction potential of H_2O_2 is $E_0'(1/2H_2O_2, H^+/H_2O) = 1.32$ V,[25] $E_0'(Asc^{\bullet -}, H^+/HAsc^-) = 0.282$ V[26] and $E_0'(dG^{\bullet}, H^+/dG) = 1.29$ V.[27] Calculating $\Delta G_0'$ for each half-cell reaction according to $\Delta G_0' = -nFE_0'$ gives (after changing signs for $HAsc^-$ and dG **1**, which are oxidized) for the total reaction (2): $\Delta G_0' = -2F(1.32) + F(0.282) + F(1.29) = -F(1.068) = -23.06(1.068) = -24.63$ kcal mol^{-1}. Also, $dG^{\bullet +}$ (**2**) and OH^- could spontaneously react, thereby pulling the reaction. O_2 or Fe^{3+} could accept the electron released in reaction (3). The 8-oxodG (**4**) formation approximately doubled at pH 7.4 (Fig. 3, 37 °C) compared to pH 5.3 (Fig. 1) when dG (**1**) was incubated with H_2O_2–ascorbate. This two-electron mechanism for the reduction of H_2O_2 is much more favourable to "free" or theoretical "site-specific" produced $OH^{\bullet}$ [reaction (4)] where $\Delta G_0' = -F(0.320) + F(0.282) = -0.88$ kcal mol^{-1} as $E_0'(H_2O_2, H^+/H_2O, OH^{\bullet}) = 0.320$ V.[25] This can possibly explain the evidence against $OH^{\bullet}$ in similar systems.[28,29]

$$HAsc^- + Fe^{3+} \longrightarrow H^+ + Asc^{\bullet -} + Fe^{2+} \quad (4a)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad (4b)$$

$$\Sigma: HAsc^- + H_2O_2\ (\text{Trans. metals}) \longrightarrow Asc^{\bullet -} + OH^{\bullet} + H_2O \quad (4)$$

As the positive charge on $dG^{\bullet +}$ (**2**) may be located on carbons other than C-8, other products may also have been formed (not measured). Purine radical cations do not react rapidly with O_2.[30] Some $dG^{\bullet +}$ (**2**) may, however, be repaired back to dG (**1**) by $HAsc^-$ or $Asc^{\bullet -}$, before hydroxylation has occurred. A similar mechanism [reaction (2)] could account for the oxidation of other substances as initiation events by H_2O_2 or other peroxides with reducing agents. Further oxidation of 8-oxodG (**4**) in DNA can repair other formed base cations by electron transfer through DNA, thus forming an 8-oxodG radical cation [8-oxod$G^{\bullet +}$ (**6**)], as E_0'(8-oxod$G^{\bullet}$, H^+/8-oxodG) = 0.74 V.[31] Replacing 8-oxodG (**4**) for dG (**1**) in reaction (2) gives $\Delta G_0' = -37.31$ kcal mol^{-1}.

Experimental

Chemicals

Hydrogen peroxide, manganese(IV) oxide, potassium iodide, 2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPO), and 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO) were purchased from Aldrich (Milwaukee, WI). Catalase (*Aspergillus niger* and bovine), alkaline phosphatase, melatonin, *N*-benzylidene-*tert*-butylamine *N*-oxide (PBN), pentoxifylline, 2-deoxyguanosine (dG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were from Sigma (St. Louis, MO). Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, imidazole, ascorbic acid, zinc chloride, methanol, sodium chloride and sodium acetate were from Merck (Darmstadt, Germany). TRIS was from Amresco (Solon, OH). Nuclease P_1 was from Roche (Mannheim, Germany). Distilled water and ultrapure chemicals were used throughout. Background transition metals were intentionally left. Rat liver DNA was extracted as previously described.[20]

Incubations of dG and DNA

Details are given in the figure legends. DNA was dissolved in distilled water with ice cooling and the DNA concentration was determined by UV in 10 mM TRIS buffer (pH 7.4). All samples were prepared with ice cooling, and ascorbate was added immediately prior to incubation. Total incubation volumes were 300 μL for dG (**1**) and 271 μL for DNA (40 μg). After incubation, the DNA was precipitated with ethanol/sodium chloride and washed three times with ice-cold 70% ethanol. Then the DNA was completely dissolved with a pipette in 50 mM sodium acetate, 0.2 mM zinc chloride (pH 5.3) containing 100 μM TEMPO, and enzymatically hydrolysed at 37 °C for 1.5 h using 25 μg nuclease P_1 and 2 U alkaline phosphatase in a total volume of 120 μL. The DNA hydrolysate was ultrafiltrated (UFC3LGC, Millipore, Bedford, MA) at 14 000 rpm (0 °C) for 10 min to remove enzymes. The solutions were stored at −80 °C until analysis.

HPLC analysis

The HPLC system consisted of a zirconium mobile-phase filter (Elsico Labs, Moscow, Russia), an isocratic Scantec 650 pump (Scantec, Partille, Sweden) set at 0.8 mL min^{-1} with an extra PEEK pulse damper (Scientific Instruments, Inc., State College, PA), an injector (7725i, Rheodyne, Cotati, CA) with a 200 μL PEEK loop, a 1 mm (C 18) Opti-Guard column (Optimize, Portland, OR), and two Delta-Pak (150 × 3.9 mm id, 5 μm) columns (Waters, Milford, MA). 8-oxodG (**4**) was detected with an electrochemical detector (Coulochem II, ESA, Chelmsford, MA) with a graphite filter protected 5011 analytical cell (ESA, screen electrode: +200 mV, analytical electrode: +350 mV), and dG (**1**) was measured with a 486 UV detector (Waters) set at 290 nm. Plastic and PEEK tubing was used throughout. The HPLC buffer consisted of 10% methanol, water of Milli-Q grade (Millipore), 20 mM sodium acetate set to pH 5.3 with acetic acid and was filtered through a CN 0.2 μm filter (Nalgene, Rochester, NY). 100 μL was injected and the retention times of dG (**1**) and 8-oxodG (**4**) (detection limit ≈ 5 fmol) were 11 and 16 min respectively. Calibration curves for dG (**1**) and 8-oxodG (**4**) were made by injection four times of each standard (100 μL) on the day of analysis.

Acknowledgements

This work was supported financially by Dr L. Möller.

References

1 Y. Kuchino, F. Mori, H. Kasai, H. Inoue, S. Iwai, K. Miura, E. Ohtsuka and S. Nishimura, *Nature*, 1987, **327**, 77.
2 M. L. Wood, M. Dizdaroglu, E. Gajewski and J. M. Essigmann, *Biochemistry*, 1990, **29**, 7024.
3 M. Moriya, C. Ou, V. Bodepudi, F. Johnson, M. Takeshita and A. P. Grollman, *Mutat. Res.*, 1991, **254**, 281.
4 H. Kasai and S. Nishimura, *Nucleic Acids Res.*, 1984, **12**, 2137.
5 O. I. Aruoma, B. Halliwell, E. Gajewski and M. Dizdaroglu, *Biochem. J.*, 1991, **273**, 601.
6 J. H. Baxendale, M. G. Evans and G. S. Park, *Trans. Faraday Soc.*, 1946, **42**, 155.
7 T. Douki, S. Spinelli, J.-L. Ravanat and J. Cadet, *J. Chem. Soc., Perkin Trans. 2*, 1999, **9**, 1875.
8 F. Haber and R. Willstätter, *Ber. Dtsch. Chem. Ges.*, 1931, **64**, 2844.
9 H. Kasai, Z. Yamaizumi, M. Berger and J. Cadet, *J. Am. Chem. Soc.*, 1992, **114**, 9692.
10 P. M. Cullis, M. E. Malone and L. A. Merson-Davies, *J. Am. Chem. Soc.*, 1996, **118**, 2775.
11 R. P. Hickerson, F. Prat, J. G. Muller, C. S. Foote and C. J. Burrows, *J. Am. Chem. Soc.*, 1999, **121**, 9423.
12 E. Meggers, A. Dussy, T. Schäfer and B. Giese, *Chem. Eur. J.*, 2000, **6**, 485.
13 R. N. Goyal, N. Jain and D. K. Garg, *Bioelectrochem. Bioenerg.*, 1997, **43**, 105.
14 G. V. Buxton, C. L. Greenstock, W. P. Helman and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1988, **17**, 513.
15 V. E. Zubarev, R. Mehnert and O. Brede, *Radiat. Phys. Chem.*, 1992, **39**, 281.
16 C. Pasquier, E. Franzini, Z. Abedinzadeh, M. N. Kaouadji and J. Hakim, *Int. J. Radiat. Biol.*, 1991, **60**, 433.
17 P. Neta, S. Steenken, E. G. Janzen and R. V. Shetty, *J. Phys. Chem.*, 1980, **84**, 532.
18 O. I. Aruoma, M. J. Laughton and B. Halliwell, *Biochem. J.*, 1989, **264**, 863.
19 L. L. De Zwart, J. H. Meerman, J. N. Commandeur and N. P. Vermeulen, *Free Radical Biol. Med.*, 1999, **26**, 202.
20 T. Hofer and L. Moller, *Chem. Res. Toxicol.*, 1998, **11**, 882.
21 F. Antunes, L. R. Barclay, K. U. Ingold, M. King, J. Q. Norris, J. C. Scaiano and F. Xi, *Free Radical Biol. Med.*, 1999, **26**, 117.
22 J. E. Baur, S. Wang and M. C. Brandt, *Anal. Chem.*, 1996, **68**, 3815.
23 U. A. Nilsson, L. I. Olsson, G. Carlin and A. C. Bylund-Fellenius, *J. Biol. Chem.*, 1989, **264**, 11131.
24 J. D. Rush and W. H. Koppenol, *J. Inorg. Biochem.*, 1987, **29**, 199.
25 W. H. Koppenol, *Methods Enzymol.*, 1996, **268**, 7.
26 N. H. Williams and J. K. Yandell, *Aust. J. Chem.*, 1982, **35**, 1133.
27 S. Steenken and S. V. Jovanovic, *J. Am. Chem. Soc.*, 1997, **119**, 617.
28 G. R. A. Johnson, N. B. Nazhat and R. A. Saadalla-Nazhat, *J. Chem. Soc., Chem. Commun.*, 1985, 407.
29 W. H. Koppenol, *Free Radical Biol. Med.*, 1985, **1**, 281.
30 M. Al-Sheikhly, *Radiat. Phys. Chem.*, 1994, **44**, 297.
31 S. Steenken, S. V. Jovanovic, M. Bietti and K. Bernhard, *J. Am. Chem. Soc.*, 2000, **122**, 2373.