Electron Paramagnetic Resonance Studies of the Effects of 1:1 Electrolytes on the Action of Ionizing Radiation on Aqueous DNA

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Exposure of dilute aqueous solutions of DNA to ^{60}Co γ-rays at 77 K gives EPR spectra characteristic of $^{\bullet}OH$ radicals in ice, and DNA radicals thought to be mainly $G^{\bullet+}$ radical cations and a mixture of $C^{\bullet-}$ and $T^{\bullet-}$ radical anions. These DNA radicals are probably stabilized by rapid gain or loss of protons. The $^{\bullet}OH$ radicals form H_2O_2 in the ice-phase at *ca.* 130 K. The $T^{\bullet-}$ centres are irreversibly converted to $^{\bullet}TH$ radicals by protonation at C6 on annealing to *ca.* 200 K, but no other intermediate radical centres are clearly defined. All radical centres are lost prior to complete melting.

On the addition of LiCl or NaCl in the 0–1.0 mol dm^{-3} region, there is a two-fold increase in the yields of DNA radicals, this being mainly in the anionic centres. Trapped $Cl_2^{\bullet-}$ ions grow in with increasing concentration. On annealing, $^{\bullet}TH$ radicals are formed, but, especially for LiCl systems, these are lost increasingly rapidly as the LiCl concentration increases. Despite the increases in target volume, very little change in the yields of strand-breaks is induced by these salts. Sodium bromide gives a smaller initial increase and more rapid loss of DNA radicals on annealing. The yields of $^{\bullet}TH$ are considerably reduced indicating protection despite the increased target volume. This is reflected in a reduction in the yields of strand-breaks.

The tetroxy salt sodium perchlorate also gives rise to an increase of initial DNA radical concentration. In contrast to the halide salts, however, this is assigned to an increase in $[G^{\bullet+}]$ with little or no effect upon the concentration of anionic centres. Strand-break studies show a marked protection of DNA by the perchlorate ion.

These results are discussed in terms of the increase in effective target volume and the reactivities of the salt radicals $Cl_2^{\bullet-}$, $Br_2^{\bullet-}$ and $O^{\bullet-}$, all of which are detected by EPR spectroscopy.

In the course of our studies of radiation damage to DNA [1–8] we have examined the effects of a range of 1:1 electrolytes on the yields of radicals and strand-breaks. In some cases, very large changes have been observed, as reported herein. Our aim is to attempt to rationalize these changes. There are two major potential modes of damage to aqueous DNA, one, of overriding importance for dilute fluid solutions, being damage to water, followed by water radical attack on DNA. This mechanism has been extensively studied.[9–11] The other, of major importance for frozen or concentrated samples, and possibly for DNA in cell nuclei, is direct damage, which we[1–8] and others[12–16] have shown to involve the initial formation of cationic (electron-loss) centres, thought to be largely localized on guanine bases, and anionic (electron-gain) centres, largely localized on cytosine and thymine. In both, one of the major forms of overall damage is strand-breaks, both single (SSB) and double (DSB), the latter being relatively large compared with expectation based on random statistics. We have used estimates of SSBs and DSBs as a useful measure of the extent of overall damage, and EPR spectroscopy to measure the extent and form of the initial damage events. The unusual salt effects were discovered for the direct damage process, using frozen aqueous solutions.

We know of no previous studies of this type. However, electrolyte effects on fluid solutions have been probed in various ways, with somewhat contradictory conclusions. Ward and coworkers initially found that in acidic solution, chloride ions enhanced damage to purine and pyrimidine bases (but not thymine) as a result of attack by $Cl_2^{\bullet-}$.[17] However, they later decided that, if anything, Cl^- protected DNA and had no major effect on damage to DNA or nucleotides and nucleosides in neutral, non-deoxygenated aqueous solutions.[18]

At the cellular level, the results are very dependent upon cell type and condition, upon the salt concentration and on temperature. For example, double maxima were observed with increase in salt concentration with a minimum in the region of isotonic solutions, the maxima corresponding to radioprotection.[19] This is for attached Chinese hamster cells; single cells in suspension behave quite differently. Raaphorst and Dewey have analysed such effects in terms of treatment during and/or after irradiation, interpreting the results in terms of changes in the extent of fixation of damage and of repair.[20] Matsuyama found that all halide ions enhanced radiation-induced killing of yeast and bacterial cells. It was concluded that the extra damage involved changes in the membrane electron-transport system induced by $Cl_2^{\bullet-}$ and $ClO_4^{2\bullet-}$ radical-anions.[21] Kosaka *et al.* and Myers *et al.*, using bromide salts, have suggested that damage is caused to repair enzymes by attack on tyrosine residues.[22] Kada *et al.*[23] found that although potassium iodide sensitized damage to *Bacillus subtillis*, there was no enhanced DNA damage, and transforming DNA was not affected. It was inferred that the repair system was the site of enhanced damage, probably in the form of SH-containing enzymes.

It seems unlikely that DNA alone is directly involved in these complex changes, whereas in our work, attention is entirely focused on DNA.

Results and Discussion

EPR Results.—After exposure to γ-rays at 77 K, $^{\bullet}OH$ radicals are formed in the ice crystallites, but these are lost irreversibly on annealing to *ca.* 130 K and can be ignored. In the absence of added electrolytes, the EPR spectra can be analysed in terms of a broad singlet feature [Fig. 1(*a*)], normally assigned to $G^{\bullet+}$ electron-loss centres, but possibly including some $A^{\bullet+}$ centres.[24] These are almost certainly trapped by proton transfer to hydrogen-bonded neighbours. The electrons are trapped at the pyrimidine bases ($Py^{\bullet-}$) to give a doublet EPR spectrum [Fig. 1(*b*)], again probably with proton transfer (Fig. 2).[25] We

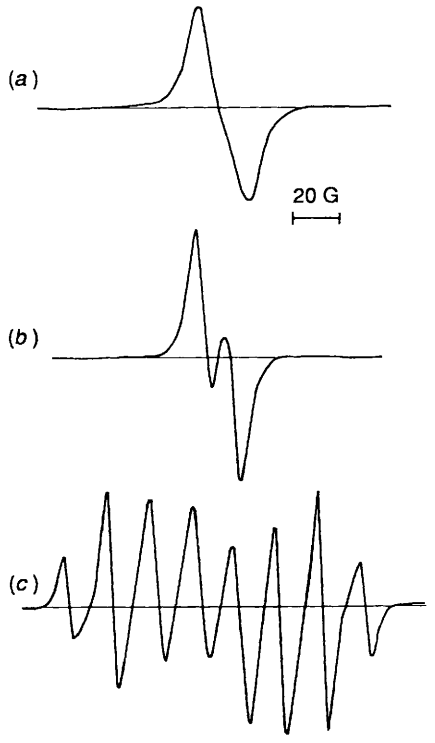


Fig. 1 First derivative X-band EPR spectra for aqueous DNA irradiated at 77 K and annealed to 130 K to remove OH• radical features. The spectrum at 130 K has been deconvoluted to give features assigned to $G^{\bullet+}$ radical-cations (*a*) and a combination of pyrimidine radical anions ($Py^{\bullet-}$) (*b*). After annealing to *ca.* 200 K features assigned to TH• radicals grow in (*c*).

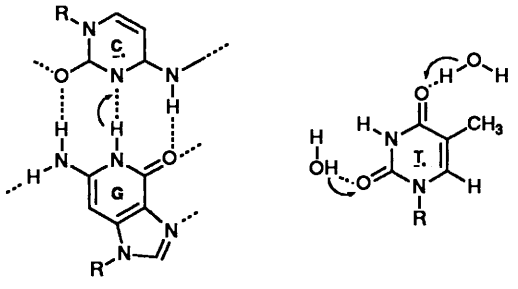


Fig. 2 Suggested modes of heteroatom protonation in DNA for (*a*) $C^{\bullet-}$ and (*b*) $T^{\bullet-}$. Protonation of $C^{\bullet-}$ most likely occurs from the N1 position of the associated guanine base within the DNA whereas $T^{\bullet-}$ is probably protonated by surrounding water molecules, at either O2 or O4.

symbolise these centres as $G^{\bullet+}$, $T^{\bullet-}$ and $C^{\bullet-}$ for convenience.

Sodium and lithium chloride. Both give a steady increase in concentration of DNA radicals as the concentration of salt increases. At *ca.* 1.0 mol dm^{-3}, the radical concentration is about double that for no salt (Fig. 3). Also, the increase in [$Py^{\bullet-}$] is greater than that in [$G^{\bullet+}$] centres as shown in Fig. 4.

At concentrations ≥ *ca.* 0.5 mol dm^{-3}, features for $Cl_2^{\bullet-}$ radicals grow in (Fig. 5). These spectra are best seen at relatively high microwave powers. We use the septet of parallel lines with $A_{||}(^{35}Cl)$ *ca.* 102 G as being characteristic of these centres. Other weaker features are probably due to Cl–$OH^{\bullet-}$ intermediates.[26] On annealing, these features are lost at *ca.* 155 K, which is the temperature at which the decay of the $Py^{\bullet-}$ doublet becomes rapid. These $Cl_2^{\bullet-}$ features are relatively broad, suggesting a glassy medium. However, for [NaCl] > *ca.* 1 mol dm^{-3} relatively sharp $Cl_2^{\bullet-}$ features are also detected, which we suggest come from a crystalline salt hydrate phase. There is also some infrared evidence for this third phase (see below).

On annealing the aqueous and NaCl samples to *ca.* 200 K

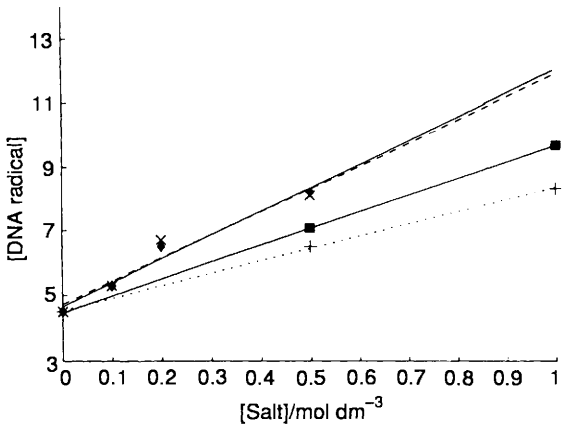


Fig. 3 Trends in the concentration of DNA radicals at 130 K as a function of salt concentration: ■, NaCl; +, LiCl; ×, $NaClO_4$; ◆, NaBr

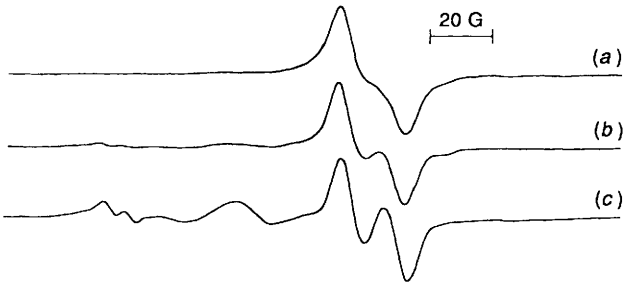


Fig. 4 First derivative X-band EPR spectra for aqueous DNA irradiated at 77 K and annealed to 130 K, (*a*) in the absence of electrolytes, (*b*) in 1 mol dm^{-3} LiCl and (*c*) in 10 mol dm^{-3} LiCl showing the increase in the $Py^{\bullet-}$ doublet with increasing [LiCl] (extra low field features are assigned to $Cl_2^{\bullet-}$ radicals)

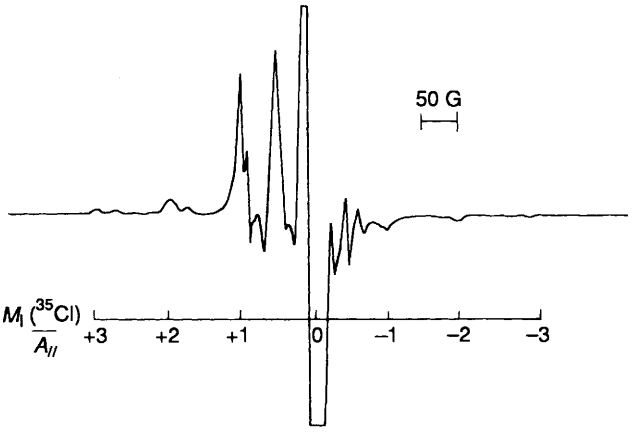
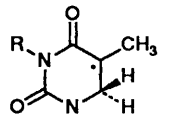


Fig. 5 First derivative X-band EPR spectrum for 50 mg cm^{-3} DNA containing 10 mol dm^{-3} LiCl after exposure to ^{60}Co γ-rays at 77 K, showing features assigned to $Cl_2^{\bullet-}$ radicals (high microwave power was used to accentuate these features)



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there is a steady increase in features assigned to TH• radicals (**1**) formed by protonation at C6 [Fig. 1(*c*)]. Above this temperature, these radicals also decay (Fig. 6). Sodium chloride induces a steady increase in the maximum [TH•] with increase in concentration, but LiCl has little effect. However, for LiCl the amount of TH• formed as a fraction of the initial DNA radical

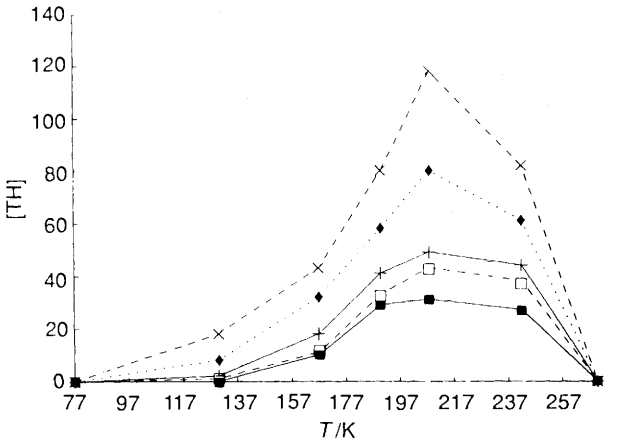

Fig. 6 Temperature profiles for the gain and loss of TH• radicals (arbitrary units) at various concentrations of NaCl: ■, STD; □, 0.5; +, 1.0; ◆, 2.0; ×, 5.0 mol dm^{-3}

concentration drops with increasing salt concentration. Also, the rate of loss of TH• with increase in temperature is enhanced relative to the frozen aqueous standards. Indeed, for very high [LiCl], *i.e.* 10 mol dm^{-3}, there is very little accumulation of TH•, the concentration maximum having shifted to *ca.* 160 K compared with *ca.* 200 K for the aqueous system.

Sodium bromide. For NaBr in the 0–0.5 mol dm^{-3} range, there was again an increase in DNA radicals (Fig. 3). However, on annealing, these decayed more rapidly than for the aqueous standard. Again, at high salt concentrations, features for $Br_2^{\bullet -}$ characterized by seven parallel lines with $A_{\parallel}(^{81}Br)$ *ca.* 480 G were well defined.[26]

Sodium perchlorate. In this case, although there was a similar increase in total DNA radical yield, this was mainly an increase in $G^{\bullet +}$ rather than in $Py^{\bullet -}$. The maximum yield of TH• was actually less than that in the absence of salt. For $[NaClO_4] \geqslant$ *ca.* 0.5 mol dm^{-3}, a $g = 2.08$ feature that has been assigned to $O^{\bullet -}$ [27,28] was clearly defined at high microwave powers. This is thought to be formed from ClO_4^- by electron addition. The $ClO_4^{2\bullet -}$ radical ion[29] is unstable in water and breaks down to give solvated $O^{\bullet -}$ ions and ClO_3^- ions,[27] even at 77 K [eqns. (1) and (2)].

$$ClO_4^- + e^- \longrightarrow ClO_4^{2\bullet -} \quad (1)$$

$$ClO_4^{2\bullet -} \longrightarrow ClO_3^- + O^{\bullet -} \quad (2)$$

Since hole-centres for perchlorate glasses are not extensively trapped, they must be effectively mobile. This mobility is presumably achieved by electron-transfers such as eqn. (3) leading ultimately to reaction with DNA [eqn. (4)] (Fig. 7).

$$ClO_4^- + ClO_4^{\bullet} \rightleftharpoons ClO_4^{\bullet} + ClO_4^- \quad (3)$$

$$ClO_4^{\bullet} + G \longrightarrow ClO_4^- + G^{\bullet +} \quad (4)$$

Infrared Studies.—These will be reported fully elsewhere.[30] Here we simply stress that they are based on the fact that, using HOD in D_2O rather than H_2O, the O–H stretch band to HOD in ice crystals is a relatively sharp single band. However, HOD molecules in glassy regions and in solvation sites contribute to a much broader feature whose maximum is shifted to higher frequencies. Thus a semi-quantitative assessment of the extent of phase-separation can be made. These results support the concept that as [salt] increases, so [ice] falls and the target volume of solvated DNA + solvated salt increases. They also revealed the formation of a separate salt hydrate phase for sodium chloride at > *ca.* 1.0 mol dm^{-3}.

Strand-breaks.—These will also be described fully elsewhere.[31] Here we note that plasmid DNA is used and the supercoiled form separated from the circular form (SSB) and the linear form (DSB) by gel-electrophoresis. This gives a sensitive measure of SSB but a relatively poor measure of DSB. Qualitatively, NaCl and LiCl in the 0–1.0 mol dm^{-3} range have very little effect on the [SSB] or [DSB]. Some experiments gave small increases but others gave small decreases in yields. Sodium bromide gave clear protection. In contrast, $NaClO_4$ gave clear increases in both SSB and DSB. These changes were comparable in magnitude to those in the EPR spectra indicated in Fig. 3.

Possible Mechanisms.—The results are interpreted in terms of several competing factors. One is an increase in target volume. We consider that radicals formed in the ice phase cannot reach the DNA and can be ignored. However, as salt is added, this accumulates in the glassy DNA phase rather than forming a third phase (except for NaCl at high concentrations). If electrons, holes or radicals formed within the glass phase can migrate to the DNA prior to undergoing other types of reaction, the number of damage centres will increase (Fig. 7).

Chloride salts. Apart from glassy solvent cavities, which give trapped electrons,[32] there are no chemical traps for e^- and hence the yield of $Py^{\bullet -}$ should increase. This is indeed observed. However, electron-loss (hole) centres, namely $H_2O^{\bullet +}$ and $Cl^{\bullet}$, are rapidly converted into •OH and $Cl_2^{\bullet -}$ radicals, which are both trapped in the glass at low temperatures. Our results suggest that $Cl_2^{\bullet -}$ radicals are the major trapped-hole centres under the conditions used. These are probably formed *via* reactions (5–7).

$$^{\bullet}OH + Cl^- \longrightarrow HO\text{–}Cl^{\bullet -} \quad (5)$$

$$HO\text{–}Cl^{\bullet -} + Cl^- \longrightarrow Cl_2^{\bullet -} + OH^- \quad (6)$$

$$Cl^{\bullet} + Cl^- \longrightarrow Cl_2^{\bullet -} \quad (7)$$

Thus we do not expect any large initial target volume effect for $G^{\bullet +}$ yields, since, in contrast with the electrons, the holes are efficiently trapped. This is in good agreement with our EPR results.

One problem is how to explain the negligible effect of the chlorides on strand-breaks. We tentatively suggest that it is the relative stability of the $Cl_2^{\bullet -}$ radicals at low temperatures that is responsible. These radicals can react with activated C–H hydrogen, but the reactions are slow at room temperature,[33] being *ca.* 10^4 times slower than those of chlorine atoms.[34] However, these radicals are very good electron acceptors, so it is possible that they might react with $C^{\bullet -}$ and $T^{\bullet -}$ *via* eqn. (8), thereby reducing the number of strand-breaks.

$$Py^{\bullet -} + Cl_2^{\bullet -} \longrightarrow Py + 2\,Cl^- \quad (8)$$

This reaction has low probability because it is between two species in low concentration. However, we can discover no other reasonable way of accommodating the EPR and strand-break results.

Sodium bromide. In this case, there is an overall final protective (*i.e.* fewer strand-breaks) effect despite the initial target volume enhancement. We suggest that electron transfer from Br^- to $G^{\bullet +}$ centres can occur on annealing, giving $G + Br_2^{\bullet -}$ [eqn. (9)]. This will enhance the yields of $Br_2^{\bullet -}$ which, in turn, can act as an electron accepter, as in the case of $Cl_2^{\bullet -}$. Both reactions serve to protect the DNA.

$$G^{\bullet +} + 2\,Br^- \longrightarrow G + Br_2^{\bullet -} \quad (9)$$

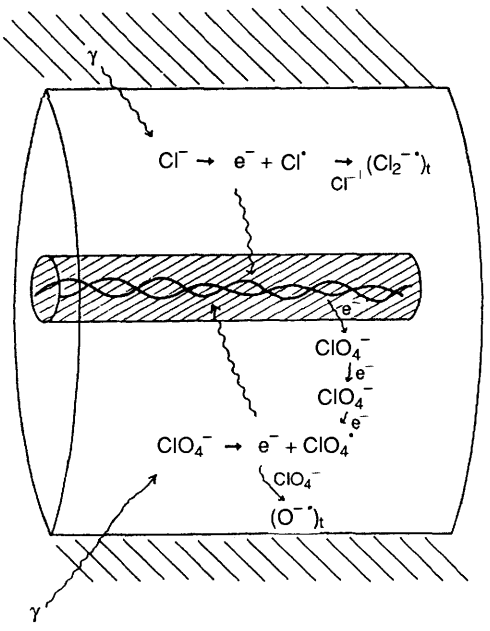

Fig. 7 Effect of increased target volume, induced by added electrolytes (outer cylinder), in the total radiation damage to DNA. The shaded cylinder represents the standard volume for frozen aqueous DNA, within which all damage is thought to reach the DNA. The outer shading represents the pure ice region.

In fact, such processes are just those that we have been looking for in possible radiation-protection redox catalysts.[5] By donating electrons to $G^{\bullet+}$ and accepting them from $T^{\bullet-}$ and $C^{\bullet-}$, a relatively large overall protection is observed. This is larger than the results suggest superficially because the enhanced target volume should have led to considerably increased damage.

Sodium perchlorate. The perchlorate ion is an electron acceptor despite its negative charge. That it does, in fact, trap electrons is shown by the appearance of the $O^{\bullet-}$ radical signal and by the reduction in the proportion of the doublet species ($Py^{\bullet-}$) in the centre of the EPR spectra. However, it is a poor electron donor and hence is not expected to suppress formation of $G^{\bullet+}$ centres in DNA. There is some EPR evidence for •OH radicals trapped in the glass, but their concentration is low. The EPR results show small increases in the yields of $T^{\bullet-}$ ($TH^{\bullet}$) and large increases in the yields of $G^{\bullet+}$. Also, in this case, there is a considerable enhancement in the yields of strand-breaks which agrees well with the EPR data and expectation.

Conclusions

We conclude that there is a marked target volume effect, even in these solid systems. This serves to increase the concentrations of DNA radicals selectively. For the chlorides, the increase is primarily for DNA radical anions. The solvent traps the hole centres ($Cl_2^{\bullet-}$) which, on annealing, remove electrons from DNA so that there is no enhancement in the number of strand-breaks. For the bromides, electron-loss centres in DNA ($G^{\bullet+}$) may react to give G and $Br_2^{\bullet-}$. In turn, the $Br_2^{\bullet-}$ radicals can again accept electrons from $C^{\bullet-}$ or $T^{\bullet-}$ centres. Thus there is overall protection.

Perchlorate acts as an electron trap, giving $O^{\bullet-}$ which will be converted into •OH radicals on melting. These may then attack the DNA. Nearby holes migrate to DNA giving enhanced yields of $G^{\bullet+}$ and an overall increase in damage.

Acknowledgements

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References

1 P. J. Boon, P. M. Cullis, M. C. R. Symons and B. W. Wren, *J. Chem. Soc., Perkin Trans. 2*, 1984, 1393.
2 P. J. Boon, P. M. Cullis, M. C. R. Symons and B. W. Wren, *J. Chem. Soc., Perkin Trans. 2*, 1985, 1057.
3 P. M. Cullis, M. C. R. Symons, B. W. Wren and S. Gregoli, *J. Chem. Soc., Perkin Trans. 2*, 1985, 1819.
4 P. M. Cullis, M. C. R. Symons, M. C. Sweeney, G. D. D. Jones and J. D. McClymont, *J. Chem. Soc., Perkin Trans. 2*, 1986, 1671.
5 M. C. R. Symons, *J. Chem. Soc., Faraday Trans. 1*, 1987, **83**, 1.
6 P. M. Cullis, G. D. D. Jones, J. S. Lea, M. C. R. Symons and M. C. Sweeney, *J. Chem. Soc., Perkin Trans. 2*, 1987, 1907.
7 P. M. Cullis, S. Langman, I. D. Podmore and M. C. R. Symons, *J. Chem. Soc., Faraday Trans.*, 1990, **86**, 3267.
8 M. C. R. Symons, *Int. J. Radiat. Biol.*, 1990, **58**, 93.
9 C. Von Sonntag and H.-P. Schuchmann, *Int. J. Radiat. Biol.*, 1986, **49**, 1.
10 C. Von Sonntag, *Radiat. Phys. Chem.*, 1987, **30**, 313.
11 S. Steenken, *Chem. Rev.*, 1989, **89**, 503.
12 A. Gräslund, L. Ehrenberg, A. Rupprecht, G. Ström and H. Crespi, *Radiat. Res.*, 1975, **61**, 488.
13 W. A. Bernhard, *Adv. Radiat. Biol.*, 1981, **9**, 199.
14 S. Gregoli, M. Olast and A. Bertinchamps, *Rad. Res.*, 1979, **77**, 417.
15 S. Gregoli, M. Olast and A. Bertinchamps, *Rad. Res.*, 1982, **89**, 238.
16 J. Hüttermann, K. Voit, H. Oloff, W. Köhnlein, A. Gräslund and A. Rupprecht, *Faraday Discuss. R. Soc. Chem.*, 1984, **78**, 135.
17 J. F. Ward and I. Kuo, *Int. J. Radiat. Biol.*, 1970, **18**, 381.
18 J. F. Ward and V. O. Mora-Arellano, *Int. J. Radiat. Oncol., Biol. Phys.*, 1984, **10**, 1523.
19 P. G. Moggach, J. R. Lepock and J. Kruuv, *Int. J. Radiat. Biol.*, 1979, **36**, 435.
20 G. P. Raaphorst and W. C. Dewey, *Int. J. Radiat. Biol.*, 1979, **36**, 303.
21 A. Matsuyama, M. Mamiki and Y. Okazawa, *Radiat. Res.*, 1967, **30**, 687.
22 T. Kosaka, I. Kaveko and F. Koide, *Int. J. Radiat. Biol.*, 1990, **58**, 417.
23 T. Kada, T. Noguti and M. Namiki, *Int. J. Radiat. Biol.*, 1970, **17**, 407.
24 M. D. Sevilla, D. Becker, M. Yan and R. Summerfield, *J. Phys. Chem.*, 1991, **95**, 3409.
25 S. Steenken, *Chem. Rev.*, 1989, **89**, 503.
26 I. S. Ginns and M. C. R. Symons, *J. Chem. Soc., Dalton Trans.*, 1972, 143.
27 M. D. Sevilla, J. B. D'Arcy, K. M. Morehouse and M. L. Engelhardt, *Photochem. Photobiol.*, 1979, **29**, 37.
28 M. J. Blandamer, L. Shields and M. C. R. Symons, *J. Chem. Soc.*, 1964, 4352.
29 M. D. D. Bloom, R. S. Eachus and M. C. R. Symons, *J. Chem. Soc. A*, 1970, 1235.
30 C. Blackburn, H. Pelmore and M. C. R. Symons, unpublished results.
31 P. M. Cullis, D. Elsey, S. Fan and M. C. R. Symons, unpublished results.
32 M. C. R. Symons, *Chem. Soc. Rev.*, 1976, **5**, 337.
33 K. Hasegawa and P Neta, *J. Phys. Chem.*, 1978, **82**, 854.
34 B. C. Gilbert, J. K. Stell, W. J. Peet and K. J. Radford, *J. Chem. Soc., Faraday Trans. 1*, 1988, **84**, 3319.

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