The Effects of Ionising Radiation on Deoxyribonucleic Acid. Part 4.[1] The Role of Hydrogen Peroxide

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The direct and indirect mechanisms for ionising-radiation-induced damage to deoxyribonucleic acid (DNA) have been probed by e.s.r. spectroscopy and by analysis for strand breaks. Irradiation of frozen aqueous DNA was shown by e.s.r. spectroscopy to give guanine and thymine ion radicals ($G^{+\bullet}$ and $T^{-\bullet}$) together with hydroxyl radicals. The latter are trapped in the ice crystallites and *do not* interact with DNA even on annealing. Addition of H_2O_2 has been shown to affect the e.s.r. spectrum of the DNA phase. H_2O_2 competes with T for electron capture as indicated by reduction to TH$^{\bullet}$. The production of $^{\bullet}OH$ close to DNA is inferred by the appearance of e.s.r. features assigned to sugar radicals. On annealing, the sugar radicals were lost at temperatures significantly below those at which the DNA radicals centred on G and T normally react; this provides an explanation for the fact that sugar radical intermediates have not hitherto been detected in the decay of these primary DNA radicals. At high H_2O_2 concentrations $HO_2^{\bullet}$ was detected. Two pathways for its formation are proposed: (i) direct reaction of $^{\bullet}OH$ with H_2O_2 and (ii) reaction with $G^{+\bullet}$. In support of the latter, we observed reduction of $G^{+\bullet}$ in the presence of H_2O_2. Concurrent strand-break analyses using plasmid DNA (pBR 322) under conditions similar to those of the e.s.r. experiments showed that the presence of H_2O_2 leads to a *radiation-dependent* enhancement of single- and double-strand breaks. The significance of these results to experiments in which the ionising radiation is fractionated as part of a freeze–thaw cycle is discussed.

This study is part of our general investigations into the mechanism of radiation damage to DNA and DNA–histone complexes and how this is modified by the presence of various additives.[1-3] The long-term motivations for this work are the hopes that (i) we can design specific compounds that will protect DNA from radiation damage, which might ultimately be useful in radiation protection, and (ii) that we can find drugs that will sensitise DNA to such damage, which might prove useful in cancer therapy.

It is generally agreed that there are two extremes of mechanism. One is the indirect process in which primary damage is to water molecules and attack on DNA occurs almost exclusively by hydrogen atoms (H$^{\bullet}$), hydrated electrons (e^-_{aq}), and especially hydroxyl radicals ($^{\bullet}OH$). This mechanism, which is appropriate to dilute aqueous DNA at room temperature has been extensively studied using pulse radiolysis and product analysis.[4-6] The other limiting mechanism is direct damage, in which primary electron loss and electron capture occur within the DNA molecule. This mechanism appears to operate in frozen systems. We have chosen to study frozen systems since this enables us to use e.s.r. spectroscopy to probe qualitatively and quantitatively the initial stages of direct damage, and to some extent follow the subsequent reactions of the radicals. We believe that the direct damage mechanism contributes significantly to the damage to DNA *in vivo* (see later).

E.s.r. results suggest that the first detectable radical centres are localised guanine and thymine ion radicals ($G^{+\bullet}$ and $T^{-\bullet}$), the spectra of which resemble closely those of the parent guanine cations and thymine anions.[1,7-9] When dilute aqueous solutions are frozen, phase separation occurs. The $^{\bullet}OH$ radicals formed in bulk water are trapped in the ice crystals and $T^{-\bullet}$ and $G^{+\bullet}$ centres are trapped in the solvated DNA phase.[1] The $^{\bullet}OH$ radicals react within the ice crystals to give hydrogen peroxide. This is liberated on thawing but causes no short-term damage to the DNA in the absence of redox-active transition metal ions.

The relevance of these observations to the situation *in vivo* is perhaps not obvious. However, within the cell nuclei of eukaryotes the concentration of water is relatively low, most of the water being solvating water rather than normal 'bulk' water. The latter is separated from the DNA by the nuclear membrane; this situation is functionally the same as that in the frozen phase-separated systems that we study. Thus the 'direct' mechanism may well be of real significance in cellular systems. Furthermore, as we have suggested elsewhere,[1-3] whilst attack by $^{\bullet}OH$ is likely to be indiscriminate and hence to lead primarily to single-damage sites such as single-strand breaks (SSBs), the 'direct' mechanism is thought to lead to damage centres in pairs. The separation of these pairs is thought to be in the range 20—60 Å. Hence the probability of forming double-strand breaks (DSBs) is greatly enhanced. If, as is often stated, DSBs are less readily repaired than SSBs, this difference may mean that more importance should be attached to the direct mechanism of damage *in vivo*.

As already stressed, our studies are concerned primarily with solvated DNA. I.r. studies[10] and our own n.m.r. studies[11] suggest that the concentration of water of solvation is *ca.* 10—12 molecules per base. The fate of ionisation events within this solvating water is unclear, but $H_2O^{+\bullet}$ must be one of the primary cation centres under our conditions. These may either transfer a proton to give $^{\bullet}OH$ radicals, or gain an electron from DNA to re-form H_2O. (They are not expected to be trapped as such.)

A major aim of the present study was to assess the former possibility. Although $G^{+\bullet}$ and $T^{-\bullet}$ are the only detectable paramagnetic centres present after annealing to *ca.* 130 K, the possibility remains that $^{\bullet}OH$ radicals, formed from H_2O^+, react with DNA to give radicals which for some reason make no clear contribution to the e.s.r. spectra. By adding hydrogen peroxide we hoped to be able to assess the effect of $^{\bullet}OH$ radicals formed in the DNA phase by electron capture under our conditions. Another aim was to explore a possible explanation for a curious effect recorded by Loman and his co-workers,[12] which, in our view, may be caused by hydrogen peroxide formation. They found that, for a given radiation dose, the extent of DNA damage was enhanced if, instead of irradiating continuously at 77 K, they interrupted the irradiation periodically and allowed

the system to warm to room temperature, then re-cooled it to 77 K before continuing the irradiation. It occurred to us that this observation might be a consequence of the accumulation of hydrogen peroxide initially formed in the irradiated ice crystals, and that this could be checked by the present experiments.

Experimental

Materials.—Plasmid DNA (pBR 322) was isolated according to a modification of the procedure of Birnboim and Doly.[13] Experiments were conducted with a pBR 322 DNA preparation containing *ca.* 80% of the superhelical Form I DNA. Tris(hydroxymethyl)methylamine (Tris) buffer, ethylenedinitrilotetra-acetic acid (EDTA), 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide), and calf thymus DNA (for e.s.r. studies) were obtained from the Sigma Chemical Company. Agarose-ME was obtained from the Miles Laboratory. Hydrogen peroxide was purchased from British Drug Houses (Poole).

γ-Irradiation and Assays for DNA Breaks.—The assay used has been described in our previous publications.[1-3] Variations were introduced in order to optimise the separation of the linear Form III DNA from the relaxed Form II DNA. A solution of hydrogen peroxide (10 μl) was added to the plasmid DNA solution (90 μl; 80 μg ml^{-1}) in Tris hydrochloride buffer (10mM; pH 7.6) containing EDTA (1mM) to give the appropriate final peroxide concentration. Samples with and without additive were γ-irradiated in a Vickrad ^{60}Co γ-ray source under the appropriate conditions. Oxygenated and deoxygenated samples were prepared by purging with oxygen or oxygen-free nitrogen for 60 min, then sealed and irradiated. After irradiation, a dye–EDTA mixture (6 μl; 56% glycerol v/v; 50mM-EDTA; 0.05% Bromophenol Blue w/v) was added and portions were removed for analysis by agarose gel electrophoresis.

Gel Electrophoresis.—Samples of irradiated DNA (0.7—1 μg) were layered onto agarose slab gels (1.3%) and subjected to electrophoresis in a horizontal gel apparatus at 40 V for *ca.* 16 h at room temperature, using Tris buffer (90mM, pH 8.3) containing boric acid (90mM) and EDTA (2.5mM). After electrophoresis the gels were stained with ethidium bromide (2.5 μg ml^{-1}) in electrophoresis buffer for at least 15 min and the excess was removed by washing. The stained gels were then excited with a transilluminator and photographed with a Polaroid MP-4 Land Camera using a red filter (Kodak Wratten filter No. 9) and Polaroid type 55 film. The negative films of gels were used for densitometric scanning.

Quantitation of Single- and Double-strand Breaks.—The negative films of the ethidium bromide-stained gels were scanned with a u.v.–visible spectrophotometer with gel-scanning attachment (Pye Unicam SP8-100). The superhelical Form I of the plasmid takes up less ethidium bromide than the other two Forms. We used a staining efficiency of 80% (as demonstrated[14] for pBR 322) to normalise our data. No further uptake of ethidium bromide was observed on prolonged incubations. All samples were analysed in duplicate and average values are reported in the Results section.

γ-Irradiation and E.s.r. Measurements.—Frozen samples were prepared by cooling in liquid nitrogen a Pyrex tube containing a solution of DNA (50 mg ml^{-1}; Type I sodium salt DNA, Sigma) with and without hydrogen peroxide (0.04—40mM). Samples were irradiated as already described, and the e.s.r. spectra were recorded (X-band Varian E-109 spectrometer; 100 KHz field modulation) at 77 K. The samples were annealed to a particular temperature for 2 min through the use of a second spectrometer

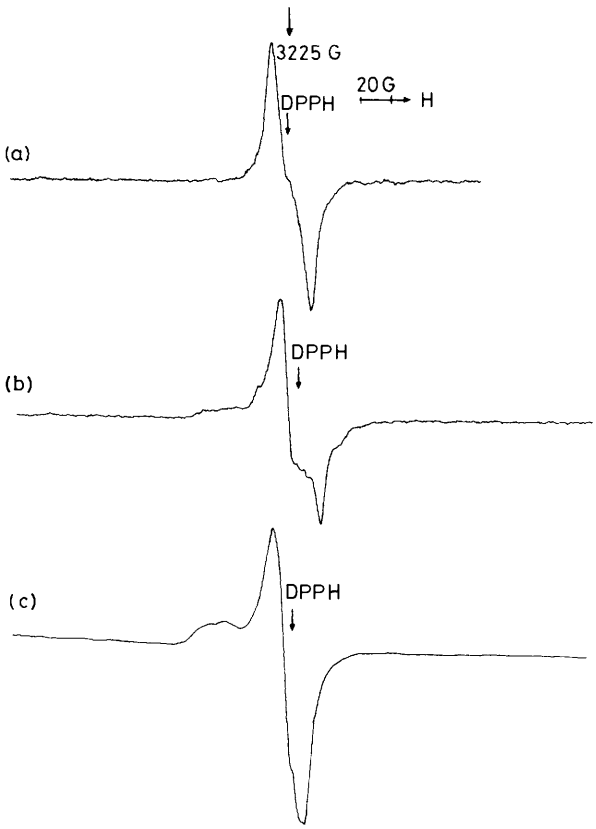


Figure 1. (a) E.s.r. spectrum of an anoxic DNA solution (50 mg ml^{-1}) after irradiation (^{60}Co γ-rays; *ca.* 1.5 Mrad; 77 K), then warming to 130 K for 2 min, and subsequent cooling to 77 K. The spectrum, recorded at a low microwave power (0.02 mW), shows features assigned to $T^{-\bullet}$ and $G^{+\bullet}$. (b) E.s.r. spectrum, again recorded at low power (0.02 mW), of an anoxic DNA solution (50 mg ml^{-1}) in the presence of hydrogen peroxide (40mM), after an irradiation and annealing cycle as outlined in (a), showing features assigned collectively to species 'X' (see text). (c) As (b) with the spectra recorded at a high microwave power (15 mW), showing parallel and perpendicular features assigned to $HO_2^{\bullet}$

(a Varian E-3 X-band) equipped with a variable-temperature accessory. The samples were then re-cooled (77 K) prior to recording of the e.s.r. spectra.

To judge the decay of the spectral features of the DNA–H_2O_2 samples with temperature, a bead containing tris(acetylacetonato)di-iron(III) was studied in tandem with the DNA samples under identical e.s.r. conditions (except for an increase in scan range to ±2 000 G) and the result was employed as an internal intensity standard. In conjunction with the Fe^{III} bead, samples of DNA without additive, co-irradiated with the DNA–H_2O_2 samples, were employed to normalise the DNA–additive spectra.

Storage, manipulations, and double integrations of spectra were performed with a Hewlett-Packard 9835B computer interfaced with the E-109 spectrometer and a Hewlett-Packard 9845A external flexible disc memory. All spectra were digitised at 1 024 equidistant points and the results were stored on disc. *G*-Values (numbers of radicals or strand breaks per 100 eV) were estimated by comparison with the double integral value taken from a spectrum of dibutylaminoxyl of known spin concentration.

Results

In all cases, otherwise identical solutions containing DNA only and DNA + H_2O_2 were compared. Care was taken to ensure

Table 1. E.s.r. parameters for $HO_2^{\bullet}$ in various environments

Host	^{1}H Hyperfine Coupling (G)[a]			g Values		
	x	*y*	*z*	*x*	*y*	*z*
$Na^{\bullet} + O_2/H_2O$, 77 K[b]		av. 11.5		2.035	2.0039	2.003
H_2O_2/H_2O glass + *hν*/77 K[c]	13.8	3.6	15.7	2.0353	2.0081	2.0042
$H^{\bullet} + O_2$/Ar, u.v., 4 K[d]	13.5	8.6	8.6	2.0393	2.0160	2.0044
$SrCl_2 \cdot 6H_2O$/77 K[e]	17.2	6	12.7	2.0355	2.0076	2.0031
$H_2O_2/H_2O/KBF_4$ glass 77 K + γ-rays	13.5	*f*	*f*	2.037	*f*	*f*
H_2O_2/H_2O phase-separated/77 K + γ-rays	14	*f*	*f*	2.045	*f*	*f*
H_2O_2/H_2O/DNA/77 K + γ-rays	13.5	*f*	*f*	2.038	*f*	*f*

[a] 1 G = 10^{-4} T. [b] J. E. Bennett, B. Mile, and A. Thomas, Proceedings of the 11th International Symposium on Combustion, 1967, p. 853. [c] S. J. Wyard, R. C. Smith, and F. J. Adrian, *J. Chem. Phys.*, 1968, **49**, 2780. [d] F. J. Adrian, E. L. Cochran, and V. A. Bowers, *J. Chem. Phys.*, 1967, **47**, 5441. [e] R. C. Catton and M. C. R. Symons, *J. Chem. Soc. A*, 1969, 446. [f] Central features hidden.

Table 2. Summary of the changes of the e.s.r. spectral features of DNA on irradiation in the presence of increasing concentrations of peroxide

$[H_2O_2]$/mM	A:BP†	Comments
0.04	1:*ca.* 1 900	Little or no effect on $G^{+\bullet}/T^{-\bullet}$ at 130 K; $TH^{\bullet}$ formation largely unaffected; $HO_2^{\bullet}$ and $X^{\bullet}$ features not apparent; DNA radicals persist to >255 K
0.4	1:*ca.* 190	Little detectable effect on $G^{+\bullet}/T^{-\bullet}$ at 130 K; $TH^{\bullet}$ formation 25—35% of normal; $HO_2^{\bullet}$ and $X^{\bullet}$ present but weak; a few DNA radicals persist to ≥255 K; a steeper annealing decay curve for radicals was observed
4	1:*ca.* 19	A lowering of the temperature (to *ca.* 190—210 K) at which DNA radicals are lost was noted; at 130 K, a significant loss of $T^{-\bullet}$ is observed with $X^{\bullet}$ features apparent; near total loss of $TH^{\bullet}$ formation; $HO_2^{\bullet}$ and X features now well established on annealing to 165 K with e.s.r. features in accord with loss of $G^{+\bullet}$; all radicals lost by *ca.* 215 K with loss of $X^{\bullet}$ features by 195 K
40	1:*ca.* 1.9	Substantial lowering of the critical temperature at which DNA radicals are lost was observed; at 130 K a large alteration in profile is observed indicative of substantial $T^{-\bullet}$ loss with large contributions from $X^{\bullet}$ and $HO_2^{\bullet}$; no $TH^{\bullet}$ observed; features from $X^{\bullet}$ and $HO_2^{\bullet}$ persist to *ca.* 195 K with apparent loss of $G^{+\bullet}$; all radicals lost by 200 K with loss of $X^{\bullet}$ features by *ca.* 195 K

† Additive:base pair.

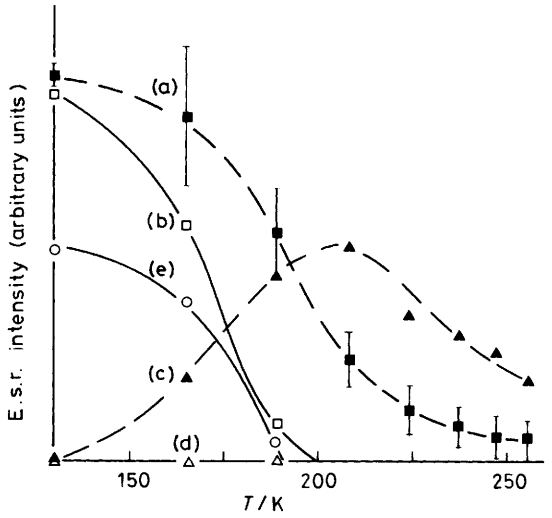


Figure 2. Temperature-dependent decay of the e.s.r. spectral features of DNA following irradiation (see text and legend to Figure 1). The intensity of the central features ($G^{+\bullet}$, $T^{-\bullet}$, 'X') of the DNA spectra produced (a) in the absence and (b) in the presence of H_2O_2 (40mM) as a function of the annealing temperature is shown together with the growth and decay of $^{\bullet}TH$ signals (as measured through the relative intensity of the seventh line of the octet at *g ca.* 1.9731 × 5) (c) in the absence and (d) in the presence of H_2O_2 (40mM). Also shown (e) is the temperature-dependent decay of wing features (measured between *g* 2.0115 and *ca.* 1.4869) attributed to the species 'X'

the absence of transition metal ions which might catalyse the decomposition of the peroxide; studies on unirradiated systems established that this was not significant. We also studied the e.s.r. spectra of $H_2O + H_2O_2$ systems in the presence and absence of glass promoters for comparative purposes.

It has been fully established [1,7–9] that in the absence of H_2O_2 three radicals are detectable by e.s.r. spectroscopy at 77 K. Hydroxyl radicals are formed in the pure ice crystallites, and $G^{+\bullet} + T^{-\bullet}$ in the solvated DNA phase. In the presence of H_2O_2 the central e.s.r. features, observed at low microwave powers, were considerably modified and at high powers extra features assignable to $HO_2^{\bullet}$ radicals were observed. These changes were far better defined after annealing to *ca.* 130 K, at which temperature the $^{\bullet}OH$ radicals in the ice are lost. Typical e.s.r. spectra at low and high powers are shown in Figure 1.

Computer analysis of the spectra at low powers shows that as $[H_2O_2]$ is increased, so the concentration of DNA radicals falls and new features collectively described as 'X' appear. These features are similar to those observed from DNA systems in the presence of iodoacetamide.[3]

The features observed at high powers comprise doublets (*x*, *y*, and *z*) characteristic of $HO_2^{\bullet}$ (Table 1). They are similar to those observed in $H_2O + H_2O_2$ glassy systems, obtained, for example, by adding KBF_4, but differ surprisingly from the features obtained from polycrystalline H_2O containing H_2O_2 in concentrations similar to those used in the DNA experiments.

The various effects on the e.s.r. spectra of increasing $[H_2O_2]$ are summarised in Table 2 and Figure 2.

Concurrent with the e.s.r. studies we have investigated reactions leading to strand breaks. We have previously established that, under conditions where $T^{-\bullet}$ and $G^{+\bullet}$ are the only detectable primary radicals, strand breaks persist at high levels. Additives that influence the yield or nature of the primary

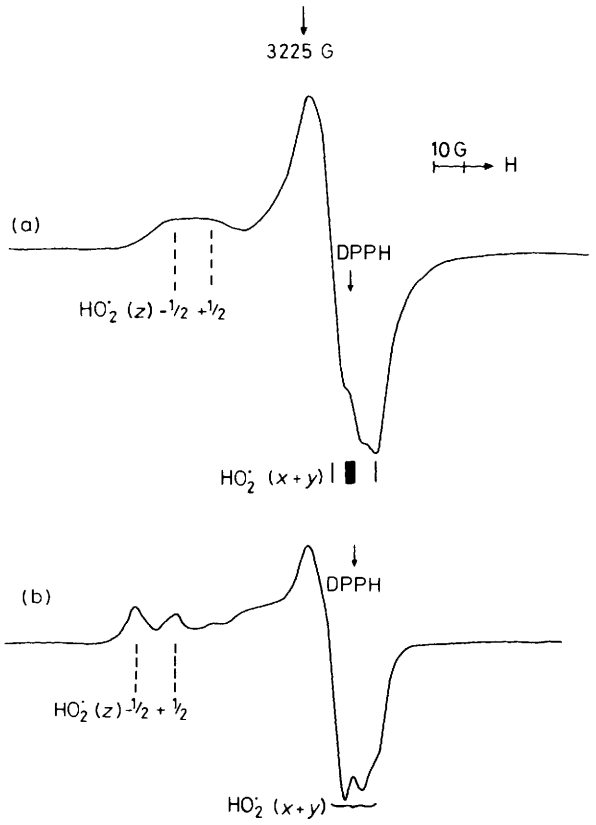


Figure 3. Comparison of e.s.r. features of $HO_2^{\cdot}$ formed (a) in frozen DNA solutions with those formed (b) from dilute frozen aqueous solutions

radicals are expected therefore to have some influence on the yield of strand breaks.

The effects of H_2O_2 on the yield of strand breaks are shown in Figures 4 and 5. In the absence of γ-irradiation (and transition metals), H_2O_2 does not significantly alter the yield of strand breaks. However, on γ-irradiation the yield of strand breaks is appreciably enhanced. The magnitude of the effect has been shown unequivocally to depend on the peroxide concentration (Figure 5). The yields of DSBs are linear with dose up to *ca.* 300 krad, but at higher doses cleavage of the plasmid into small fragments is responsible for the apparent curvature (Figure 4).

Discussion

Formation of 'OH *Radicals.*—It is important to note that in our experiments, H_2O_2 is expected to be largely in the solvated DNA phase rather than in the ice crystals. That the $HO_2^{\cdot}$ radicals are indeed formed in such a phase is strongly supported by the fact that their e.s.r. spectrum is similar to that for $HO_2^{\cdot}$ in aqueous glasses and is clearly distinguishable from the species formed from dilute aqueous solutions (Figure 3). We do not, at present, understand why these spectra are so markedly different, but we use the result empirically to establish the location of the $HO_2^{\cdot}$ radicals.

As in all our studies,[1-3] damage to the ice crystals can be ignored since the hydroxyl radicals in the ice crystallites are lost on annealing without change in nature or yield of the DNA radicals. The fact that $[T^{-\cdot}]$ and $[TH^{\cdot}]$ are greatly diminished at relatively low $[H_2O_2]$ shows that H_2O_2 is acting as an efficient electron trap, as in reaction (1). These 'OH radicals are

$$H_2O_2 + e^- \longrightarrow {}^{\cdot}OH + OH^- \qquad (1)$$

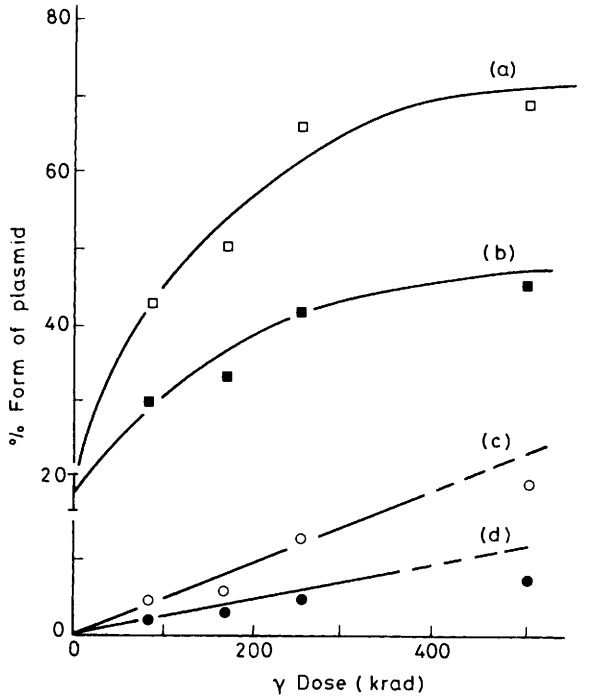


Figure 4. The effect of H_2O_2 on the number of strand breaks induced by γ-irradiation of plasmid DNA (pBR 322) at 77 K. The percentage of Form II indicates single-strand breaks produced (a) in the presence and (b) in the absence of H_2O_2 (4mM). Double-strand breaks formed (c) in the presence and (d) in the absence of H_2O_2 are indicated by Form III

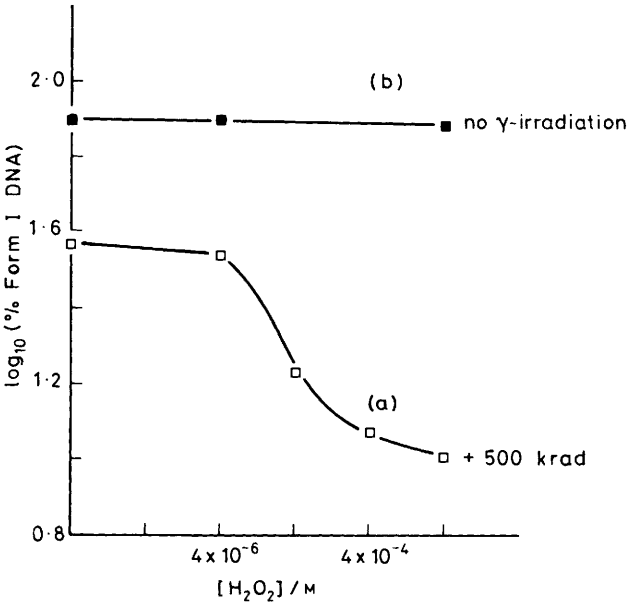


Figure 5. The effect of increasing hydrogen peroxide concentration on the radiation-dependent reactions leading to strand breaks. Samples of plasmid DNA (supercoiled pBR 322; *ca.* 80 μg ml^{-1}) in the presence of various concentrations of hydrogen peroxide were subjected to a constant dose of γ-irradiation (500 krad) at 77 K (a). Control experiments (b) in which the γ-irradiation was omitted were also performed

not detected by e.s.r. spectroscopy. If they are trapped, their contribution to the e.s.r. spectrum is expected to be poorly defined,[15] and would be masked by the intense, well defined signals from 'OH radicals in ice. Certainly after annealing to 130 K they must have reacted, since no signals assignable to 'OH

radicals could be detected. The appearance of features collectively described as 'X' strongly suggests that these 'OH radicals have attacked the DNA molecules to give carbon-centred radicals.

Radicals 'X'.—The outer lines indicated in Figure 1 closely resemble features obtained from DNA in the presence of iodoacetamide, to which we previously assigned the symbol 'X'.[3] In that study, we suggested that these are mainly radicals formed by hydrogen abstraction by $H_2\dot{C}CONH_2$ from various sites in the deoxyribose units. One of the reactions exhibited by 'OH at ambient temperatures is such hydrogen-atom abstraction;[4,5] therefore similar reactions are expected in this study. Another important reaction in fluid solutions is addition to the base moieties to give the respective OH adducts, *e.g.* 'GOH.[4,6] These may have been formed in our work but, if so, their features must be poorly defined and cannot make a major contribution to the spectra.

The assignments of various features comprising 'X' to different sugar radicals were discussed fully in Part III,[3] and are not further considered herein. If the radicals responsible for the features 'X' are indeed sugar radicals, this explains the occurrence of extensive strand breaks. Indeed, the fact that the number of strand breaks is enhanced by H_2O_2 strongly supports this assignment. It is important to note that sugar radicals, *when formed*, can be detected in frozen aqueous solution. Our studies with various additives, particularly iodoacetamide and H_2O_2, have shown that sugar radicals are formed under special circumstances for which we can propose reasonable mechanistic pathways.

However, we stress that the features 'X' are not observed in the e.s.r. spectra obtained from DNA in the absence of additives. This is an important contrast since it means that these radicals are not formed significantly in the temperature range 130—190 K in the absence of additives. This, in turn, means that the concentration of 'OH radicals formed in the vicinity of the DNA (*i.e.* from solvating water molecules) must be very low, confirming our previous conclusions.[1] This presumably means that electron transfer to H_2O^+ is faster than proton transfer from H_2O^+ under our conditions.

Another important result is that features 'X' are lost at lower temperatures (*ca.* 190 K) than those at which $G^{+\bullet}$ and TH' signals are lost (Figure 2). Hence, if, as we propose,[1-3] sugar radicals are formed in the pathway leading from $G^{+\bullet}$ and TH' to strand breaks, we would be unable to detect them by e.s.r. spectroscopy. This explains our failure to detect sugar radicals in the absence of additives.[1]

Formation of $HO_2^{\bullet}$ *Radicals.*—At high microwave powers, signals from $HO_2^{\bullet}$ radicals dominate the e.s.r. spectra. This is because the signals from organic radicals are strongly saturated whilst saturation has not set in for the $HO_2^{\bullet}$ radicals. The data are compared with those for $HO_2^{\bullet}$ radicals formed in other matrices in Table 1; there can be little doubt about the identification. As already stressed, the results show that these radicals are formed in the DNA phase rather than in the ice phase.

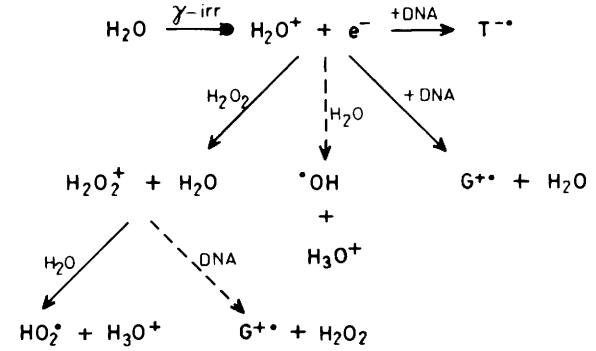
They can be formed by at least three possible mechanisms, namely direct (2) or indirect (3) electron loss followed by proton

$$H_2O_2 \longrightarrow H_2O_2^+ \longrightarrow HO_2^{\bullet} + (H^+) \quad (2)$$

$$G^+ + H_2O_2 \longrightarrow G + HO_2^{\bullet} + (H^+) \quad (3)$$

$$H_2O_2 + {}^{\bullet}OH \longrightarrow HO_2^{\bullet} + H_2O \quad (4)$$

loss, or attack of 'OH radicals (4). Furthermore, the fact that yields of $G^{+\bullet}$ are reduced requires that reactions (2) and/or (3)



Scheme 1.

are of some importance, and we conclude that except for relatively concentrated solutions, these reactions dominate.

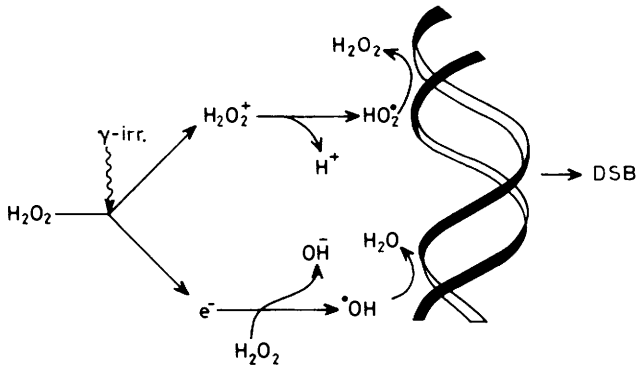
The radical cation $H_2O_2^{+\bullet}$ has not been studied by e.s.r. spectroscopy, but recent work on the di-t-butyl derivative shows that this must have the expected π^* structure.[16] Since H_2O_2 has a lower ionisation potential than H_2O, $H_2O_2^{+\bullet}$ might well be formed indirectly from $H_2O^{+\bullet}$. On the other hand, $H_2O_2^{+\bullet}$ is expected to be a strong acid, so deprotonation should be rapid, thereby inhibiting further electron-transfer processes (Scheme 1).

The fact that $HO_2^{\bullet}$ radicals are lost at temperatures higher than those required for loss of species 'X' means that if, as we postulate, they ultimately react to form mainly sugar radicals, we would not be able to detect these extra radicals during the decay of the $HO_2^{\bullet}$ signal. That such hydrogen-atom abstraction should occur under these conditions is expected, since intermediates $RO_2^{\bullet}$ formed in oxygenated systems almost certainly react in this way.[1,5]

Strand Breaks.—As $[H_2O_2]$ increases there is an increase in the number of both single-strand breaks and double-strand breaks (Figure 4). This observation is in accord with our expectations since 'OH and $HO_2^{\bullet}$ will, amongst other reactions, abstract hydrogen from sugar units, and the resulting sugar radicals are known to cause strand breaks.[17] Importantly, the modest magnitude of the H_2O_2 enhancement implies that the direct pathways (*via* $G^{+\bullet}$ and $T^{-\bullet}$) leading to strand break are similar in efficiency to the indirect pathways mediated through oxygen-centred radicals ('OH and $HO_2^{\bullet}$).

An important conclusion of our earlier work is that direct damage causes a disproportionately high degree of DSBs.[1-3] The exclusive formation of the radical anion of thymine and the radical cation of guanine implies some charge migration, although we have argued that this is not extensive. The high incidence of DSBs was explained in terms of a distribution of pair trapping sites for $G^{+\bullet}$ and $T^{-\bullet}$ such that many $G^{+\bullet}$ and $T^{-\bullet}$ centres are trapped within *ca.* 50 Å of each other. Provided that there is a reasonable probability that both centres can lead to chain scission, it follows that DSBs will occur far more frequently than expected from an initial random statistical distribution of radical cations and anions. (The probability of a DSB arising from two *independent* coincident SSBs can be shown to be negligible for the doses used in this work.) It should be emphasised that this proposal suggests that DSBs arise from a *single* ionising event in which the electron and the 'hole' that are initially formed together have a reasonable chance of being trapped close together.

In the presence of higher concentrations of H_2O_2, DSBs are enhanced, since under our constrained conditions 'OH and $HO_2^{\bullet}$ are largely trapped close to DNA molecules, and many also lead to an excess of pair trapping, in contrast with fluid systems. Thus, for example, an electron ejected from H_2O_2,



Scheme 2.

leaving a trapped $HO_2^•$ radical, could react with a nearby H_2O_2 molecule yielding an $^•OH$ radical. These radicals are expected to react with DNA units close to the trapping sites since mobility is restricted and these events may lead to DSBs under the right conditions (see Scheme 2).

We stress that the enhancement partially arises because the efficiency of strand breaks is enhanced, as shown by the increase in SSBs, but it may also reflect the fact that neutral radicals can be trapped closer together than the charged centres, since the latter can back-react *via* electron-transfer when they are too close.

Annealing Cycles.—Our result that the presence of H_2O_2 enhances the overall yield of SSBs provides at least a qualitative explanation for the small increases in damage observed when several thermal cycles are included in radiolyses of DNA.[12] Thus, each time the irradiated ice crystals melt, H_2O_2 molecules formed from $^•OH$ radicals are liberated from the ice crystals, and on re-freezing these molecules will be incorporated largely into the DNA phase, as our results establish. Direct damage to these H_2O_2 molecules will give some $HO_2^•$ and $^•OH$ radicals which will attack the DNA and lead to a modest increase in SSBs. This seems to us to be the simplest explanation of the increases observed.

Conclusions

(i) The addition of H_2O_2 switches the mechanism from direct damage to a pathway in part mediated through oxygen-centred radicals analogous to the indirect mechanism.

(ii) This only results in a modest increase in the number of SSBs. This, in turn, means that the efficiency of conversion of $G^{+•}$ and $T^{-•}$ centres into SSBs does not differ markedly from that of the formation of SSBs *via* attack of $^•OH$ and $HO_2^•$.

(iii) Under our trapping conditions, pair-trapping remains important, but this is unlikely to be the case for dilute fluid solutions in which indirect damage dominates.

(iv) A simple explanation for the catalytic effect of freeze–thaw cycles during exposure to ionising radiation is that H_2O_2, formed in ice crystallites, is repeatedly transferred to the DNA phase and hence can increase the extent of damage, by the proposed pathway.

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