Effects of Ionizing Radiation on Deoxyribonucleic Acid. Part 7. Electron Capture at Cytosine and Thymine†

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Exposure of a range of DNA samples in various media to ^{60}Co γ-rays at 77 K gives electron-capture centres characterized by an EPR doublet with $A(^1H)$ of *ca.* 16 G. Since the electron-adducts of C and T give very similar doublet EPR spectra in irradiated DNA, it is difficult to judge the proportions of $C^{\bullet -}$ and $T^{\bullet -}$ formation by inspection. The possibility, suggested by others, that computer fits can be used to give a quantitative measure of these species is discussed. However, in view of the variability of the features directly assignable to $C^{\bullet -}$ and $T^{\bullet -}$ units in different environments, we suggest that this approach has only qualitative significance.

The alternative method involves annealing to convert $T^{\bullet -}$ into $TH^{\bullet}$ radicals in which a hydrogen atom is added to C_6, the resulting radical having a completely characteristic octet EPR spectrum. It is argued that the ejected electrons move through the stacked DNA bases, becoming trapped at C or T depending upon the relative rates at which $C^{\bullet -}$ and $T^{\bullet -}$ are protonated to give $C^{\bullet -}(H^+)$ (protonated at N_3) and $T^{\bullet -}(H^+)$ (protonated on oxygen). If this is correct, interconversion between $C^{\bullet -}$ and $T^{\bullet -}$ on annealing is unlikely, and only $T^{\bullet -}$ can lead to $TH^{\bullet}$ formation.

This is also not accurate, since $TH^{\bullet}$ decay sets in within the same temperature range as it is being formed. By generating $TH^{\bullet}$ from frozen aqueous DNA with ultraviolet light, the pure decay annealing curve has been obtained, and using this we have been able to extrapolate the data for $TH^{\bullet}$ from the γ-irradiated samples to give the real yields of $TH^{\bullet}$. The results show that *ca.* 36% of the doublet must be due to $T^{\bullet -}$ centres, the remainder (64%) being assigned to $C^{\bullet -}$ centres.

The ratio of $C^{\bullet -}$ to $T^{\bullet -}$ varies with the DNA source, and with the environment. We suggest that it is largely governed by the relative rates of protonation to give $C^{\bullet -}(H^+)$ and $T^{\bullet -}(H^+)$, and the factors controlling these rates are discussed. The use of lithium chloride glasses completely suppresses the formation of $G^{\bullet +}$ centres, leaving well-defined radical-anion spectra, but on annealing, conversion to $TH^{\bullet}$ is negligible despite the rapid, and complete, loss of the doublet species. This result is discussed in terms of reaction with $Cl_2^{\bullet -}$ radicals formed in abundance in these glasses.

Studies designed to detect any site-specificity in the DNA damage leading to strand breaks suggest that all possible sites are damaged. These results strongly support the postulate that yields of $C^{\bullet -}$ and $T^{\bullet -}$ are comparable. The possibility that some $A^{\bullet +}$ cations are formed in addition to $G^{\bullet +}$ cations is also considered in the light of these results.

In our studies of radiation damage to DNA,[1–7] we have largely confined our attention to DNA itself because of the extensive work previously carried out by others using single crystals or frozen solutions of bases, nucleosides, nucleotides and small oligomers.[8–11] The system that we selected was frozen aqueous DNA, since others had shown that good yields of primary DNA radical centres were trapped at 77 K and could be readily studied by EPR spectroscopy.[12,13] For our EPR spectroscopic studies, calf thymus DNA was generally used since other sources of high molecular weight DNA have been shown to give similar results.

These systems are phase separated on cooling into a pure ice phase and a DNA phase containing glassy water which is thought to correspond to fully aquated DNA in the liquid state. The only radical detected in the ice phase at 77 K is $^{\bullet}OH$, which has a characteristic EPR spectrum[14] and is largely converted into hydrogen peroxide on annealing to *ca.* 130 K. These radicals are not involved in any reactions of DNA.

When aqueous solutions of DNA are exposed to ionizing radiation at low temperatures, two major signals from DNA radicals are detected by EPR spectroscopy. One, a broad singlet, is generally assigned to some form of electron-loss centre based on guanine, and the other, a doublet, is assigned to centres derived either from cytosine radical-anions or thymine radical-anions.

In earlier EPR studies of frozen irradiated DNA it was generally assumed that the doublet feature is assignable to $T^{\bullet -}$ electron-gain centres, and the singlet to $G^{\bullet +}$ electron-loss centres, although Bernhard warned that the former assignment could be wrong. We stress that this is a surprising result. Ionizing radiation generally damages indiscriminately. So initial electron-loss centres should include $H_2O^{\bullet +}$ (from solvating water), $(RO)_2PO_2^{\bullet}$, sugar radical-cations, and all four base radical-cations. Similarly, one might expect that the electrons would react at phosphate groups or with any of the four bases. The results require migration of holes and electrons at rates greater than those for proton or group loss. They also require that trapping in some form is ultimately specific and effective. This is almost certainly induced by proton gain and loss from $(base)^{\bullet -}$ and $(base)^{\bullet +}$ centres.

The $T^{\bullet -}$ assignment was given because of conversion of the 'doublet' centre into the completely characterized $TH^{\bullet}$ radical on annealing.[15] The warning was given because the 'powder' EPR spectrum for '$C^{\bullet -}$' was found to be almost indistinguish-

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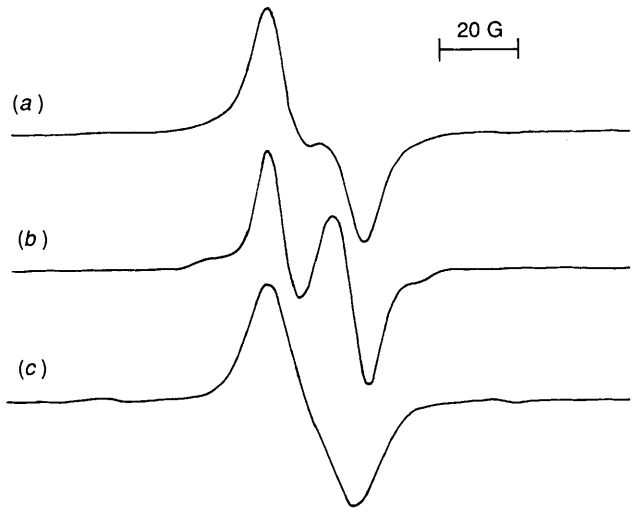


Fig. 1 X-band EPR spectra of (*a*) γ-irradiated frozen aqueous DNA annealed to 130 K and recorded at 77 K using a microwave power of 0.01 mW; (*b*) the DNA radical-anion spectrum generated by subtraction of the high power spectrum (0.2 mW) from the low power spectrum (0.01 mW) and (*c*) the DNA radical-cation spectrum generated by subtraction of the low power spectrum from the high power spectrum

able from that for $T^{\bullet -}$. This warning was reinforced by studies of oriented DNA ribbon.[16,17] The results were interpreted in terms of $G^{\bullet +}$ and $T^{\bullet -}$ but Bernhard stressed that the well resolved spectrum assigned to $T^{\bullet -}$ gave results which were not in good accord with those for this radical trapped in single crystals.[11]

This issue has now been reopened, largely as a result of renewed attacks on the problem by Bernhard and co-workers,[18–20] Sevilla *et al.*,[21] and Hüttermann and co-workers.[22] All these groups have come to the conclusion that the major anionic centre formed in DNA at low temperatures is actually $C^{\bullet -}$ not $T^{\bullet -}$. Our aim has been to endeavour to assess the proportion of $T^{\bullet -}$ and $C^{\bullet -}$ centres formed in the systems that we have normally used.

We, and others, generally represent these primary centres as $G^{\bullet +}$, $T^{\bullet -}$, $TH^{\bullet}$ and $C^{\bullet -}$, and we use this symbolism herein. However, we stress that rapid gain or loss of protons probably occurs, even at very low temperatures. For $G^{\bullet +}$, various single crystal studies of G have shown that proton-loss is indeed efficient. This occurs from nitrogen, but opinions differ as to which proton is involved in the solid state.[23,24] For our purposes this is simply represented as $G^{\bullet +}(-H^{+})$ since we are unable to contribute to the identification.

The first results that we obtained, which indicated that the $G^{\bullet +} + T^{\bullet -}$ theory was an over-simplification, came from strand-break specificity studies.[25] We found no evidence for preferential breaks in G, T regions under direct damage conditions. This work is described in detail herein.

Experimental

Thymidine-5′-monophosphate (TMP), 2′-deoxycytidine-5′-monophosphate (dCMP), thymidylyl (3′-5′) deoxycytidine (TpdC), polydeoxyadenylic–thymidylic acid (Poly[dA–dT]·Poly[dA–dT]), polydeoxyguanylic–deoxycytidylic acid (Poly-[dG–dC]·Poly[dG–dC]), deoxyribonucleic acid (DNA) from salmon testes, *Escherichia coli*, *Micrococcus lysodeikticus* and *Clostridium penfringens* were all obtained from Sigma Chemical Co. Calf thymus DNA was obtained from BDH. Lithium chloride and $K_3Fe(CN)_6$ were obtained from Aldrich Chemical Co. These materials were used without further purification. The H_2O was obtained from a Millipore 'Multi-Q' purifier.

Procedure.—Two media were employed for this investigation, aqueous lithium chloride glasses and frozen aqueous solutions. The lithium chloride glasses were made using nucleotide and dinucleotide concentrations of 50 mmol dm^{-3}. For DNA, (Poly[dG–dC]·Poly[dG–dC]) and (Poly[dA–dT]·Poly[dA–dT]) glasses were prepared by mixing 50 mg cm^{-3} of solute with an equal volume of saturated LiCl solution. Cylindrical 'pellets' were made by freezing the solutions in tin foil moulds. Frozen aqueous DNA (50 mg cm^{-3}) were prepared by freezing *ca.* 0.3 cm^3 of solution in a Pyrex tube.

Samples were γ-irradiated at 77 K using a Vickrad ^{60}Co γ-ray source; irradiations were carried out using doses of *ca.* 1 Mrad for the glasses and 4 Mrad for the frozen aqueous DNA. UV irradiations were carried out at 77 K using a Hanovia high pressure mercury arc. EPR spectra were recorded at 77 K on a JEOL JES-RE1Z X-band spectrometer, interfaced with an Archimedes computer. The radical-anion EPR spectra in aqueous lithium chloride glasses were obtained directly at 77 K. To test whether electron adducts do form, and that no solute radical-cations are formed in the lithium chloride glass, a powerful electron scavenger [$K_3Fe(CN)_6$] was employed. Complete suppression of radical-anion formation was obtained with 8 mg cm^{-3} ferricyanide with no evidence of solute radical-cations. Samples were annealed using a copper block cryostat. Typically, they were allowed to warm to a predetermined temperature which was maintained for 8 min before recooling to 77 K.

Pellets of the γ-irradiated frozen aqueous DNA samples were annealed to *ca.* 130 K to remove $^{\bullet}OH$ radicals present in the ice phase. Radical growth and decay studies were achieved by stepwise annealing up to 270 K. The EPR spectra of the UV-irradiated frozen aqueous DNA were obtained directly at 77 K and the $TH^{\bullet}$ decay profile was measured upon annealing using the height of the seventh line to measure the relative amount of $TH^{\bullet}$.

Gel Electrophoresis.—Samples of irradiated DNA (20 mm^3) were layered onto agarose slab gels (1.4%) and electrophoresed in a horizontal gel apparatus at 120 V for *ca.* 1.5 h at room temperature using TBE buffer (98 mmol dm^{-3} Tris, 98 mmol dm^{-3} boric acid and 2.5 mmol dm^{-3} EDTA, pH 8.3). After electrophoresis the gels were stained with ethidium bromide (2.5 μg cm^{-3}) in electrophoresis buffer and then washed. The stained gels were then excited with a transilluminator and photographed and the negatives used for densitometric scanning.

Results and Discussion

Isolation of EPR Spectra for Individual Primary Centres.—The primary radical centres under direct damage conditions in DNA are radical-anion ($T^{\bullet -}/C^{\bullet -}$) and radical-cation ($G^{\bullet +}/A^{\bullet +}$) centres giving rise to poorly resolved composite EPR spectra (Fig. 1). The radical-anion doublet (Fig. 1) can be isolated by subtraction of spectra obtained at high and low microwave powers.[12,13,21] This method depends on the fact that the doublet spectrum saturates more readily than the 'singlet' (usually assigned to $G^{\bullet +}$) so that at high powers the singlet dominates (Fig. 1). Alternatively, purine radical-cations can be generated by γ-irradiation of the purine and $K_3Fe(CN)_6$ in 10 mol dm^{-3} LiCl.[21] The ferricyanide acts as an electron capture agent and upon annealing to 155 K the purine undergoes one-electron oxidation by $Cl_2^{\bullet -}$. Subtraction of the radical-cation spectra from the primary frozen aqueous DNA spectrum at 130 K yields the radical-anion doublet.

Alternatively, good radical-anion spectra, largely free of cation signals, can be obtained by radiolysis of calf thymus DNA and the polynucleotides {Poly(dG–dC) and Poly(dA–

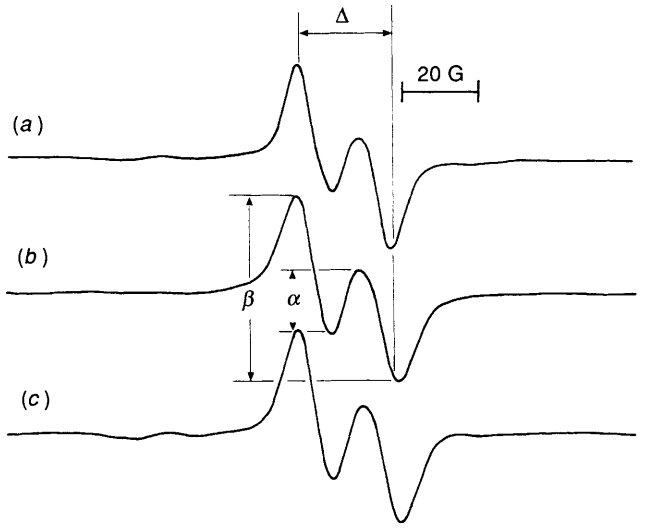


Fig. 2 X-band EPR radical anion 'benchmark' spectra of: (*a*) (Poly-[dA–dT]•Poly[dA–dT])[$T^{\bullet -}$]; (*b*) (Poly[dG–dC]•Poly[dG–dC])[$C^{\bullet -}$] and (*c*) DNA [$DNA^{\bullet -}$]. The radical-anions were generated by γ-irradiation in $LiCl/H_2O$ glasses. All spectra were recorded at 77 K and are shown with the solvent signal subtracted.

Table 1 The separation between points of maximum slope (*Δ*) for the $C^{\bullet -}$, $T^{\bullet -}$ and $DNA^{\bullet -}$ doublets generated by γ-irradiation in 10 mol dm^{-3} LiCl. A trend between *Δ* and % GC content should be expected, but is not evident

Compound	% GC	*Δ*	α/β
Poly(dG–dC)	100	25.3	0.35
Poly(dA–dT)	0	22.8	0.30
Clostridium perfringens DNA	30	25.3	0.35
Salmon testis DNA	41	25.0	0.33
Calf thymus DNA	42	25.0	0.37
E. coli DNA	50	26.0	0.33
M. lysideikticus DNA	72	25.0	0.31

dT)} in 10 mol dm^{-3} LiCl glasses. In these systems, electron-loss centres are primarily $Cl_2^{\bullet -}$ radicals and the radical-anion centres have well resolved doublet EPR spectra (Fig. 2). Of the four possible radical-anions the two purine radical-anions $A^{\bullet -}$ and $G^{\bullet -}$ have singlet spectra which suggests that their contribution to the anionic spectra of the polymers is negligible. Electron affinities of the pyrimidines appear to be much greater than those of the purines,[26–29] consequently, spectra from Poly(dA–dT)$^{\bullet -}$ and Poly(dG–dC)$^{\bullet -}$ in LiCl glasses may be considered as benchmark spectra of $T^{\bullet -}$ and $C^{\bullet -}$ respectively, in LiCl glasses.

From Fig. 2 it is apparent that in DNA we are faced with the problem of distinguishing between two very similar doublet spectra. One is assigned to $T^{\bullet -}$ or its protonated form $T^{\bullet -}(H^+)$ (Fig. 3).[30] The other doublet is assigned to $C^{\bullet -}(H^+)$ where the proton has added to N(3) (Fig. 4) rather than to the $-NH_2$ group as occurs in the free base and its derivatives.[31,32] The hyperfine coupling to the N(3) proton is too small to detect so only a doublet, from coupling to the C_6-H proton is resolved. We stress that non-polymeric cytosine derivatives in D_2O also

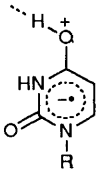


Fig. 3 Structure of $T^{\bullet -}(H^+)$

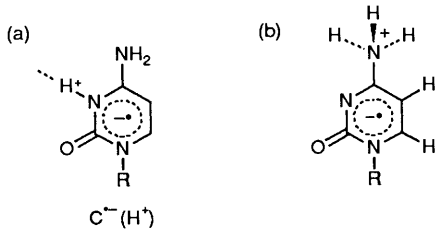


Fig. 4 Possible structure of $C^{\bullet -}(H^+)$. (*a*) Proton attached to N(3). (*b*) Proton attached to NH_2.

give similar doublet spectra. However, these are due to the $-ND_3^+$ species and should not be used as a measure of the $C^{\bullet -}(H^+)$[N(3)-H^+] species found in DNA and other duplex systems, since the two radicals must surely differ.

As is evident from Fig. 2 the task of differentiating between the $T^{\bullet -}/T^{\bullet -}(H^+)$ and $C^{\bullet -}(H^+)$ doublets is a formidable one. Comparisons of $DNA^{\bullet -}$ spectra with computed mixtures of the $C^{\bullet -}$ and $T^{\bullet -}$ doublets suggest the contribution of $C^{\bullet -}$ to be ≥90%. A simple and reproducible measure of comparison is the separation between points of maximum slope (*Δ*) (Fig. 2), which is in agreement with the visual comparison. However, in contrast to this measure, comparison of the depth of the switchback (α/β) (Fig. 2) does not indicate such a large $C^{\bullet -}/T^{\bullet -}$ ratio (*ca.* 60:40), but owing to the variability of this factor from sample to sample we place more weight on the measure of *Δ*. For DNA samples of different GC content some trend in *Δ* values between the two extremes should be expected, however, this does not occur and the *Δ* values remain almost constant (Table 1).

Two groups[18,21] have used 'benchmark' spectra for $C^{\bullet -}$ and $T^{\bullet -}$ with least squares computer analysis to break down the DNA doublet into its $C^{\bullet -}$ and $T^{\bullet -}$ components. This method certainly provides clear answers. The question remains as to how reliable these answers are. The problem is that the doublets obtained from authentic $C^{\bullet -}$ and $T^{\bullet -}$ centres may themselves be quite variable for different systems and in different environments. We suggest that such computational procedures should not be taken as giving accurate measures of the relative concentrations of $C^{\bullet -}$ and $T^{\bullet -}$ centres in DNA. Another reason for making this suggestion is that there are clearly environmental effects on the EPR parameters. These will be exaggerated for $C^{\bullet -}$ and $T^{\bullet -}$ centres in DNA rather than in benchmark spectra since there will be a wide range of different local environments in DNA which will tend to broaden the spectra and hence make any differentiation even more difficult. Because of the extreme and apparently random variations in the centre regions of these spectra (Table 1), computer fits, in our hands, are also scattered with no significant trends. Consequently, we feel that no clear distinction between the $C^{\bullet -}$ and $T^{\bullet -}$ centres can be made.

Electron Competition in Thymine and Cytosine Nucleotides and Dinucleotides.—Our results establish that for irradiated frozen aqueous DNA, the yield of TH$^{\bullet}$ radicals is at least 36% based on the initial radical-anion yield at 130 K (it is assumed that there is a 50/50 distribution of radical-anions and radical-cations at this temperature). Thus either the primary yield of $T^{\bullet -}$ is at least 36% or some electron transfer from $C^{\bullet -}$ to $T^{\bullet -}$ must occur prior to the formation of TH$^{\bullet}$. Such transfer is, of course, invoked in the initial stage of capture of the mobile electrons and, if it were to occur, it surely represents a slow extension of this process.

If the computer analyses[18,21] of the initial EPR doublet assigned to anionic species are accepted, it becomes necessary to assume a slow, thermally activated step in which $C^{\bullet -} \longrightarrow T^{\bullet -}$ occurs. In this section we examine the probability of such a slow step.

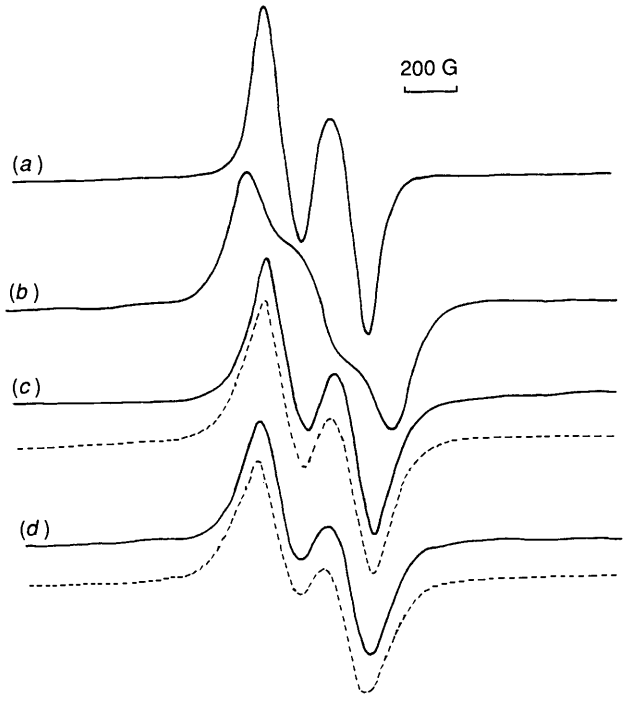
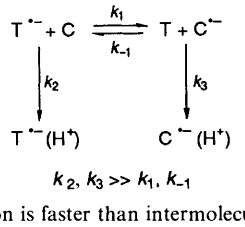


Fig. 5 X-band EPR radical anion spectra of (*a*) TMP, (*b*) dCMP, (*c*) an equimolar mixture of TMP and dCMP with simulation, and (*d*) TpdC with simulation. The radical-anions were produced by γ-irradiation in LiCl glasses and all spectra were recorded at 77 K.

We have recently shown that cytosine and various derivatives thereof give rise to triplet EPR spectra in aqueous (H_2O) solvents because of an extra 12 G splitting from an added proton at the amino group.[31,32] This mode of protonation on the $-NH_2$ group does not seem to occur for duplex systems[19,21,27] ostensibly due to G–C hydrogen bonding. Fortuitously, this triplet improves our ability to distinguish between the two species.

In our competition studies we have used TMP and dCMP in equal concentration in 10 mol dm^{-3} LiCl glasses at 77 K. After radiolysis, the EPR spectra comprised central features for a $C^{\bullet -}$ triplet and a $T^{\bullet -}$ doublet which, using computer simulation were shown to be present in equal concentrations (Fig. 5). Even on annealing to *ca.* 140 K (signals for $Cl_2^{\bullet -}$ are not lost at this temperature), the concentrations remained equal. This result is unusual. What is expected is that even if e^- capture is initially statistical, fast electron-transfer then ensues between neighbouring units, so that the species with high electron affinity gains electrons at the expense of the other species (Scheme 1).

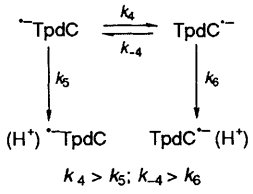


Scheme 1 Protonation is faster than intermolecular electron-transfer in isolated nucleotides

This behaviour can be modified if only one species is rapidly protonated since the protonated species will be favoured. However, if protonation of both anions is very rapid, the initial distribution of electrons should remain fixed, secondary electron-transfer being totally prevented.

We conclude that, in the present case, both the parent anions must be protonated. There is no doubt that $C^{\bullet -}$ is protonated since the added proton leads to an extra hyperfine splitting. If $T^{\bullet -}$ were not protonated, electron-transfer would occur to favour $C^{\bullet -}(H^+)$ formation. Since this is not found even on warming to 140 K, we conclude that $T^{\bullet -}$ must also be protonated, presumably at the O_4 carbonyl oxygen.

In contrast, if the T and C bases are held close together, as in TpdC, intramolecular electron-transfer will be very much faster than the intermolecular transfer discussed above and might be expected to compete effectively with protonation (Scheme 2).



Scheme 2 Intramolecular electron-transfer and protonation in dinucleotides

Hence, it should be driven by the differences in electron affinities. In this case, computer simulation of the electron addition spectrum of TpdC at 77 K using the individual base spectra indicate the electron distribution to be approximately 37% $T^{\bullet -}/T^{\bullet -}(H^+)$ and 63% $C^{\bullet -}(H^+)$ (Fig. 5), which implies that cytosine has the greater electron affinity. It also implies that protonation of $T^{\bullet -}$ still competes to some extent with electron-transfer, since transfer to C is by no means complete. An alternative explanation may be that the $C^{\bullet -}/T^{\bullet -}$ ratio does not pertain to the relative electron affinities of C and T but rather reflects a more rapid protonation of $C^{\bullet -}$. We stress that in TpdC, $C^{\bullet -}$ is again protonated in such a manner that it gives a triplet EPR spectrum, making analysis easy and confirming that protonation does still occur.

Qualitatively, these results are significant to the DNA problem. They confirm the importance of electron-transfer and, in particular, of protonation in the process of final electron trapping and that both base-anions become protonated. They also show that cytosine has a higher electron affinity than thymine and/or protonation at $C^{\bullet -}$ is faster than at $T^{\bullet -}$.

There are major differences compared with the bases in DNA. One is that $C^{\bullet -}$ protonates at an alternative site. Another is that C and T bases are no longer necessarily adjacent. Also in DNA relative electron hole migration is extensive prior to trapping.[33] Finally, base-stacking in the dinucleotide is unlikely to be as efficient as in DNA. Thus quantitative comparison of TpdC and DNA is of no use. The major conclusion, however, is that, in order that electrons be permanently trapped to give $T^{\bullet -}$, it almost certainly becomes protonated otherwise migration to C would be essentially complete. We stress that *O*-protonation of $T^{\bullet -}$ occurs readily at 4 K in crystalline thymidine,[10] so there is good precedent for this postulate even though it is not favoured by others.[34] It has been claimed that *O*-protonation of $T^{\bullet -}$ does not occur in neutral glasses.[11,19] This is based upon the fact that line-width broadening of the doublet, presumably due to *O*-protonation, is observed in acidic glasses. However, this is not a compelling argument since the –O–H coupling is expected to be small and a function of the orientation of the O–H bond relative to the radical plane, falling to zero close to this plane.

Formation of TH• Centres in Frozen Aqueous DNA.—When systems containing $T^{\bullet -}$ centres are annealed the doublet is lost and an octet grows in.[13,15] That this species is the hydrogen adduct is fully accepted by all workers in the field since interpretation of the EPR spectrum is unambiguous. If both anionic centres are initially protonated, the probability of thermally-induced electron-transfer between C and T bases in DNA seems to us to be low. In that case, it should be possible

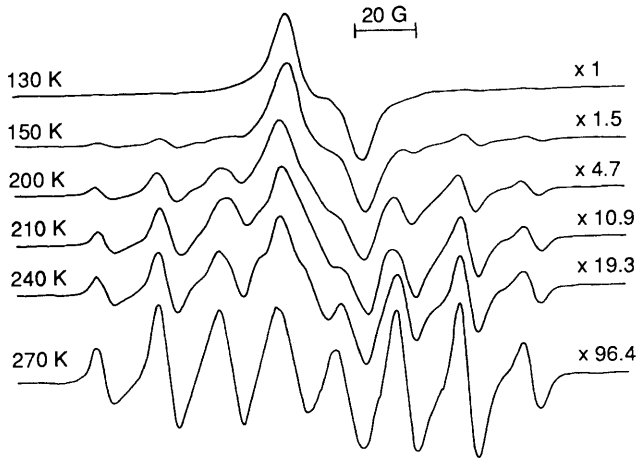


Fig. 6 X-band EPR spectra of γ-irradiated frozen aqueous DNA (H_2O) after annealing to successively higher temperatures. All spectra were recorded at 77 K after annealing to the prescribed temperature for 8 min. In each case, the modulation amplitude was 3.2 G and the microwave power 0.005 mW.

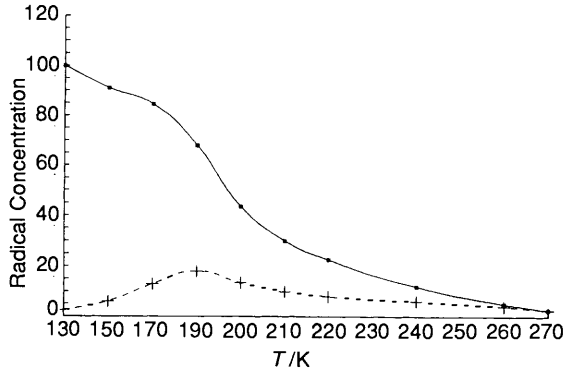


Fig. 7 Thermal anneal curves of frozen aqueous DNA showing the decay of total DNA radicals (■) and the growth and decay of TH$^{\bullet}$ (+) which maximizes at *ca.* 190 K forming approximately 18% of the initial radical yield at 130 K

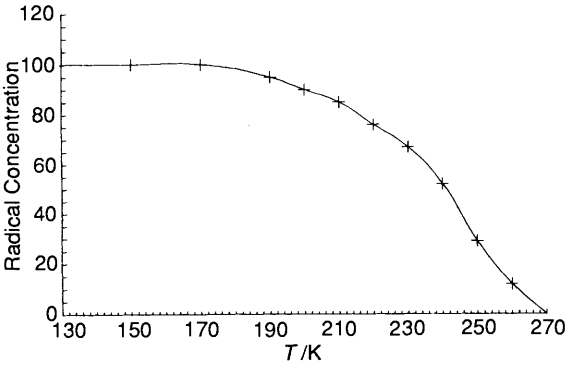


Fig. 8 Thermal decay curve of TH$^{\bullet}$ generated by UV–VIS irradiation of frozen aqueous DNA at 77 K

to use the relative yields of TH$^{\bullet}$ radicals as a measure of the initial ratio of $C^{\bullet -}$ and $T^{\bullet -}$ centres.

Annealing frozen aqueous γ-irradiated DNA pellets to 130 K removes all ice-related EPR signals leaving a mixture of DNA radical-cation and -anion centres which is close to 50%. Further annealing leads to the growth of TH$^{\bullet}$ features (Fig. 6). Thermal decay curves are shown in Fig. 7. The major difficulty with this method of assessing $[TH^{\bullet}]_{max}$ is that decay of TH$^{\bullet}$ may set in before formation from $T^{\bullet -}$ or $T^{\bullet -}(H^+)$ is complete. Also, of course, there may well be other pathways of reaction for the thymine radical anion other than C_6 protonation. The extent of concurrent formation and decay of TH$^{\bullet}$ on annealing can be estimated by generating TH$^{\bullet}$ in frozen aqueous DNA at 77 K using ultraviolet light. This gives TH$^{\bullet}$ radicals in good yield, and the thermal decay curve can be monitored (Fig. 8). By matching this with the high temperature region of the rise and decay curve for γ-irradiated TH$^{\bullet}$ systems it is evident that a reasonable estimate of the total yield of TH$^{\bullet}$ can be obtained. In this manner we have calculated that the maximum experimental yield of TH$^{\bullet}$ at *ca.* 200 K is 18 ± 1%. The contribution of $T^{\bullet -}$ to the overall anionic doublet must therefore be at least 36 ± 2% assuming 50% radical-anion contribution to the 130 K DNA spectrum.

Thermally-induced Transfer of e$^-$ from C$^{\bullet -}$ Units to T in DNA.—In order to reconcile the computer results which require almost complete $C^{\bullet -}$ formation at 130 K with our results for TH$^{\bullet}$ which require less than 64% $C^{\bullet -}$ formation, it is necessary to postulate a thermally-induced electron-transfer mechanism from $C^{\bullet -}$ to T. If this does occur, it simply introduces a more complicated route to the 64% value that we deduce. In the light of our results, the process shown in Scheme 3 is required.

$$\text{Step (1)}\quad C^{\bullet -}(H^+) \overset{\text{heat}}{\rightleftharpoons} C^{\bullet -} + (H^+)$$

$$\text{Step (2)}\quad C^{\bullet -} + T \rightleftharpoons C + T^{\bullet -}$$

$$\text{Step (3)}\quad T^{\bullet -} + H^+ \longrightarrow TH^{\bullet}$$

Scheme 3 Thermal migration of electrons from $C^{\bullet -}$ centres to T

Step (1) requires loss of a proton from $C^{\bullet -}(H^+)$ but since $C^{\bullet -}$ has a high proton affinity this stage should have a very low probability. Step (2) is the normal process that occurs in the primary ionization stage. Presumably, it can involve considerable movement of e^-, or just transfer to an adjacent unit. This reaction is reversible and since protonation of $C^{\bullet -}$ is extremely fast [step (−1)] the lifetime of $T^{\bullet -}$ will be short. Step (3) must be slow since it cannot proceed *via* movement within hydrogen bonds, these being of no importance in carbon protonation. Thus it will hardly compete with step (−2) and step (−1). These considerations make Scheme 3 unlikely. Another aspect of this mechanism is that, if it is the main route to TH$^{\bullet}$, why is not all of the $C^{\bullet -}$ ultimately removed to give TH$^{\bullet}$? Also, if the electron can enter into thermally-induced transfer, we would expect to detect some electron return to the cation centres. This will result in thermoluminescence which is an extremely sensitive test for electron return. We know of no reports for this in the required temperature range despite very careful studies. In our work we have not been able to pick up any light emission.

Thus, although we cannot rule out Scheme 3, we strongly favour our proposed mechanism which fixes electrons on T by protonation, this being followed by a tautomeric change to form TH$^{\bullet}$, with no interchange between $C^{\bullet -}$ and T. This conclusion is at complete variance with those of others [18,19,21,34] and is also at variance with our own attempts to estimate the relative contributions of $T^{\bullet -}$ and $C^{\bullet -}$ to the EPR doublet of $DNA^{\bullet -}$ in 10 mol dm^{-3} LiCl by computer analysis. Some justification for questioning the computer results is therefore required.

Computer Estimates of the Concentrations of C$^{\bullet -}$ and T$^{\bullet -}$ Centres.—When a computer is used to analyse similar spectra, it will generally lead to an apparently clear conclusion. The trouble is that the greater the similarity between the two spectra, the smaller are the changes required, anywhere in the spectrum, to alter the computed relative contributions. We find that very small changes are required to move from the 83/17 result of Sevilla *et al.*[21] to our result of 64/36 based on TH$^{\bullet}$ formation.

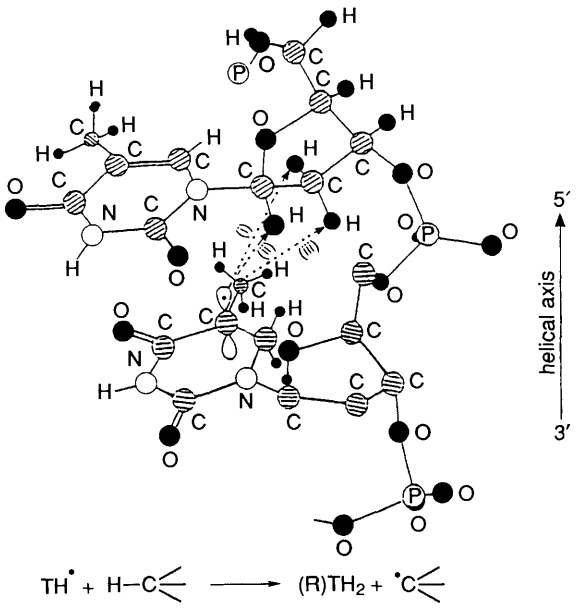


Fig. 9 Local structure around a TH• radical showing how a 'poised' hydrogen can be transferred to give a sugar-centred radical. (i) C5 to C′ 2-Ha 3.93 Å; (ii) C5 to C′ 1-H 3.49 Å (iii) C5 to C′ 2-Hb 3.29 Å.

Table 2 Distances, in Angstroms, from the 5-thymyl (C5) radical site of TH• to the C′-H groups of the deoxyribose sugars 5′ and 3′ to the radical-bearing nucleotide, and also to the C′-H groups of the sugar (N′) that is directly attached to the 5-thymyl base [*cf.* ref. 42][a]

5′-deoxyribose	Distance/Å
C5 to C′ 1-H	3.49
C5 to C′ 2-Ha	3.93
C5 to C′ 2-Hb	3.29
C5 to C′ 3-H	5.79
C5 to C′ 4-H	6.69
C5 to C′ 5-Ha	6.97
C5 to C′ 5-Hb	7.52
N′-deoxyribose	
C5 to C′ 1-H	4.40
C5 to C′ 2-Ha	3.73
C5 to C′ 2-Hb	4.78
C5 to C′ 3-H	5.81
C5 to C′ 4-H	6.05
C5 to C′ 5-H	4.72
C5 to C′ 5-Hb	5.82
3′-deoxyribose	
C5 to C′ 1-H	7.86
C5 to C′ 2-Ha	7.51
C5 to C′ 2-Hb	8.76
C5 to C′ 3-H	9.05
C5 to C′ 4-H	8.44
C5 to C′ 5-Ha	6.30
C5 to C′ 5-Hb	7.71

[a] For C′2, Ha is assigned to the hydrogen atom above the ribosyl plane, *i.e.* on the same side as the base residue and the C′5 moiety, and Hb to the hydrogen below the ribosyl plane. For C′5, Ha is assigned to the hydrogen atom closest to the stacked base interior of the DNA, and Hb to the hydrogen furthest away.

Difficulties include (*i*) the presence of low concentrations of other species that have not been allowed for correctly, (*ii*) site effects and/or solvent effects on the EPR parameters, and (*iii*) the translation of spectra obtained from small units to DNA itself. Our studies suggest that such variables can make spectral changes that are as large as the differences between the two 'benchmark' spectra that are recommended. There is clearly a limit at which such computer analysis of similar spectra must fail. The trouble is that an apparently good answer may still be forthcoming. We conclude that the issue remains an open one but, in view of all the results presented herein, we favour the clear-cut results for TH• formation as an indicator of the extent of $T^{\bullet -}$ formation.

TH• Yields in LiCl Glasses.—The maximum yield of the TH• octet after γ-radiolysis of TMP in LiCl glasses is small and the signal is lost at much lower temperatures compared with frozen aqueous solutions. These glasses show intense EPR features for $Cl_2^{\bullet -}$ and probably $ClOH^{\bullet -}$ radicals after irradiation.[35] These features are lost in the same temperature range as those for $T^{\bullet -}$ and we suggest that reaction (1) may be responsible. Such a

$$T^{\bullet -} + Cl_2^{\bullet -} \longrightarrow T + 2\,Cl^- \qquad (1)$$

reaction is less probable for DNA/LiCl systems since $Cl_2^{\bullet -}$ can react as an electron acceptor, especially with the purines.[36] In fact, we have shown that $Cl_2^{\bullet -}$ is able to cause one-electron oxidation of DNA.[37] Nonetheless, in view of a similar rapid loss of $DNA^{\bullet -}$ on annealing, it seems that reaction (2) may still be

$$DNA^{\bullet -} + Cl_2^{\bullet -} \longrightarrow DNA + 2\,Cl^- \qquad (2)$$

important. In any case, the very low yields of TH• (5–10%) cannot be taken as evidence for or against an increase in the $C^{\bullet -}/T^{\bullet -}$ ratio with added salt.

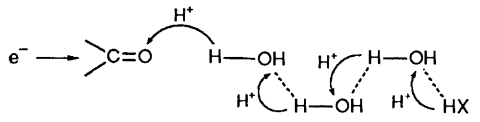
Factors Affecting the Relative Yields of $T^{\bullet -}$ and $C^{\bullet -}$.—Using the TH• yield criterion, our results show that as the concentration of water is reduced below the 100% humidity value, the relative yield of $T^{\bullet -}$ is reduced, such that, for 'dry' DNA, $C^{\bullet -}$ dominates. This accords with the results for oriented DNA ribbon, which are quite definitive in showing that $T^{\bullet -}$ is a minor component.[16,17] These results can most readily be interpreted in terms of a change in the relative protonation rates for $C^{\bullet -}$ and $T^{\bullet -}$. In duplex DNA, the degree of hydrogen bonding for cytosine, particularly at N(3), within the C–G base pair, is independent of water concentration, whereas the hydration of the C=O group in thymine is expected to decrease as water is removed. We suggest that this is the factor which is responsible for the relative increase in $[C^{\bullet -}]$. As water is removed, so the rate of protonation of $T^{\bullet -}$ is reduced, whilst that for $C^{\bullet -}$ remains relatively constant. Hence trapping at cytosine becomes progressively more important.

When salts (LiCl and NaCl) are added to aqueous DNA there appears to be a reduction in the relative yield of TH• even at low salt concentrations. The addition of these salts is known to dehydrate the DNA and cause conformational changes.[38] In these cases, we suggest that the C=O groups of thymine become partially dehydrated, so that $T^{\bullet -}$ protonation is inhibited thereby altering the extent to which the electrons are captured to give $T^{\bullet -}(H^+)$ centres. The large apparent $C^{\bullet -}/T^{\bullet -}$ ratio for DNA in 10 mol dm^{-3} LiCl glasses may possibly be a result of this phenomenon, in which case these salt solutions would not be applicable as a measure of the electron distribution in the frozen aqueous systems.

We stress that for the protonation of $T^{\bullet -}$ we are not postulating the simple reaction (3), since this would probably

$$T^{\bullet -} + H_2O \rightleftharpoons T^{\bullet -}(H^+) + OH^- \qquad (3)$$

favour the reverse process. We suggest that a chain of water molecules is required, as in Scheme 4, thereby removing the OH^- ion from the vicinity of the $T^{\bullet -}(H^+)$ unit, with the formation of a remote X^- anion. Any of the more acidic protons in the neighbourhood should suffice. Clearly, loss of water will



Scheme 4

break the chain and hence inhibit $T^{\bullet -}(H^+)$ formation. For cases involving radical-cations and -anions in fairly close proximity, the proton donor could well be the radical-cation.

Sites of Strand Cleavage in Irradiated DNA.—We have previously suggested that under conditions of direct damage to DNA strand breaks must arise from the initially formed radical-anion and/or -cations. Furthermore, if both of these radicals are capable of initiating strand breaks we were able to propose a novel mechanism to account for the surprisingly large numbers of double strand breaks that arise. Although we discussed our earlier results in terms of $G^{\bullet +}$ and $T^{\bullet -}$, with respect to the origin of single and double strand breaks the same arguments apply even if the initial radical population is more heterogeneous, as now seems likely.

For base-centred radicals to give rise to strand breaks we have previously proposed that an intramolecular hydrogen atom abstraction from a neighbouring sugar residue must at some point take place. Computer modelling has suggested that C2′-H_b and C1′-H on the 5′-deoxyribose residue next to the base radical are the nearest sugar C-H groups for DNA in the B conformation (Fig. 9 and Table 2). Based upon this we reasoned that sites adjacent to either T or G should suffer greater damage than those with A or C as neighbours. To test this hypothesis we have looked at the site specificity of strand cleavage on irradiation of frozen aqueous solutions of DNA of known sequence (restriction fragments from pBR322).

The ^{32}P 3′-end-labelled 346bp *BanHI/HindIII* restriction fragment from the plasmid pBR322 was prepared and irradiated in frozen aqueous solution. The products were analysed by gel electrophoresis on polyacrylamide sequencing gels using the sequencing techniques of Gilbert and Maxam[39] to relate cleavage sites to the sequence. For comparison the same DNA fragment was separately irradiated at room temperature which is known to promote random strand cleavage through the indiscriminate attack of hydroxyl radicals.[40] Under *both* irradiation conditions the gel analysis showed a uniform ladder with bands of similar intensities at each of the possible sites. We conclude that under conditions of direct damage single strand breaks arise in a largely non-sequence-specific manner.

We believe that the most reasonable and straightforward explanation of these observations is that the initial base radical population is considerably more heterogeneous than has often been assumed. The formation of $G^{\bullet +}$, $T^{\bullet -}$ and $C^{\bullet -}$ centres would probably account for our results since within this fragment there are few sites without neighbouring G, T or C. We can discount any explanation in terms of pathways mediated by hydroxyl radicals on several grounds. Firstly, when we promote such a pathway by inclusion of H_2O_2, sugar radicals are readily detected by EPR spectroscopy[6] but under normal circumstances these are not detected. Secondly, we have previously reported that the 3′-end groups generated under conditions of direct damage differ from those arising through hydroxyl radical attack, which is highly suggestive of different pathways. The former are predominantly phosphates[41] whilst the latter have been shown to be a roughly 1:1 mixture of phosphates and phosphoglycollates. There remains the possibility that H-atom abstraction does not only occur from the neighbouring sugar and therefore that high base specificity in the formation of the radical-cation and -anion would not be followed by subsequent sequence specificity in the development of strand breaks. We do not feel this is likely since we believe that a major driving force for the hydrogen atom transfer must be its favourable juxtaposition with respect to the base radical. Inspection of models suggest that the C-H's of the neighbouring sugar are appropriately poised.

It is difficult to interpret these data quantitatively in terms of the ratios of the initial base radicals because of the considerable uncertainties surrounding the rates and efficiencies by which each of the base radicals can proceed on to a strand break. However, these results do provide strong support to the growing body of evidence that in DNA irradiated under conditions of direct damage $C^{\bullet -}$, $T^{\bullet -}$, $G^{\bullet +}$ and possibly some $A^{\bullet +}$ are formed.

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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 and B. W. Wren, *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 P. M. Cullis, M. C. R. Symons, G. D. D. Jones, J. S. Lea and M. C. Sweeney, *J. Chem. Soc., Perkin Trans. 2*, 1987, 1907.
6 P. M. Cullis, S. Langman, I. D. Podmore and M. C. R. Symons, *J. Chem. Soc., Faraday Trans.*, 1990, **86**, 3267.
7 M. C. R. Symons, *J. Chem. Soc., Faraday Trans. 1*, 1987, **83**, 1.
8 H. C. Box and E. E. Budzinski, *J. Chem. Phys.*, 1975, **62**, 197.
9 D. M. Close and W. A. Bernhard, *J. Chem. Phys.*, 1970, **70**, 210.
10 E. Sagstuen, E. D. Hole, W. H. Nelson and D. M. Close, *J. Phys. Chem.*, 1989, **93**, 5974.
11 W. A. Bernhard, *Adv. Radiat. Biol.*, 1981, **9**, 199.
12 S. Gregoli and A. Bertinchamps, *Int. J. Radiat. Biol.*, 1972, **21**, 75.
13 S. Gregoli, M. Olast and A. Bertinchamps, *Radiat. Res.*, 1982, **89**, 238.
14 H. Riederer, J. Hüttermann, P. Boon and M. C. R. Symons, *J. Magn. Reson.*, 1983, **54**, 54.
15 R. A. Holroyd and J. W. Glass. *Int. J. Radiat. Biol.*, 1968, **14**, 445.
16 A. Gräslund, A. Ehrenberg, A. Rupprecht and G. Ström, *Biochim. Biophys. Acta*, 1971, **254**, 172.
17 A. Gräslund, A. Ehrenberg, A. Rupprecht and G. Ström, *Radiat. Res.*, 1975, **61**, 488.
18 W. A. Bernhard, *J. Phys. Chem.*, 1989, **93**, 2187.
19 W. A. Bernhard and A. Z. Patrzalek, *Radiat. Res.*, 1989, **117**, 370.
20 J. Barnes, W. A. Bernhard and K. R. Mercer, *Radiat. Res.*, 1991, **126**, 104.
21 M. D. Sevilla, D. Becker, M. Yan and S. R. Summerfield, *J. Phys. Chem.*, 1991, **95**, 3409.
22 J. Hüttermann, R. Röhrig and W. Köhnlein, *Int. J. Radiat. Biol.*, 1992, **61**, 299.
23 B. Ravkin, J. N. Herak, K. Voit and J. Hüttermann, *Radiat. Environ. Biophys.*, 1987, **26**, 1.
24 D. M. Close, E. Sagstuen and W. H. Nelson, *J. Chem. Phys.*, 1985, **82**, 4386.
25 P. M. Cullis, G. D. D. Jones, M. C. Sweeney, M. C. R. Symons and B. W. Wren, *Free Rad. Res. Commun.*, 1989, **6**, 149.
26 M. D. Sevilla, R. Failor, C. Clark, R. A. Holroyd and M. Pettei, *J. Phys. Chem.*, 1976, **80**, 353.
27 S. Gregoli, M. Olast and A. Bertinchamps, *Radiat. Res.*, 1977, **70**, 255.
28 S. Gregoli, M. Olast and A. Bertinchamps, *Radiat. Res.*, 1979, **77**, 417.
29 M. E. Malone, I. D. Podmore and M. C. R. Symons, unpublished results.
30 M. C. R. Symons, *Int. J. Radiat. Biol.*, 1990, **58**, 93.
31 P. M. Cullis, I. D. Podmore, M. Lawson, M. C. R. Symons, B. G. Dalgarno and J. D. McClymont, *J. Chem. Soc., Chem. Commun.*, 1989, 1003.
32 I. D. Podmore, M. E. Malone, M. C. R. Symons, P. M. Cullis and B. G. Dalgarno, *J. Chem. Soc., Faraday Trans.*, 1991, **87**, 3652.
33 P. M. Cullis, J. D. McClymont and M. C. R. Symons, *J. Chem. Soc., Faraday Trans.*, 1990, **86**, 591.

34 S. Steenken, *Chem. Rev.*, 1989, **89**, 503.
35 I. S. Ginns and M. C. R. Symons, *J. Chem. Soc., Dalton Trans.*, 1972, 143.
36 M. D. Sevilla, D. Suryanarayana and K. M. Morehouse, *J. Phys. Chem.*, 1981, **85**, 1027.
37 M. E. Malone and M. C. R. Symons, unpublished results.
38 B. Wolf and S. Hanlon, *Biochem.*, 1975, **14**, 1661.
39 A. M. Maxam and W. Gilbert, *Methods Enzymol.*, 1980, **65**, 499.
40 W. D. Henner, S. M. Grunberg and W. A. Haseltine, *J. Biol. Chem.*, 1982, **257**, 11750.
41 W. D. Henner, S. M. Grunberg and W. A. Haseltine, *J. Biol. Chem.*, 1983, **258**, 15198.
42 G. D. D. Jones, Ph.D. Thesis, Leicester University, 1987.

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