EPR Spin-trapping Studies of Radical Damage to DNA

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EPR spectroscopy has been employed, in conjunction with spin-trapping [using 2-methyl-2-nitrosopropane dimer (MNP)] to study the attack of $^{\bullet}OH$ (generated by the Fe^{2+}–EDTA/H_2O_2 couple) on deoxyribonucleic acid (DNA) and its components. The results confirm that for pyrimidine deoxyribonucleosides and deoxyribonucleotides the alkenic C_5–C_6 double bond of the base moiety is the predominant site of attack (as with the pyrimidine bases, nucleosides and nucleotides) though weak signals from sugar-derived radicals are also observed. Both sugar and base radicals have also been detected for purines (both ribose and deoxyribose derivatives), providing evidence that rapid transfer occurs from the initially formed base radicals to the sugar–phosphate backbone.

The spectra of the adducts from double-stranded (ds) and single-stranded (ss) DNA are found to vary with pH and $^{\bullet}OH$ flux. With dsDNA at pH 7, an anisotropic spectrum due to a slowly tumbling adduct of a high molecular weight polymer radical-adduct is accompanied by a more isotropic signal due to a mobile, low molecular weight radical-adduct, the relative yield of which increases as the pH is lowered. Close similarities are observed between the spectra of dsDNA at pH 2 and ssDNA at pH 7, with a greater occurrence of strand-breakage under these conditions. Treatment of the partially-immobilised DNA adducts formed at pH 6–7 either with the enzyme DNase or with acid releases more mobile adducts enabling information to be obtained about sites of initial attack (at pyrimidine bases) and mechanisms of transfer of damage.

It is widely believed that oxidative damage to DNA plays a crucial role in carcinogenesis induced by ionizing radiation both *via* direct ionization of the bases and indirectly *via* hydroxyl radical generation from solvating water molecules, and by a wide range of chemical compounds.[1–6] There is a considerable body of evidence to support the hypothesis that one of the major lesions that is known to occur, strand-breakage, results from radical generation on the sugar–phosphate moieties and subsequent rearrangement reactions (see *e.g.* ref. 6). A number of previous studies lead to the proposal that the major site of initial damage, induced by both $^{\bullet}OH$ and direct ionization, is at the nucleotide base, which suggests that processes must exist which result in the transfer of damage from the base to the sugar–phosphate.[6–12] The mechanisms of these transfer processes are not clear, though under certain circumstances peroxyl radicals or base radical cations may be involved:[13–15] for example, we have previously employed direct EPR observation in flow experiments to demonstrate that protonation of hydroxyl radical adducts to the C_5–C_6 double bond of the pyrimidine bases can lead to loss of H_2O and hence radical-cation formation, which results ultimately in transfer of the radical centre to the C_2' site in the ribose ring in uridine and cytidine.[16]

Small-scale EPR spin-trapping methods (*e.g.*, in conjunction with HPLC analysis) have been developed and employed to study radical damage induced by pulse radiolysis and X-irradiation to cytosine and poly(C),[17] some oligonucleotides[18] and adenosine, guanosine and poly(A)[19] as well as DNA itself.[20] We have previously used the spin-trapping technique {with 2-methyl-2-nitrosopropane dimer (MNP) [$(Bu^tNO)_2$] as trap}[21] to detect first-formed radicals obtained on reaction of $^{\bullet}OH$ (produced from metal ion–hydrogen peroxide couples) with pyrimidine bases, ribonucleosides, ribonucleotides, polyribonucleotides [*e.g.* poly(U)] and RNA. In the investigation to be described here we set out to use this approach to examine the corresponding reactions in metal–peroxide systems with the 2′-deoxyribose derivatives and hence DNA itself, particular emphasis being placed on the possible mechanisms of damage-transfer to the 2′-deoxyribose ring (and hence chain-breakage); transfer of damage to the (deactivated) C_2' site would not be expected to be favoured with these substrates.

Results and Discussion

Reactions of the Pyrimidine 2′-Deoxynucleosides.—The pyrimidine 2′-deoxynucleosides were incubated with the Fe^{2+}–EDTA/H_2O_2 couple in the presence of the spin-trap, MNP and EPR spectra were recorded over a period of time. The procedure employed was typically as follows (concentrations are those in the final mixture); the substrate (3×10^{-2} mol dm^{-3}) and Fe^{2+}–EDTA complex ($1–4 \times 10^{-3}$ mol dm^{-3} Fe^{2+}, with EDTA present in a two-fold excess) were mixed and combined with a mixture of H_2O_2 ($1–4 \times 10^{-3}$ mol dm^{-3}) and MNP ($0.5–2.5 \times 10^{-2}$ mol dm^{-3}), immediately prior to recording the EPR spectra.

The reaction of 2′-deoxyuridine (**1**) with $^{\bullet}OH$ at pH 7 gave a spectrum [see Fig. 1(*a*)] very similar to that observed previously with uridine and uridine 5′-phosphate (5′UMP) at pH 7.[21] This is analysed in terms of two triplets of doublets, which have been previously assigned to the spin-trapped C_5–OH and C_6–OH adducts of the base moiety, [**2** (●) and **3** (○)] respectively, the wider doublet being assigned to the spin-trapped C_5–OH adduct (*i.e.* the spin-trapped C_6-yl radical) by comparison with the data from continuous-flow and spin-trapping experiments reported previously[21] (see Table 1: *n.b.* the individual assignments are not totally unambiguous). The ratio of spin-trapped radicals C_5–OH : C_6–OH is estimated (*via* computer simulation) as *ca.* 1:2 (though this ratio may not reflect accurately the initial ratio of attack on the substrate because the rates of radical trapping may be different). The presence of one or more additional features in the centre of each set of features, which was also noted in the reactions of uridine and 5′UMP, is believed to indicate the formation and subsequent trapping of sugar-derived radicals. Varying the concentration of the trap appeared to have no effect on the relative ratios of the species present and at low pH the central feature of each set of signals

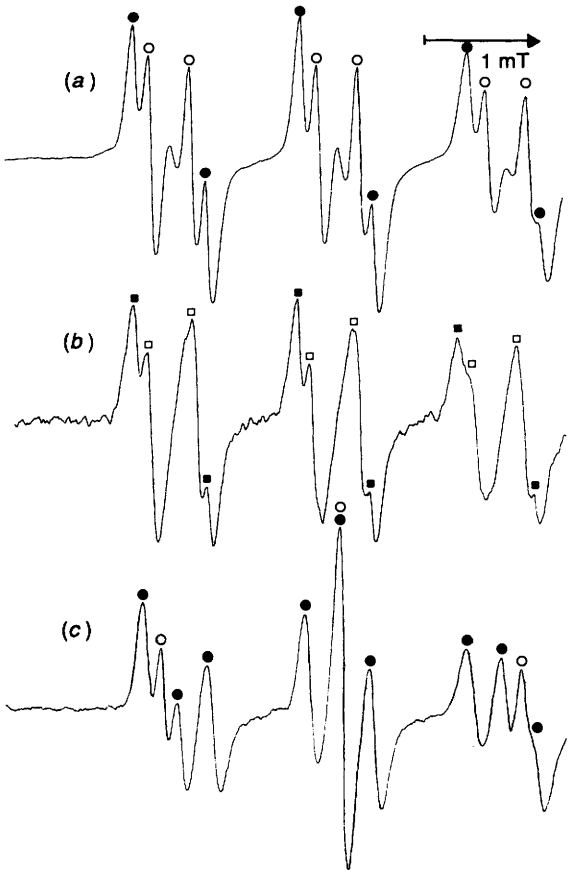


Fig. 1 (*a*) EPR spectra of **2** (●) and **3** (○) obtained from the reaction of $^{\bullet}OH$ with 2′-deoxyuridine and subsequent addition of the C_5–OH and C_6–OH base adducts to the spin-trap MNP at pH 7 with [2′-deoxyuridine] 3 × 10^{-2} mol dm^{-3}, [Fe^{2+}–EDTA] 1 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3} and [MNP] 1 × 10^{-2} mol dm^{-3} (see Table 1 and text). (*b*) As (*a*), but with 2′-deoxycytidine; spectra assigned to **5** (■) and **6** (□) (see Table 1 and text). (*c*) As (*a*), but with thymidine; spectra assigned to **8** (●) and **9** (○) (see Table 1 and text).

decayed rapidly. It is thought that this sugar radical may be generated either *via* transfer of the radical-centre from the base moiety to the sugar–phosphate backbone, or by direct attack of $^{\bullet}OH$ on the sugar moieties; the clear triplet signal observed with 2′-deoxyuridine suggests that the radical centre is present at either the C_3′ or C_4′ position on the sugar (see later).

Reaction of an equimolar mixture of uracil and 2′-deoxyribose with $^{\bullet}OH$ under identical conditions however yielded only the spectra of uracil-derived adducts (as observed previously), which suggests that transfer of the initial centre from the base to the sugar is the major route by which sugar radicals are formed from $^{\bullet}OH$ in reaction of the nucleotides; possible assignments will be discussed later.

Reactions of 2′-deoxycytidine (**4**) with $^{\bullet}OH$ at pH 7 [see Fig. 1(*b*)] gave very similar results to 2′-deoxyuridine, with the observed spectra dominated by two species: the spin-trapped C_5–OH and C_6–OH adducts of the base moiety [**5** (■) and **6** (□), respectively], in a ratio of *ca.* 1:1.3. The presence of shoulders on these lines indicates the presence of other radicals, presumably sugar-derived species. (The spectra bear a resemblance to those reported by Kuwabara and co-workers,[17] following pulse radiolysis of cytidine and related compounds. They too suggest the presence of C_5 and C_6 adducts; our spectra do not provide evidence for the cyclic species derived by attack of the C_5′ sugar radical on the C_5–C_6 double bond.) An equimolar mixture of cytosine and 2′-deoxyribose again yielded only the spectra of the base-derived adducts, suggesting that, as with 2′-deoxyuridine, transfer of the radical centre from the base to the sugar moiety is the predominant mechanism of formation of sugar radicals. As the concentration of MNP was lowered, the overall intensity of the spectra decreased; as the pH was lowered to *ca.* 2, the spectra became better resolved and there was a noticeable change in the β-hydrogen splitting of the C_5–OH adduct (to *ca.* 0.55 mT); this is believed to be due to the protonation of the C_4 amino group.

Experiments with thymidine (**7**) at pH 7 [Fig. 1(*c*)] led to the detection of only the spin-trapped C_5–OH and C_6–OH adducts [**8** (●) and **9** (○) in the ratio of *ca.* 2:1], the former with a clear β-nitrogen splitting (as with the Ti^{III}–H_2O reaction[21]); a similar spectrum is obtained after X-irradiation.[20] On lowering the pH to 2, the concentration of the spin-trapped C_6–OH adduct decreased. As with the reactions of uracil and cytosine described above, $^{\bullet}OH$ attack on a mixture of thymine and 2′-deoxyribose yielded only the spectra of spin-trapped base radicals.

These spin-trapping results together with those obtained previously from continuous-flow experiments with both the ribose and the 2′-deoxyribose derivatives,[6,16] confirm the suggestion that addition of $^{\bullet}OH$ to the electron-rich C_5–C_6 double bond of the base moieties is the predominant mode of attack and that addition at this site may result in transfer of damage to the sugar ring in some cases.

Reactions of the Purine Bases, Nucleosides and Nucleotides.—Guanine, its derivatives and related sugars. Purine bases and their ribose and deoxyribose derivatives were incubated with Fe^{2+}–EDTA, H_2O_2 and MNP at pH 2–7. Guanine (**10**) reacted with $^{\bullet}OH$ at pH 7 to give a signal with a dominant triplet of triplets (1:1:1) and a further weaker triplet [see Fig. 2(*a*)]. The former (■) cannot be unambiguously assigned, but would be consistent with the trapping of the C_4–OH radical (as the adduct **11**) on the basis of the extra nitrogen splitting observed and the pulse radiolysis results obtained by Steenken and co-workers.[22] The weak triplet (□) may be from the spin-trapped C_5–OH or the C_8–OH adducts (see *e.g.* **12** and **13**). Only very weak signals were obtained from guanosine. Here, as in other experiments, we cannot rule out the occurrence of reactions of radical intermediates with Fe^{2+} or Fe^{3+}, which would serve to reduce (trapped) radical concentrations.

Very different signals were obtained from some water-soluble guanosine phosphates and deoxyribose derivatives [see Fig. 2(*b*)–(*e*)]; these are attributed to sugar-derived species (see below). For guanosine 5′-phosphate (**14**), for example, the spectrum is believed to be composed of superimposed signals from two radical adducts, each with a characteristic doublet splitting [*a*(β-H) 0.18 and 0.30 mT, respectively; see Table 1 and Fig. 2(*b*)]; the 3′-phosphate (**15**) behaved similarly, with the major species giving rise to a triplet of doublets [with *a*(β-H) 0.38 mT]. In contrast, the 3′:5′-cyclic monophosphate (**16**) gave only a triplet spectrum. With the deoxyribose derivatives, the spectra obtained from both the 5′- and 3′-phosphates (**17**) and (**18**) are dominated by a triplet of doublets [the splitting being much greater in the latter: see Fig. 2(*c*) and (*d*)] and a triplet-based spectrum was observed for the 3′:5′-cyclic monophosphate derivative (**19**) [see Fig. 2(*e*)]. Somewhat different spectra have been reported (largely unassigned) following HPLC separation of aminoxyls from some related purines and nucleosides:[19] again it is not clear whether this reflects the difference between methods of radical generation or time dependent phenomena.

With a mixture of guanine and 2′-deoxyribose, only sugar radicals are seen and so it is thought that intermolecular transfer may play an important role in their formation, although direct attack at the sugar moieties cannot be ruled out. It has previously been shown that intramolecular radical transfer can occur from the base to the sugar in some pyrimidine nucleo-

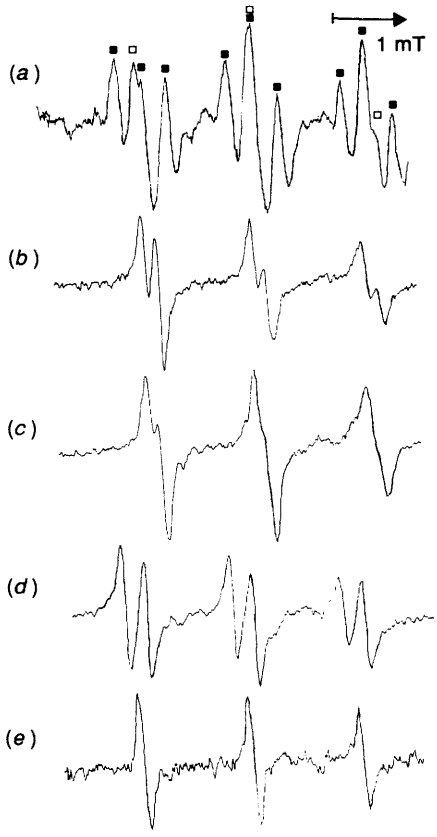
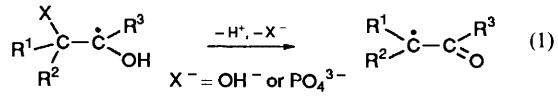


Fig. 2 (*a*) EPR spectra of **11** (■) and **12** or **13** (□) obtained from the reaction of $^{\bullet}OH$ with guanine (saturated) at pH 7 in the presence of MNP [Fe^{2+}–EDTA] 1 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3} and [MNP] 1 × 10^{-2} mol dm^{-3} (see text). (*b*) As (*a*), but with [guanosine 5′-phosphate] 3 × 10^{-2} mol dm^{-3}, [Fe^{2+}–EDTA] 2 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3} and [MNP] 2.5 × 10^{-2} mol dm^{-3}. (*c*) As (*a*), but with [2′-deoxyguanosine 5′-phosphate] 3 × 10^{-2} mol dm^{-3}. (*d*) As (*a*), but with [2′-deoxyguanosine 3′-phosphate] 3 × 10^{-2} mol dm^{-3}, [Fe^{2+}–EDTA] 2 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3} and [MNP] 2.5 × 10^{-2} mol dm^{-3}. (*e*) As (*a*), but with [2′-deoxyguanosine 3′:5′-phosphate], saturated solution, [Fe^{2+}–EDTA] 2 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3} and [MNP] 2.5 × 10^{-2} mol dm^{-3}.

sides, *via* reactions of the base radical cation.[16] This appears to be encouraged by the presence of α-OH groups in the incipient radicals and it has been clearly established for C_2'-H in the ribose derivative. It is expected that this internal abstraction will be followed by elimination of a β-leaving group, either acid-catalysed loss of HO^- or rapid phosphate loss, a process which results in strand-breakage [see reaction (1)]; the radicals



(1)

trapped may thus be secondary radicals produced by rearrangement (see *e.g.* ref. 6).

We have examined the validity of these interpretations by studying the reactions of some ribose- and deoxyribose-phosphates themselves (see Table 1): these give narrower spectra dominated by a triplet of doublets, similar to those seen from guanosine and its derivatives. In particular, ribose 5′-phosphate (**20**) gave at least two triplets of doublets and a triplet [see Fig. 3(*a*)]: the doublets are assigned to the C_5' radical obtained by hydrogen abstraction at this site and to one at least of the rearranged radicals following attack at C_2' and C_3' (the individual radicals cannot be unambiguously assigned). The

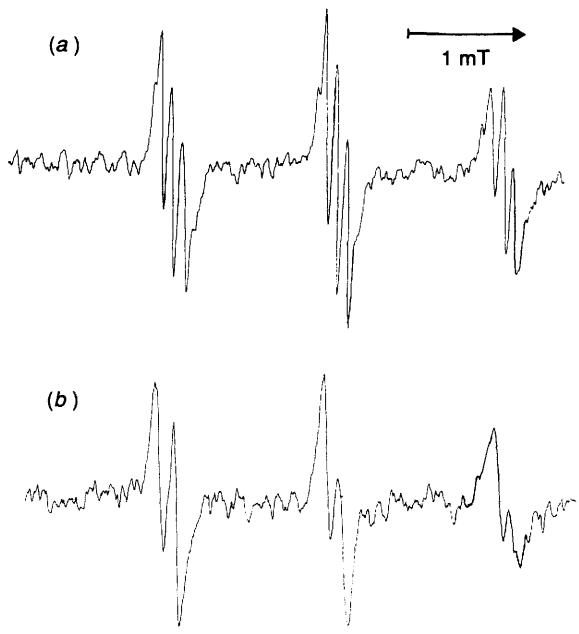


Fig. 3 (*a*) EPR spectra obtained from the reaction of $^{\bullet}OH$ with D-ribose 5′-phosphate in the presence of MNP at pH 7, with [D-ribose 5′-phosphate] 9 × 10^{-2} mol dm^{-3}, [Fe^{2+}–EDTA] 1 × 10^{-3} mol dm^{-3}, [H_2O_2] 1 × 10^{-3} mol dm^{-3} and [MNP] 1 × 10^{-2} mol dm^{-3}. (*b*) As (*a*), but with [2′-deoxyribose 5′-phosphate] 7 × 10^{-2} mol dm^3.

triplet may be from the C_4' radical (if this does not undergo rearrangement rapidly). With 2′-deoxyribose 5′-phosphate (**21**) (see Fig. 3) the dominant doublet is likely to be due to the C_5'-abstraction radical. Attack at the C_2' position (inactivated) would not be expected to be significant.

Returning now to the related spectra from guanosine derivatives, it is believed that with guanosine 5′-phosphate (**14**) the major radicals detected are C_5'- and the rearranged-radicals formed from attack at C_2' and/or C_3'; this is also the case with guanosine 3′-phosphate (**15**). With the 3′:5′-cyclic monophosphate (**16**), it is believed that the triplet species can be assigned to a spin-adduct from a radical centred on C_2', C_3' or C_4' (phosphate is evidently not an effective leaving group in this case). With the 2′-deoxyribose derivatives, the spectra of both the 5′- and 3′-phosphates (**17**) and (**18**) are also dominated by a triplet of doublets assigned to attack at C_5' (a minor component may be from attack at C_2'); the triplet seen with the 3′:5′-cyclic monophosphate (**19**) is identical to that seen with the ribose derivative and is evidently due to attack at C_3' or C_4'.

Adenine and its derivatives. With adenine and its derivatives at pH 7 better spectra were generally obtained using the Ti^{3+}/H_2O_2 couple for generating $^{\bullet}OH$, rather than Fe^{2+}/H_2O_2 (see Fig. 4); the spectra are dominated by triplets (1:1:1) of triplets (1:1:1) (*cf.* guanine). For example, adenine itself (**22**, R = H) gave a spectrum [Fig. 4(*a*)] analysed in terms of two triplets of triplets [with β-N splittings of 0.51 mT (●) and 0.24 mT (□)] and a triplet of singlets (■). For adenosine, similar spectra, but with lower β-N splittings [0.29 mT (●) and 0.19 mT (□)], were observed [Fig. 4(*b*)] and with adenosine 5′-phosphate and 2′-deoxyadenosine, spectra were dominated by a triplet of singlets (■) and a triplet of triplets (□), with a β-N splitting of 0.29 mT for the latter, [see Fig. 4(*c*)] (similar complex spectra have been observed before, but not individually assigned following X-irradiation of adenine and some derivatives[19]). Again we suggest that the triplet of triplets spectra correspond to the C_4–OH and/or the C_8–OH adducts [see *e.g.* (**23**) and **24**)] as expected from the pulse-radiolysis studies of Steenken and coworkers;[18] where a triplet of singlets dominates, this may be the C_5–OH adduct (**25**). At low pH, weaker signals, which decayed

Table 1 EPR parameters for aminoxyls obtained by spin-trapping radicals from 'OH and pyrimidine, purine and sugar derivatives in the presence of MNP

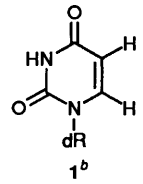
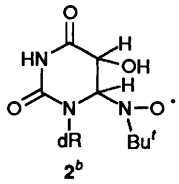
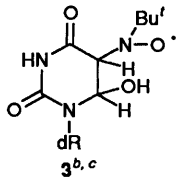
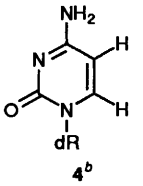
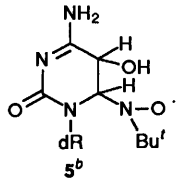
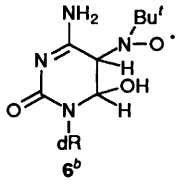
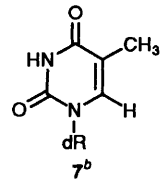
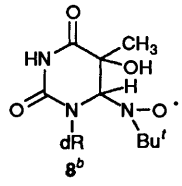
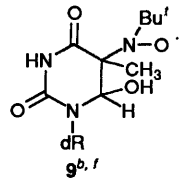
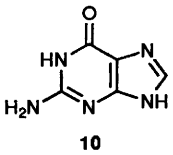
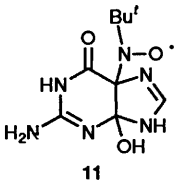
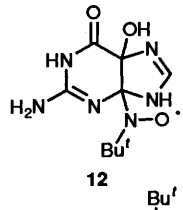
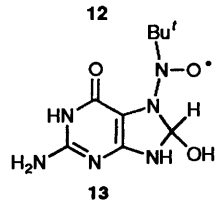
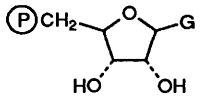
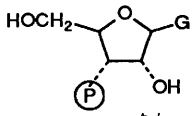
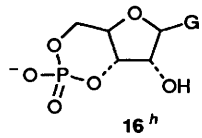
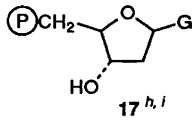
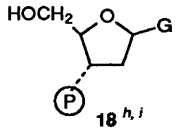
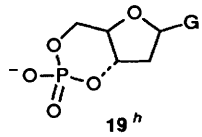
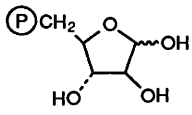
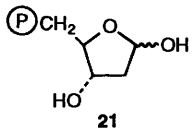
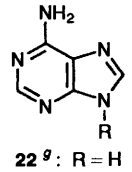
Substrate	Radical(s)	pH	Hyperfine splittings/mT	
			$a(\alpha\text{-N})^a$	$a(\text{other})^a$
 **1**[b]	 **2**[b]	7	1.50	0.67
	 **3**[b,c]		1.51	0.37
			1.51[d]	
 **4**[b]	 **5**[b]	2–7	1.49	0.67[e]
	 **6**[b]		1.48	0.41
			1.52[d]	0.21[d]
 **7**[b]	 **8**[b]	7	1.44	0.29(β-N)
	 **9**[b,f]		1.57	—
 **10**	 **11**	7	1.54[g]	0.33(β-N)[g]
	or  **12**		1.61[g]	—
	 **13**			

Table 1 (*continued*)

Substrate	Radical(s)	pH	Hyperfine splittings/mT a(α-N)[a]	a(other)[a]
 **14** [h,i]		2–7	1.53[e] 1.52[e]	0.18[e] 0.30[e]
 **15** [h,i]		7	1.48 1.49	0.38 0.26
 **16** [h]		7	1.53[j]	—
 **17** [h,i]		2–7	1.53[ej]	0.14[ej]
 **18** [h,i]		7	1.50	0.31
 **19** [h]		7	1.53[j]	—
 **20**		2–10	1.47 1.50 1.49	0.37 0.23 —
 **21**		3–10	1.53[j]	0.16[j]
 **22**[g]: R = H		7	1.50 1.50 1.61	0.50(β-N) 0.24(β-N) —
	22[g]: R = ribose		1.46 1.47 1.47	0.29(β-N) 0.19(β-N) —
	22[g]: R = ribose 5′-phosphate		1.46 1.43	0.40(β-N) —
	22[g]: R = 2′-deoxyribose		1.46 1.50	0.29(β-N) —

[a] Typically ±0.005 mT, except where indicated otherwise. The β-splittings are proton splittings, except where indicated otherwise. [b] dR = deoxyribose. [c] Concentration increases at low pH. [d] This signal decays rapidly at low pH. [e] Parameters measured at pH 7; minor variation noted at low pH. [f] Concentration decreases at low pH. [g] See text for possible assignments. [h] G = guanine. [i] Ⓟ = PO_4^{2-}. [j] Predominant species.

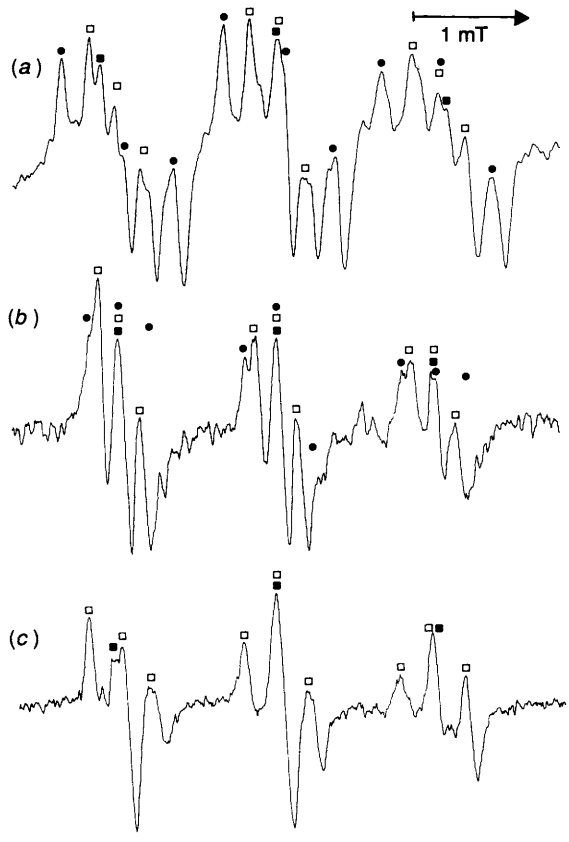
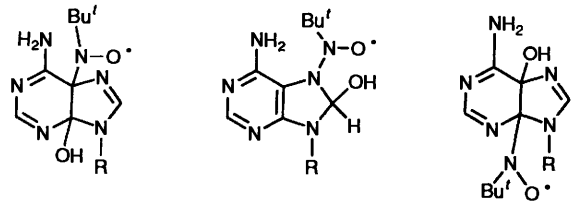


Fig. 4 (*a*) EPR spectra of **23**, **24** and **25** [(●), (□) and (■), respectively] obtained from the reaction of 'OH with adenine in the presence of MNP at pH 7 with [adenine] saturated solution, $[Ti^{3+}]$ 1×10^{-3} mol dm^{-3}, $[H_2O_2]$ 8×10^{-3} mol dm^{-3} and [MNP] 4×10^{-3} mol dm^{-3}. (*b*) As (*a*), but with [adenosine] 1×10^{-1} mol dm^{-3}, $[Ti^{3+}]$ 2×10^{-3} mol dm^{-3}, $[H_2O_2]$ 9×10^{-3} mol dm^{-3} and [MNP] 6×10^{-3} mol dm^{-3}. (*c*) As (*a*), but with [2'-deoxyadenosine] 2×10^{-1} mol dm^{-3}, $[Ti^{3+}]$ 1×10^{-3} mol dm^{-3}, $[H_2O_2]$ 8×10^{-3} mol dm^{-3} and [MNP] 6×10^{-3} mol dm^{-3}.



23 **24** **25**

rapidly, were seen for these substrates. Under these conditions, it is believed that acid-catalysed loss of OH^- leads to the formation of a radical cation, which may be readily reduced by Fe^{2+} and Ti^{3+}.[16]

Conclusions. Results from guanine itself (**10**), and from adenine and its derivatives (**22**) indicate that radical attack occurs at the base moieties; in particular, it is thought that 'OH attack occurs at the C_4–C_5 double bond to give (formally) the C_5-yl and C_4-yl radicals, respectively, and the C_8 position (though the radicals are not necessarily trapped through these positions). In marked contrast, with the guanine nucleosides and nucleotides, the only radicals seen correspond to those detected from sugars and sugar–phosphates and, by comparison with data from pulse radiolysis studies,[6–12] and EPR observation,[21] it seems likely that rapid transfer of the initially formed base radical to the sugar–phosphate backbone has occurred (though direct attack at the sugar moiety cannot be completely ruled out); only minor differences are noted between the ribose and 2'-deoxyribose derivatives.

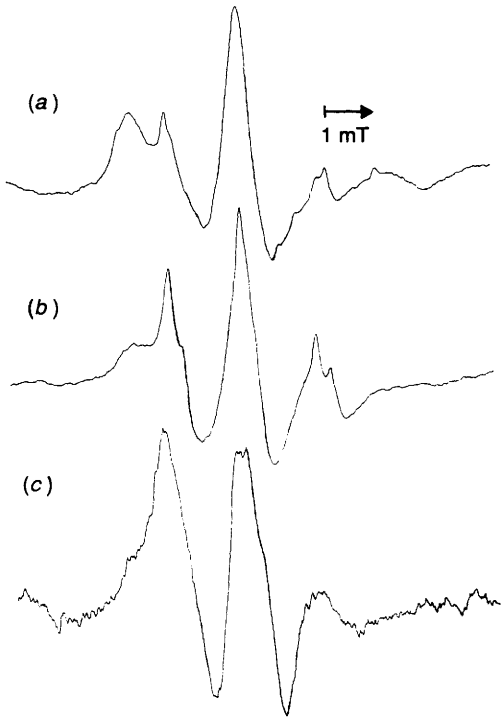


Fig. 5 (*a*) EPR spectra of the highly immobilised and more mobile spin-adducts formed in the reaction between 'OH and dsDNA at pH 7 with [dsDNA] 6 mg cm^{-3}, $[Fe^{2+}$–EDTA] 1×10^{-3} mol dm^{-3}, $[H_2O_2]$ 2×10^{-3} mol dm^{-3} and [MNP] 1×10^{-2} mol dm^{-3}. (*b*) As (*a*), but at pH 3, showing an increase in the relative proportion of the more isotropic species. (*c*) As (*a*), but at pH 2, showing a greater increase in the relative proportion of the more isotropic species.

'OH *Attack on DNA.*—Following hydroxyl-radical attack on DNA, the resulting spectra showed a marked dependence upon pH, 'OH flux and the type of DNA used (highly polymerised *vs.* partially degraded lower molecular weight fragments and single-stranded *vs.* double-stranded, as described below).

Highly polymerised dsDNA (calf thymus). Reactions of highly polymerised dsDNA (5–6 mg cm^{-3}) with Fe^{2+}–EDTA and H_2O_2 in the presence of MNP at pH 7 resulted in the observation of spectra consisting of signals from at least two radical adducts, a large, highly immobilised spin adduct with $A_{\|} = 3.2$ mT and a more mobile species [see Fig. 5(*a*)]. These adducts were relatively stable; no decay of the spectrum was observed after 1 h at room temperature. However, incubating the DNA solution at 37 °C for 2 h prior to use resulted in the observation of less anisotropic spectra, attributed to smaller, more mobile radical adducts (see below). It is suggested that autoxidation of low (micromolar) concentrations of Fe^{2+} present in the buffer generates oxygen radicals, which are responsible for the degradation of the DNA.

On repeating the initial experiment at pH 6, the relative concentration of the isotropic spectrum increased and further hyperfine splitting could be discerned. As the pH was lowered the decrease in concentration of the immobilised species was coupled with an increase in the relative concentration of this isotropic adduct [see Fig. 5(*b*) and (*c*)]. Assignment of the isotropic signals (see below) provides information about the initial site(s) of radical attack.

When the dsDNA adducts generated at pH 7 were treated with acid, isotropic spectra were obtained, which are very similar to those generated at low pH. These isotropic adducts formed by acid-catalysed cleavage were unstable; they completely decayed within 15 min.

To assess the effect of exposure of DNA to low pH before radical attack, dsDNA (pH 7) was treated with acid (pH 2) and then neutralised. When the DNA adducts were then formed

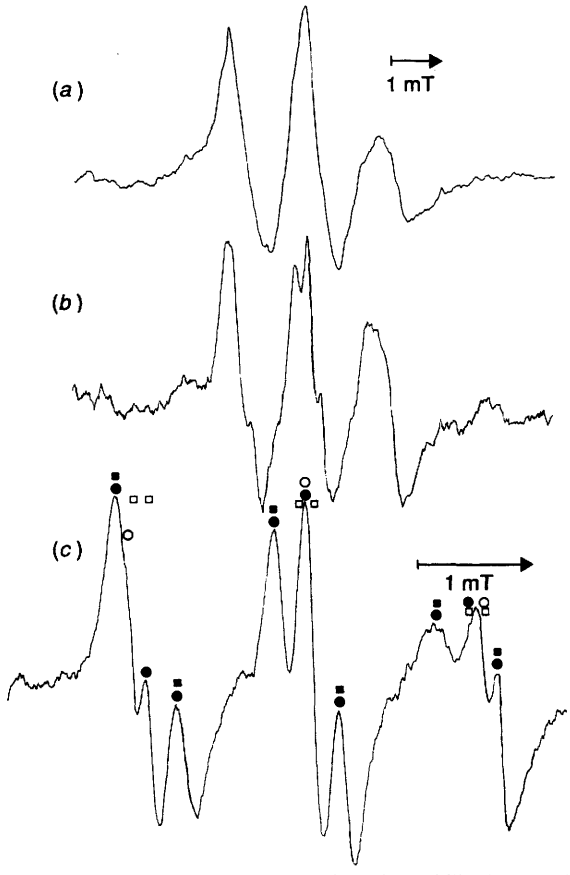


Fig. 6 (*a*) EPR spectrum of the partially immobilised spin-adduct formed in the reaction between $^{\bullet}OH$ and ssDNA at pH 7 with [ssDNA] 6 mg cm^{-3}, [Fe^{2+}–EDTA] 1 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3} and [MNP] 2.5 × 10^{-2} mol dm^{-3}. (*b*) EPR spectra obtained after enzymatic digestion of the immobilised spin-adduct formed in the reaction between $^{\bullet}OH$ and dsDNA at pH 7 with [dsDNA] 6 mg cm^{-3}, [Fe^{2+}–EDTA] 1 × 10^{-3} mol dm^{-3}, [H_2O_2] 2 × 10^{-3} mol dm^{-3}, [MNP] 2.5 × 10^{-2} mol dm^{-3} and [DNase] 1.6 mg cm^{-3}, incubated at 37 °C for 60 min. (*c*) As (*b*), with [dsDNA] 4 mg cm^{-3} and [Fe^{2+}–EDTA] 2 × 10^{-3} mol dm^{-3}, incubated at 37 °C for 240 min and assigned to **5** (■), **8** (●), **9** (○) and sugar-derived radicals (□).

very little of the anisotropic signal remained and the spectra resembled those previously detected at low pH and after treatment with acid. Under all these conditions, acid-catalysed strand-breakage is evidently occurring: at low pH significant degradation of the backbone can occur before subsequent radical damage.

The effect of altering the concentrations of the reactants was also investigated; increasing the concentrations of both Fe^{2+}–EDTA and H_2O_2 (to increase the radical flux) increased the intensity, but did not alter the nature of the spectra, with Fe^{2+}–EDTA (1 × 10^{-2} mol dm^{-3}) and H_2O_2 (5 × 10^{-3} mol dm^{-3}) being the optimum conditions; this suggests that there is not a significant increase in the number of EPR-detectable double-strand breaks. Further increases in the concentration of H_2O_2 (1 × 10^{-2} mol dm^{-3}) resulted in a decrease in intensity.

$^{\bullet}OH$ attack on highly polymerised ssDNA (calf thymus), partially degraded DNA (herring testes) and fully degraded DNA. With ssDNA (5–6 mg cm^{-3}) at pH 7, the spectrum of a partially immobilised spin-adduct was obtained with a sharper spectrum superimposed [see Fig. 6(*a*)]. At pH 6, the spectrum was more intense and the isotropic signal showed further splitting. As the pH was decreased, the concentration of the partially immobilised species decreased and a number of mobile DNA-derived adducts became clearly visible, although at pH 3–4 the broad high-field peak indicates that some of the anisotropy is still present. The adducts detected at low pH are similar to those seen with dsDNA and resemble those detected following X-irradiation of DNA[20] and are assigned below.

The occurrence of strand-breakage resulting from radical attack is clearly greater in ssDNA compared with dsDNA (deduced from the greater proportion of isotropic adducts with the former: see also ref. 23). The close similarities noted between the spectra of ss and dsDNA at low pH suggest that under these conditions, dsDNA may be partially unwound, thus increasing the accessibility of the base moieties to radical attack.

The spectra obtained from experiments with partially degraded DNA were dependent upon $^{\bullet}OH$ flux; with final concentrations of Fe^{2+}–EDTA (1 × 10^{-3} mol dm^{-3}) and H_2O_2 (2 × 10^{-3} mol dm^{-3}), the major signal observed was an isotropic species with an extra splitting [*cf.* Fig. 6(*b*) below]. With fully degraded DNA, the spectrum could also be attributed to several species, assigned on the basis of enzymatic experiments described below.

The effect of the addition of DNase. Enzymatic cleavage of the dsDNA adducts at pH 7 with DNase gave a spectrum that is similar to those detected from both ss and dsDNA at low pH [see Figs. 6(*b*) and 5(*c*)], although the former is better resolved. On the basis of comparisons between the isotropic spectra obtained and those generated from the 2′-deoxynucleotides and detailed analysis of high-resolution expanded spectra, the signals from the DNA-derived adducts are assigned in terms of a mixture of radicals from different sites of attack. The two major radicals [(●) and (○)] are believed to be the spin-trapped C_5–OH and C_6–OH adducts of thymidine [see Figs. 1(*c*) and 6(*c*)] (as also noted in X-irradiated DNA[20]); in addition, there are contributions believed to be from the spin-trapped C_5–OH adduct of 2′-deoxycytidine (■) and traces of a triplet of doublets (□) believed to be derived from the trapping of sugar radicals formed either by rapid transfer of the initial base radicals to the DNA backbone or by direct attack on the sugar [see Fig. 6(*b*) and (*c*)].

Conclusions

The spin-trapping experiments reported here provide evidence for the generation of free hydroxyl radicals by the Fe^{2+}–EDTA/H_2O_2 couple and the subsequent rapid reaction of these intermediates primarily with the base moieties of 2-deoxyribonucleosides, 2-deoxyribonucleotides and DNA itself. Though the signals obtained with the parent macromolecule are anisotropic in nature, owing to the trapping of a high molecular weight polymer radical, we have shown that information can be obtained as to the initial sites of attack since these spin-adducts are relatively stable. Degradation of the nucleic acid adducts, either enzymatically (with DNase) or with H^+, results in the release of smaller fragments with the spin trap still attached; these low molecular weight spin adducts give rise to isotropic spectra which can be readily assigned by comparison with those obtained with model compounds. Thus clear evidence has been obtained in these experiments for addition of hydroxyl radicals at the pyrimidine bases.

The similarity of the spectra obtained with the macromolecule to those obtained with thymidine (as previously suggested by Kuwabara and co-workers[20]) and cytidine in particular, is interpreted in terms of addition of $HO^{\bullet}$ at the C_5–C_6 double bond in the base moiety and rapid reaction of these adducts with the spin trap, thereby halting further fragmentation, rearrangement or decay reactions and giving valuable information about the initial site(s) of attack.

Little evidence has been obtained in these spin-trapping experiments for attack at guanine and adenine in DNA itself, though this is believed to occur (*cf.* previous radiation studies[24]). The lack of signals from such species is, however,

consistent with our previous study with the individual homopolymers poly(A) and poly(G), for which only weak signals were obtained and experiments with poly(A)·poly(U) in which radicals derived mainly from attack at uracil were observed.[21] Radical-adduct species derived from the purines have however been detected with the isolated nucleosides and nucleotides demonstrating both that such species can be generated and that they can be readily trapped. These differences may be due to either the stability of the adducts once formed or the relative rates of trapping *vs.* radical rearrangement and transfer of damage; the latter processes are expected to be significantly affected by steric factors (steric hindrance for trapping and possible increased rates of damage transfer due to steric locking) in the macromolecule, as opposed to the isolated nucleosides and nucleotides.

Evidence for the transfer of damage from an initial site of attack on the base to the sugar has been obtained with both the intact macromolecule and, in some cases, with the isolated nucleosides. Thus the increase in the contribution to the overall spectral envelope of isotropic components (from low molecular weight fragments) which increased radical flux, alterations in pH and changing from dsDNA to ssDNA is believed to reflect, at least in part, a higher incidence of strand breaks.

Evidence has also been obtained with 2′-deoxyuridine, 2′-deoxycytidine and especially the guanine (but not adenine) derivatives, for the presence of sugar-derived spin adducts. This observation might be interpreted in terms of different rates of transfer of damage from the bases to the sugar (and hence uncompetitive reaction with the spin trap). A likely explanation for the observation for guanine derivatives is that its base adducts are either relatively slowly trapped (due to delocalisation of the radicals) or, if trapped, are very short-lived and hence not readily detected. Our experiments do, however, provide further evidence for a lower rate of damage transfer from the purine bases (at least for adenine) to the sugar moieties than with the pyrimidines, as also suggested by data from radiation experiments where the yield of strand breaks in the homopolymers was found to be much higher for poly(U) and poly(C) than poly(G) and poly(A) under identical conditions.[24]

These postulated differences in rates of damage transfer from the base to the sugar may arise from the steric and electronic constraints imposed by the transfer reaction. We have previously suggested that for ribonucleosides and ribonucleotides transfer occurs to the C_2' position (as a result of the direct detection of the rearranged C_2' radical in flow experiments).[16] With the analogous 2-deoxy compounds, transfer to this site would be expected to be disfavoured owing to the lack of the (activating) hydroxyl function which is believed to play a key role in the transfer process. With these latter substrates it is suggested that radical transfer to C_4' (which is activated by the ring oxygen) is the major pathway for strand cleavage as the presence of a radical centre at this position is known, from previous studies, to lead to rapid loss of either the C_5'- or C_3'-phosphate group with concomitant formation of the hydroxylated product (*i.e.* either C_5'-OH or C_3'-OH).[6] EPR signals have been detected with a number of substrates which may be due to the formation of such C_4' radicals; further studies to confirm the identity of these sugar-derived species and to elucidate the key role that these radicals may play in strand breakage are currently being carried out.

Experimental

Experiments were carried out on a Bruker ESP 300 and JEOL RE1X EPR spectrometers, using an aqueous sample cell as described previously.[17] The pyrimidine and purine bases, nucleosides, nucleotides and the sugars and sugar–phosphates were dissolved in phosphate buffer (50 mmol dm^{-3}) at pH 7, citrate (10 mmol dm^{-3}) at pH 3–6 and HCl–glycine buffer at (10 mmol dm^{-3}) at pH 2; samples were prepared by combining a mixture of substrate and Fe^{2+}–EDTA with a mixture of H_2O_2 and MNP, immediately prior to running the EPR spectra. The final concentrations of the reactants were: [substrate] 3×10^{-2} mol dm^{-3} for the bases, nucleosides and nucleotides and $0.3–1 \times 10^{-1}$ mol dm^{-3} for the sugars and sugar–phosphates, [Fe^{2+}–EDTA] $1–4 \times 10^{-3}$ mol dm^{-3} of Fe^{2+} with EDTA present in a two fold excess, [H_2O_2] $1–4 \times 10^{-3}$ mol dm^{-3} and [MNP] $0.5–2.5 \times 10^{-2}$ mol dm^{-3}. The MNP was left to stir in an aqueous solution of acetonitrile for at least 1 h prior to use and the final concentration of acetonitrile was *ca.* 10%.

In experiments with highly polymerised DNA from calf thymus, the dsDNA was dissolved in phosphate buffer at pH 7, citrate buffer at pH 3–6 and HCl–glycine buffer at pH 2. ssDNA was produced by taking samples of dsDNA prepared as above, incubating them at 95 °C for 20 min, followed by rapid cooling on ice; the samples were used immediately. The dsDNA [lower molecular weight from herring testes and the degraded DNA (free acid)] were both used at pH 7 without further treatment.

EPR samples were prepared in the same way as the pyridimidine and purine derivatives and the final concentrations were: [highly polymerised ssDNA] 5–6 mg cm^{-3}, [Fe^{2+}–EDTA] $1–4 \times 10^{-3}$ mol dm^{-3} of Fe^{2+}, [H_2O_2] $1–4 \times 10^{-3}$ mol dm^{-3} and [MNP] $0.1–2.5 \times 10^{-2}$ mol dm^{-2} with *ca.* 10% acetonitrile and [highly polymerised dsDNA and partially and fully degraded DNA] 2–6 mg cm^{-3}, [Fe^{2+}–EDTA] $1–2 \times 10^{-3}$ mol dm^{-3}, [H_2O_2] $1–4 \times 10^{-3}$ mol dm^{-3} and [MNP] $0.6–2.5 \times 10^{-2}$ mol dm^{-3} with *ca.* 10% acetonitrile. The DNA–Fe^{2+} was vortex mixed to ensure thorough mixing before the H_2O_2–MNP was added.

Enzymatic digestion with DNase was performed at 37 °C for up to 4 h; a solution of DNase was added to the samples after mixing to give final concentrations of [DNase] 1.6 mg cm^{-3} and [$MgCl_2$] 10 mmol dm^{-3}. Acid-induced hydrolysis was carried out by adding 0.1 mol dm^{-3} HCl to the samples after mixing and 0.1 mol dm^{-3} NaOH was used to readjust the pH to 7.

All chemicals were obtained from commercial sources; the DNA and the purine and pyrimidine derivatives were from Sigma, Aldrich and Lancaster and all were used without further purification.

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