EPR Spin-trapping studies of the reaction of radicals derived from hydroperoxide tumour-promoters with nucleic acids and their components

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EPR spin trapping experiments using the spin trap Me_3CNO have been used to identify radicals formed on reaction of $Bu^tO^\bullet$ and $PhC(CH_3)_2O^\bullet$ [generated from reaction of the tumour promoter compounds Bu^tO_2H and $PhC(CH_3)_2O_2H$ with Ti^{III}] with a variety of pyrimidine and purine derivatives, as well as RNA and DNA. The observed radical adducts can be rationalized in terms of addition of the alkoxyl radical to the C_5–C_6 double bond of the pyrimidine base and ring positions on the purine derivatives, as well as hydrogen abstraction at the sugar moiety; the latter process is believed to be a minor pathway and may occur as a result of radical transfer from the base to sugar moieties. Comparison of the experimental data with that obtained previously with $HO^\bullet$ shows that the $Bu^tO^\bullet$ and $PhC(CH_3)_2O^\bullet$ radicals exhibit different regioselectivities than the former species. Experiments carried out with DNA, RNA and polynucleosides provide direct evidence for alkoxyl radical attack on these macromolecules; such reactions may play a role in the tumour-promoting activity of these hydroperoxides.

Considerable attention has recently been focussed on the mechanism(s) through which a large number of organic peroxides, hydroperoxides and related compounds, which are used extensively in the chemical, pharmaceutical and cosmetic industries, exert cellular damage. A number of these materials have also been shown, in animal models, to act as tumour promoters in the multi-stage model of chemical carcinogenesis, though they are not initiators or complete carcinogens.[1,2] The mechanism of this promoting activity is poorly understood, though it is believed to involve clonal expansion and/or selection of an altered phenotype; this may involve either genetic damage or *via* changes in epigenetic processes.[3–5] A number of studies have provided evidence to support the hypothesis that these effects are mediated through the generation of free radicals from these compounds.[6–8]

Previous studies have demonstrated that hydroperoxides, such as Bu^tO_2H and cumene hydroperoxide, are rapidly metabolized by a large number of cell types *via* two major processes. The first of these, which is the normal metabolic route and the major detoxification pathway, involves two-electron reduction (to the corresponding alcohol) at the expense of glutathione in a reaction catalysed by the enzyme glutathione peroxidase.[9] This pathway can however be overwhelmed easily by the depletion of glutathione, inactivation of the enzyme, or the presence of high concentrations of the hydroperoxide; under these conditions one-electron reduction, to give free radicals, becomes a significant process. The generation of radicals from these compounds has been demonstrated, principally by EPR spectroscopy and spin trapping, in a number of cells and biological systems, including rat liver hepatocyte fractions (microsomes,[10,11] mitochondria,[12] isolated nuclei[12] and cytosol[14]), isolated human[15] and murine keratinocytes,[8,16] various cultured fibroblast cell lines,[17] and intact murine skin.[18] The catalytic source of these radicals appears to depend on the cell type, with evidence having been presented for radical formation by the leakage of electrons from the mitochondrial electron transport chain,[12] by either reduction or oxidation of the hydroperoxide by the cytochrome P450 enzyme family,[10,11,13] by other haemproteins,[10,11,13] and by low-molecular-weight metal-ion complexes.[14] The generation of radical species from these compounds has been directly linked with their tumour-promoting activity as a result of studies with radical-scavengers such as butylated-hydroxyanisole (BHA) and butylated-hydroxytoluene (BHT); such compounds, which have been shown to scavenge hydroperoxide-derived radicals in isolated murine keratinocytes *in vitro*,[8] have also been shown to inhibit tumour promotion *in vivo*.[6,7,19]

However, though there is now a strong causative link between tumour promotion and radical generation from these compounds, relatively little is known about the biological effects of these radicals and the key cellular targets for these species. Recent studies have demonstrated that exposure of cultured cells to hydroperoxides results in the generation of DNA strand breaks,[4,20–22] though the mechanism of damage has not been elucidated. In particular, it is not clear whether the observed damage arises from the direct interactions of the hydroperoxide-derived radicals with DNA, or whether this arises from a secondary process. Similarly it is not known whether strand-breakage is the major form of DNA damage, or whether, like $HO^\bullet$-induced damage,[23] attack at (and hence modification of) the base moiety is also important. Previous studies on the reactivity of alkoxyl radicals (such as $Bu^tO^\bullet$) suggest that this species might be expected to attack both the sugar and the base moieties;[24] whether the latter can subsequently give rise to sugar damage and hence strand breaks (*cf.*, the damage transfer processes postulated for $HO^\bullet$-induced damage to DNA) is not known.

In order to further elucidate the mechanism of DNA damage induced by radicals derived from these hydroperoxide tumour promoters, we have carried out a detailed study of the reactions of the $Bu^tO^\bullet$ radical (generated by use of a Bu^tO_2H/Ti^{III} couple[25]) with isolated DNA, RNA and their components, by use of EPR spin trapping using the spin trap 2-methyl-2-nitrosopropane (Me_3CNO, MNP), with the aim of characterizing the radicals generated on the target molecules (and hence the initial sites of attack), the subsequent reactions of these species, and therefore the mechanism(s) of damage. Comparative studies have also been carried out in some cases with the cumene alkoxyl radical [$PhC(CH_3)_2O^\bullet$; generated by a cumene-O_2H/Ti^{III} couple] in order to investigate the effect of steric bulk on these processes.

Results and discussion

Reactions of alkoxyl radicals with pyrimidine nucleobases, nucleosides and nucleotides

In the experiments reported here $Bu^tO^•$ radicals were generated by mixing nitrogen-purged solutions of Ti^{III} and Bu^tO_2H [reaction (1)]. The reactions of these radicals with various

$$Ti^{III} + Bu^tO_2H \longrightarrow Ti^{IV} + HO^- + Bu^tO^• \quad (1)$$

substrates (present in large excess) were then examined in the presence of the spin trap MNP with the pH in the range 1–7.4. EPR spectra were recorded (for a period of up to 2 h) shortly after transfer of the sample solutions to an aqueous solution sample cell. High concentrations of the substrate (0.1–1 mol dm^{-3}, or saturated solutions) were employed in all cases to ensure that the reactions under study were those of the $Bu^tO^•$ or $PhC(CH_3)_2O^•$ radicals rather than those of the methyl radical which can arise from β-scission of the initial alkoxyl radical [*e.g.* reaction (2)].[26] Previous studies have determined the rates of

$$Bu^tO^• \longrightarrow CH_3C(O)CH_3 + CH_3^• \quad (2)$$

these unimolecular scission processes as *ca.* 1.4×10^6 and 1.0×10^7 s^{-1}, respectively,[24,27,28] and it has been reported that the $Bu^tO^•$ radical reacts with thymidine and adenosine with rate constants of 3.1×10^8 and 1.4×10^8 mol^{-1} dm^3 s^{-1}, respectively;[24] it is expected that the free bases and related compounds (such as the less-hindered uridine) would react at similar rates. The rate constants for reaction of the $Bu^tO^•$ and methyl radical with MNP have also been determined (1.3×10^8 and 1.7×10^7 mol^{-1} dm^3 s^{-1}, respectively[29,30]); in the light of these values an intermediate concentration of spin trap (3.8×10^{-3} mol dm^{-3}) was chosen such that only low levels of the initial hydroperoxide-derived radicals would be trapped, whilst maintaining a sufficiently high concentration to encourage a relatively high yield of adducts derived from the substrates. For a substrate concentration of 0.1 mol dm^{-3} (typical of those employed in this study) it can be estimated, using the above figures, that *ca.* 90% of the alkoxyl radicals generated should react with the substrate rather than fragment to give methyl radicals or react directly with the spin trap.

Pyrimidine nucleobases. Reaction of $Bu^tO^•$ radicals with the spin trap MNP in the absence of added substrate, gave rise to signals from aminoxyl radicals resulting from the trapping of both $Bu^tO^•$ and $CH_3^•$ as predicted from the above kinetic analysis [with hyperfine coupling constants, as reported previously, of a(N) 2.67, and a(N) 1.71, a(3H) 1.41 mT, respectively[31]], together with short-lived signals from $Bu^tNHO^•$ [a(N) 1.42, a(H) 1.42 mT[31]] and the radical adduct of $^•CH_2CN$ [a(N) 1.63, a(2H) 1.0 mT]. The $Bu^tNHO^•$ is believed to be formed by reduction of the spin trap by Ti^{III} and subsequent protonation, whereas the $^•CH_2CN$ is thought to arise from hydrogen-abstraction from the acetonitrile used to enhance the dissolution and monomerization of the spin trap. In the presence of any of the nucleobases uracil, cytosine or thymine, these signals were diminished in intensity or completely removed, and additional signals, which can be assigned to the trapping of nucleobase-derived radicals, observed. Thus with uracil at pH 7.4 a spectrum, which can be assigned as a triplet (from the aminoxyl nitrogen) of triplet of doublets, with further partially resolved splittings, is observed; the hyperfine coupling constants of this species (which are collected in Table 1) are consistent with this species being due to the trapping of **1**, the C_6-yl radical from the base formed by addition of the alkoxyl radical at the C_5 end of the C_5–C_6 double bond. Comparison of these spectra with those obtained in the analogous reaction with $HO^•$ suggests that these two radicals

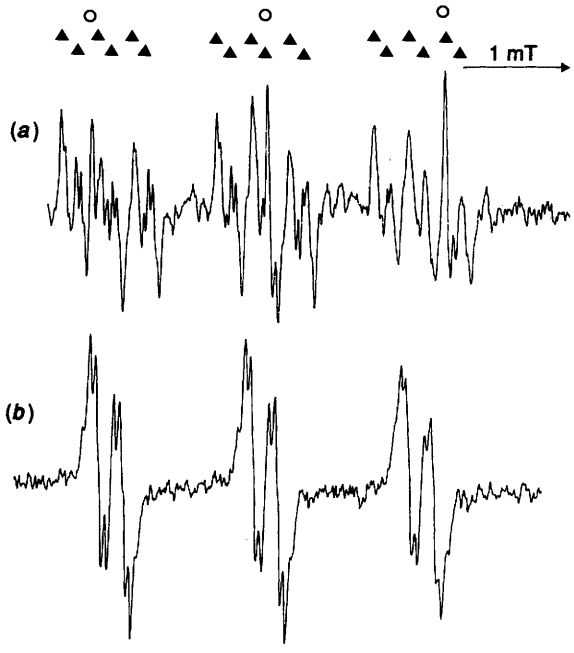
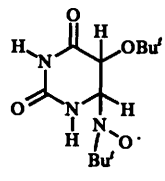
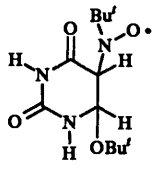


Fig. 1 (*a*) EPR spectrum observed on reaction of $Bu^tO^•$ with uracil at pH 7.4 in the presence of MNP with [uracil] 2.4×10^{-2}, [Bu^tO_2H] 4.8×10^{-3}, [Ti^{III}] 1.16×10^{-3}, [MNP] 0.4×10^{-3} mol dm^{-3}. Signals assigned to the C_6-yl C_5-OBu^t radical adduct to the spin trap; additional lines marked (○) are due to di-*tert*-butylaminoxyl formed by decomposition of the spin trap; (*b*) as (*a*) except at pH 1 and with uracil as a saturated solution, [Bu^tO_2H] 8.3×10^{-3}, [Ti^{III}] 3.0×10^{-3} and [MNP] 1.9×10^{-2} mol dm^{-3}. Signals assigned to the C_5-yl C_6-OBu^t radical adduct.



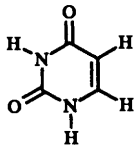
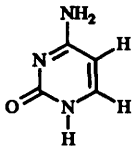
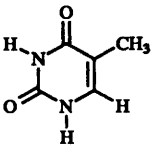
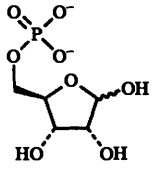
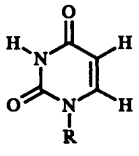
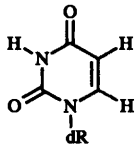
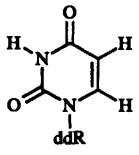
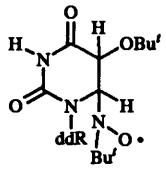
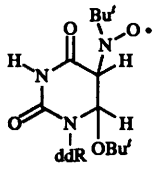
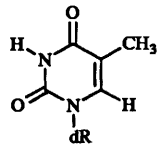
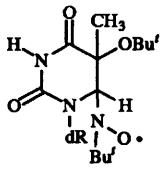
1

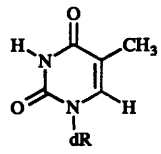
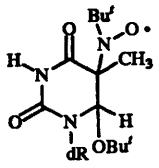
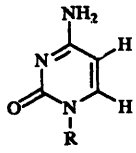
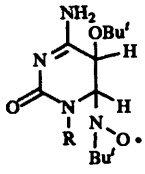
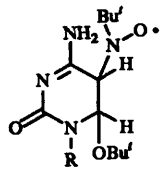
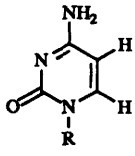
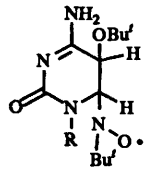
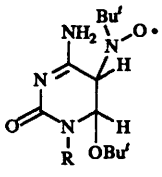
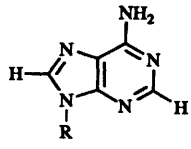
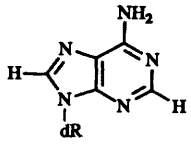


2

have, in this case, similar regioselectivities and give rise to spin adducts with similar parameters.[32,33] It should be noted that in this case, and all subsequent studies, the ratio of the spin adducts detected is dependent on (*a*) the relative rates of addition of the attacking radical at the double bond, (*b*) the rates of subsequent trapping of these (substrate-derived) radicals by the spin trap, and (*c*) the stability of the spin adducts (though in many cases these are very similar, in that they decay very slowly); the ratios of spin adducts observed may therefore not be a wholly accurate indication of the initial ratio of attack on the substrate. At lower pH the intensity of this signal decreases and the signal from a further species appears, until at pH 1 the spectrum is dominated by this second radical (**2**). A species with identical parameters, believed to be formed by addition of $HO^•$ at C_6 and trapping through the C_5 position (*i.e.* the C_6-OH C_5-yl radical adduct), is observed following attack of $HO^•$ (generated using a Ti^{III}/H_2O_2 couple[32]) on uracil at very low pH,[34] suggesting that under acid conditions either the regioselectivity of attack by $Bu^tO^•$ changes and that this second adduct is the corresponding C_6-OBu^t C_5-yl radical adduct, or that an interconversion process exists which results in the generation of hydroxylated products. Experiments carried out with a variety of carbon-centred radicals (methyl, *p*-methoxyphenyl), whose adducts would not be expected to rearrange, show a similar pH dependence,[34] suggesting that the adduct

Table 1 EPR parameters of radicals generated from reaction of Bu^{t}O$^\bullet$ with nucleobases, nucleosides, nucleotides, RNA and DNA in aqueous solution in the presence of the spin trap MNP

Substrate[a]	Radical(s)	pH	a(N)[b]	a(other)[b]
	**1**	7.4	1.50	0.345 (β-N) 0.135 (β-H)
	2	1	1.50	0.245 (β-H) 0.064 (γ-H) 0.064 (γ-N) 0.046 (γ-N)
	**3**	7.4	1.50	0.347 (β-N)[c] 0.167 (β-H)[c]
	4	2.5	1.50	0.380 (β-H)[c]
	**5**	1–7.4	1.50	0.354 (β-N)
	6	1–7.4	1.60	
	Sugar adduct	7.4	1.50	
	Sugar adduct	7.4	1.50	0.21 (β-H)
	**7**	1–7.4	1.495	0.65 (β-H)
	8	1–7.4	1.50	0.35 (β-H)
	Sugar adduct	1–7.4	1.505	
	**9**	1–7.4	1.50	0.69 (β-H)
	10	1–7.4	1.52	0.342 (β-H)
	Sugar adduct	1–7.4	1.50	
		7.4	1.51	0.68 (β-H)
		7.4	1.52	0.39 (β-H)
	Sugar adduct	7.4	1.58	
		1–7.4	1.458	0.30 (β-N)
	11			

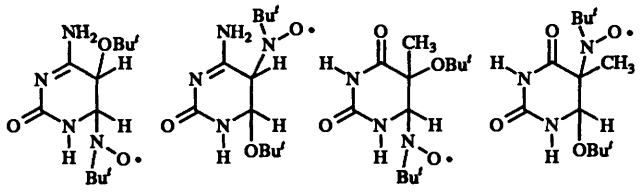
Substrate[a]	Radical(s)	pH	a(N)[b]	a(other)[b]
		1–7.4	1.60	
	12			
		5–7.4	1.493	0.46 (β-H)[c] 0.11 (β-N)[c]
	13			
		1–7.4	1.5	0.39 (β-H)[c]
	14			
		7.4	1.46	≈ 0.6 (β-H)[c,d]
	15			
		7.4	1.49	0.375 (β-H)[c]
	16			
	Sugar adduct	2, 7.4	1.40	0.14 (β-H)
	*e*	3	1.50	1.8 (β-H)
	*e*	2.5	1.49	1.7 (β-H)
Adenosine-5′-triphosphate	*e*	3	1.58	0.20 (β-H)
Poly-U	*e*	3.5, 7.4	1.5	0.35 (β-H)
Poly-C	*e*	7.4	1.5	
Poly-A-Poly-U	*e*	2.5, 7.4	1.49	0.40 (β-H)
RNA	*e*	2, 7.4	1.50	0.36 (β-H)
DNA	*e*	2, 7.4	1.60	

(cont.)

[a] Abbreviations used: R = ribose, dR = 2′-deoxyribose, ddR = 2′,3′-dideoxyribose. [b] Typically ± 0.005 mT. [c] Parameters measured at pH 7.4; these values vary at low pH where the amine group will be protonated. [d] Estimated parameters. [e] For discussion of possible assignments, see text.

seen with $Bu^tO^{\bullet}$ is indeed the C_6-OBu^t C_5-yl radical adduct and that the change in regioselectivity at these very low pH values is due to protonation of the pyrimidine ring.

Replacement of uracil with cytosine resulted in the detection, at pH 7.4, of a spin adduct whose parameters, by comparison with the corresponding $HO^{\bullet}$ reactions, identify this species as the spin trapped C_5-OBu^t C_6-yl radical (**3**). As in the case of



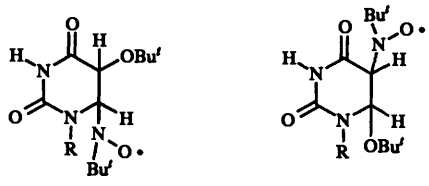
3 **4** **5** **6**

uracil, changes in the observed spectra were observed as the pH was lowered with this species replaced by a further signal with a single β-H splitting. At pH 1.5, this second species can be clearly identified as being due to the spin adduct formed by addition of the $Bu^tO^{\bullet}$ radical at the other end of the double bond [*i.e.* the C_6-OBu^t C_5-yl radical adduct (**4**)]; this change, which occurs at a much higher pH than that seen with uracil, is believed to be due to the protonation of the ring amine group, which has pK_a *ca.* 4.5. In contrast, with thymine attack at both ends of the C_5–C_6 double bond appears to occur at pH 7.4. Thus signals which can be assigned to the radical adducts **5** and **6**, in a concentration ratio of *ca.* 13:1 (obtained from computer simulation of the experimental spectra), are observed. At lower pH values, no further species are observed, though the relative intensities of the signals of **5** and **6** changes, with a ratio of *ca.* 3.3:1 calculated at pH 1.5. No evidence was obtained for the formation of a radical arising from hydrogen abstraction at the C_5 methyl group which would give an allyl species. The spectral parameters of all these adducts are collected in Table 1.

When Bu^tO_2H was replaced by $CumO_2H$ in each of the above experiments, very similar results were obtained, although the regioselectivity of attack on the C_5–C_6 bond appears to be somewhat different, with attack at C_5 less favoured; this is probably due to steric factors. Thus with thymine the ratio of the C_5-yl C_6-$O(CH_3)_2CPh$ to C_6-yl C_5-$O(CH_3)_2CPh$ adducts at pH 2.5 has been calculated, by spectral simulation, as 1 to 2 (*cf.* ratios of 1:6.7 and 1:13 for the corresponding hydroxyl and *tert*-butoxyl systems respectively at the same pH).

Ribose and 2-deoxyribose sugars. In order to investigate possible attack on the sugar moieties of nucleosides, nucleotides, DNA and RNA, studies were carried out on two sets of model sugars. Inclusion of either D-ribose or 2-deoxyribose in the above reaction systems instead of the nucleobases, at pH 7.4, resulted in the detection of complex mixtures of spin trapped sugar-derived radicals. The spectra observed with ribose can be rationalized in terms of the presence of signals from (at least) two spin adducts, whereas with 2-deoxyribose at least four species can be distinguished. Assignment of these signals, which are believed to be formed by hydrogen-abstraction, to particular radical adducts has however proved impossible due to the similarity of many of the possible intermediates; it is however clear that attack of the hydroperoxide-derived radicals on the sugar is occurring at multiple sites and is essentially random. At lower pH values more intense spectra are observed but there is little change in the relative intensities of the signals. Less well-resolved spectra were obtained using the corresponding sugar-5-phosphates, with attack of $Bu^tO^{\bullet}$ on D-ribose-5-phosphate resulting in the detection of (at least) two radical adduct species (see Table 1 for parameters). These results are qualitatively similar to those obtained on reaction of $HO^{\bullet}$ with these same substrates[33] (suggesting that common intermediates are being generated) though there are differences in the relative ratios of the observed species.

Nucleosides, 2′-deoxynucleosides and nucleotides, and 2,3-dideoxynucleosides. The above studies have demonstrated that both the base and the sugar moieties of DNA and RNA components are possible sites of alkoxyl radical attack. In order to assess the relative reactivity of these two different components, and to investigate possible transfer of damage between these sites (*cf.* previous studies with $HO^{\bullet}$ [32,33]), studies were carried out with a range of nucleosides and nucleotides. Reaction of $Bu^tO^{\bullet}$ with uridine at pH 7.4 in the presence of MNP resulted in the detection of several radical adduct species. These have been identified, by comparison with the data obtained above and in the corresponding reactions with $HO^{\bullet}$,[32,33] as being due to two base-derived adduct radicals **7** and **8** (whose spectra consist of triplets of doublets, with



7 (R = ribose sugar) **8**

relatively large doublet splittings; see Table 1), in the ratio *ca.* 0.6:1 and, at least one, further species which is believed to be due to the trapping of a sugar-derived radical. The parameters of this sugar-derived radical adduct are difficult to determine exactly due to overlap with the lines of the base-derived species; they are however similar to some of those observed on direct reaction of $Bu^tO^{\bullet}$ with the isolated sugar (see above and Table 1). Whether this species arises from direct attack on the sugar or *via* a radical transfer process from an initially formed base radical is not certain. However, in a system containing equimolar (50 mmol dm^{-3}) amounts of uracil and ribose at pH 7.4, only the uracil C_5-OBu^t C_6-yl radical was trapped and no sugar-derived species; this suggests that the sugar-derived species observed with uridine arises *via* a subsequent reaction of the base-derived species and not *via* direct attack.

On repeating the experiment with uridine at lower pH values, significant differences were observed in the relative proportions of these radical adducts, with the relative ratio of **7** to **8** changing to *ca.* 0.45:1 at pH 1. The relative yield of the sugar-derived adduct(s) also increased as the pH was lowered (from 7.4 to 1), with the ratio of (**7** + **8**):sugar changing from *ca.* 4.9:1 to *ca.* 2.8:1. With 2′-deoxyuridine very similar behaviour was observed, though in this case, in addition to the signals from the two base-derived species **9** and **10**, a triplet signal, from what is believed to be a sugar-derived radical adduct, was also detected (see Table 1). Analogous behaviour to that observed with uridine and 2′-deoxyuridine, was detected with 2′-deoxycytidine and 2,3-dideoxyuridine (i.e. two-base derived species and one, or more, sugar-derived species), whereas with cytidine and thymidine only pairs of base-derived species are detected in each case and no sugar-derived species; the formation of sugar-derived species with the latter substrate would however be difficult to detect due to the potential overlap between these signals and those from the C_6-OBu^t C_5-yl base-derived adduct, which gives a relatively strong, broad, triplet signal at similar magnetic field values.

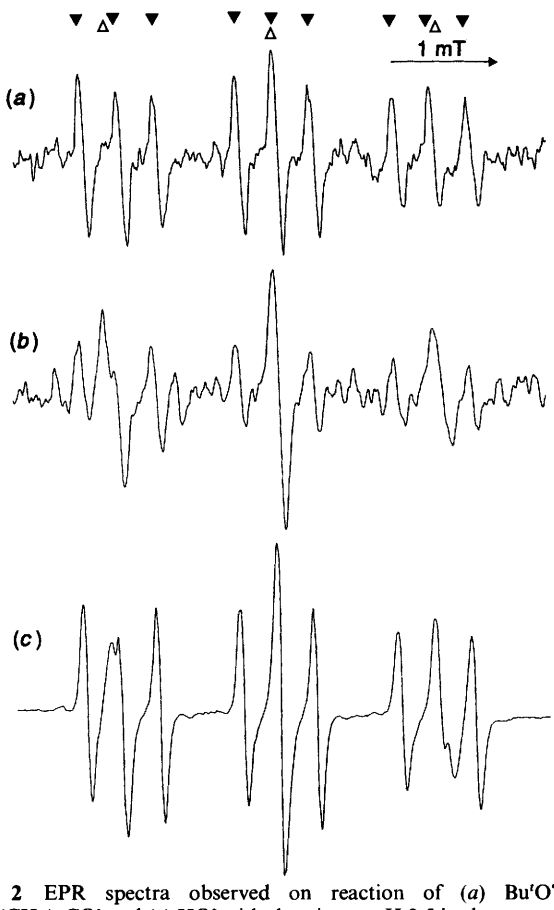
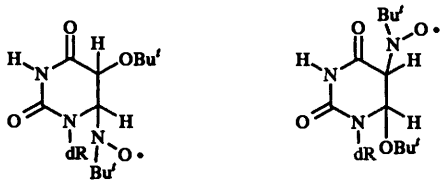


Fig. 2 EPR spectra observed on reaction of (*a*) $Bu^tO^{\bullet}$ (*b*) $PhC(CH_3)_2CO^{\bullet}$ and (*c*) $HO^{\bullet}$ with thymine at pH 2.5 in the presence of MNP with thymine as a saturated aqueous solution, $[Bu^tO_2H$, $PhC(CH_3)_2O_2H$ or $H_2O_2]$ 8.3×10^{-3}, $[Ti^{III}]$ 1.0×10^{-3} and [MNP] 3.8×10^{-3} mol dm^{-3}. Signals assigned to mixtures of the C_5-yl C_6-OR (△) and C_6-yl C_5-OR (▼) radical adducts to the spin trap with R = Bu^t, $PhC(CH_3)_2$ and H respectively. Additional weak signals are due to the $^{\bullet}CH_2CN$ radical adduct to the spin trap.



9 (dR = 2-deoxyribose sugar) **10**

Reactions of $Bu^tO^{\bullet}$ with purine nucleobases, nucleosides and nucleotides

Substitution of the pyrimidine compounds used in the above experiments with purine derivatives resulted in much poorer spectra and somewhat different behaviour. Attempts to study the reactions of $Bu^tO^{\bullet}$ with the bases themselves were frustrated by the poor solubility of these materials in aqueous solution—even at the solubility limit, the concentration of these materials was insufficient to produce detectable levels of base-derived spin adducts. However, the signals from the adducts of the $Bu^tO^{\bullet}$ and $CH_3^{\bullet}$ radicals were significantly diminished in intensity compared to those obtained in the absence of the base, suggesting that reaction does occur between these radicals and the purine bases, as suggested by pulse radiolysis experiments.[24] In contrast, weak signals were obtained with some of the much more soluble nucleosides. Although no nucleoside-derived radicals could be detected at pH 7.4 (in contrast with the case of $^{\bullet}OH$ attack on the nucleosides[33]), inclusion of adenosine or 2′-deoxyadenosine at pH *ca.* 3 gave rise to poorly resolved and weak spectra with a triplet of doublets pattern in each case (for parameters see Table 1); these may be adducts formed by trapping of base-derived species, (*cf.* pulse radiolysis experiments, which suggest that reaction of $Bu^tO^{\bullet}$ with adenosine is very rapid[24]), though exact assignments are not possible. Experiments with the corresponding nucleotide ATP, also resulted in the detection of a species with a triplet of doublets splitting, although the parameters are different to those observed with the nucleosides; this is again believed to be due to a base-derived species. In contrast with guanosine and its derivatives no substrate-derived radicals could be detected, though as with adenine evidence for the occurrence of a reaction between the $Bu^tO^{\bullet}$ radical and the base is inferred from the decrease in intensity of the signals from the $Bu^tO^{\bullet}$ and methyl radical adducts in the presence of these substrates.

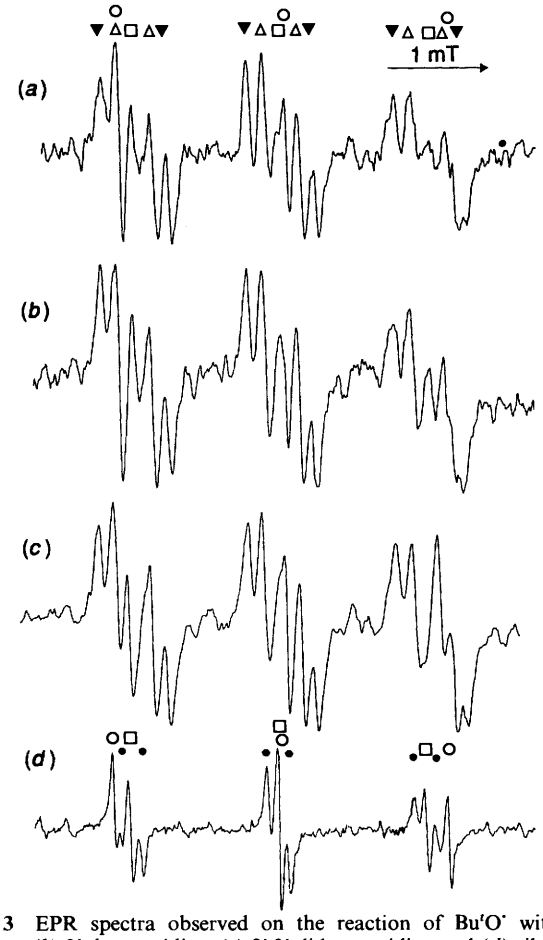


Fig. 3 EPR spectra observed on the reaction of $Bu^tO^{\bullet}$ with (*a*) uridine, (*b*) 2′-deoxyuridine, (*c*) 2′,3′-dideoxyuridine and (*d*) ribose-5-phosphate at pH 7.4 in the presence of MNP with [uridine] 1.7×10^{-1}, [2′-deoxyuridine] 1.8×10^{-1}, [2′,3′-dideoxyuridine] 6.5×10^{-2}, [ribose-5-phosphate] 1.2×10^{-1}, $[Bu^tO_2H]$ 8.3×10^{-3}, $[Ti^{III}]$ 1.0×10^{-3}, [MNP] 3.8×10^{-3} mol dm^{-3}. Signals assigned to a mixture of the C_5-yl C_6-OBu^t (△), C_6-yl C_5-OBu^t (▼) base radical adducts, together with sugar-derived radical adduct species (□); lines marked (○) are due to di-*tert*-butylaminoxyl formed by decomposition of the spin trap; additional weak signals are due to the $^{\bullet}CH_2CN$ radical adduct to the spin trap.

Reaction of $Bu^tO^{\bullet}$ with polynucleotides, RNA and DNA

The results obtained in the experiments described above suggest that damage occurs (either initially or *via* subsequent transfer processes) at both the base and the sugar moieties; the former would be expected to give rise to altered bases as products, whereas the latter might lead to strand breakage of the sugar–phosphate backbone in RNA and DNA (as well as in the appropriate homopolymers) as described previously for $HO^{\bullet}$-

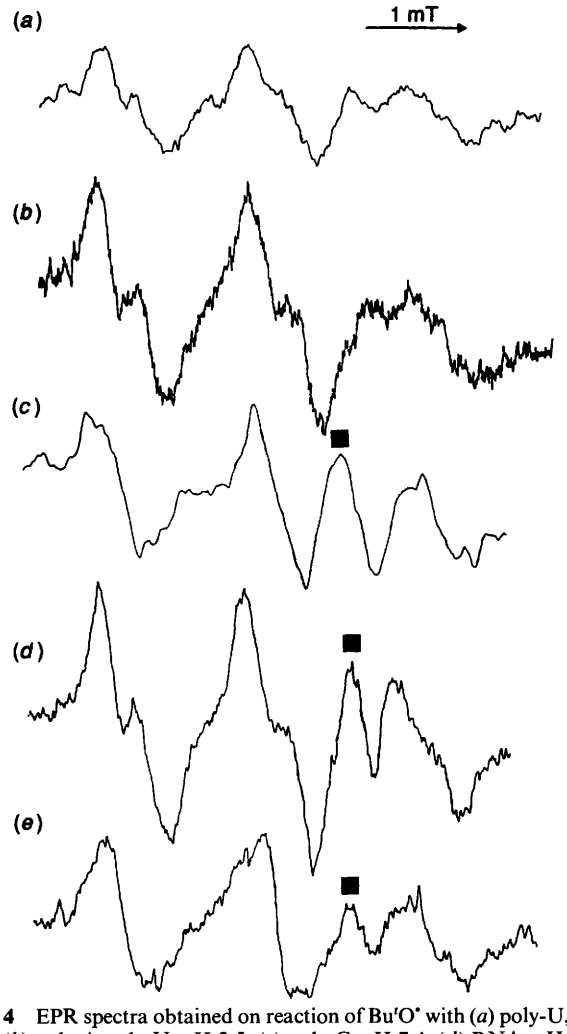


Fig. 4 EPR spectra obtained on reaction of $Bu^tO^•$ with (*a*) poly-U, pH 3.5; (*b*) poly-A-poly-U, pH 2.5; (*c*) poly-C, pH 7.4; (*d*) RNA, pH 7.4; and (*e*) DNA at pH 7.4 in the presence of MNP with (*a*) [poly U] 71 mg cm^{-3}, [Bu^tO_2H] 1.0 × 10^{-2}, [Ti^{III}] 8.6 × 10^{-4}, [MNP] 2.5 × 10^{-1} mol dm^{-3}; (*b*) [poly-A-poly-U] 100 mg cm^{-3}, [Bu^tO_2H] 9.3 × 10^{-3}, [Ti^{III}] 9.6 × 10^{-4}, [MNP] 2.3 × 10^{-1} mol dm^{-3}; (*c*) [poly-C] 16 mg cm^{-3}, [Bu^tO_2H] 4.8 × 10^{-3}, [Ti^{III}] 1.2 × 10^{-3}, [MNP] 7.5 × 10^{-4} mol dm^{-3}, (*d*) [RNA] 66 mg cm^{-3}, [Bu^tO_2H] 4.9 × 10^{-3}, [Ti^{III}] 1.2 × 10^{-3}, [MNP] 0.4 × 10^{-3} mol dm^{-3}; and (*e*) [DNA] 73 mg cm^{-3}, [Bu^tO_2H] 4.9 × 10^{-3}, [Ti^{III}] 1.2 × 10^{-3}, [MNP] 0.3 × 10^{-3} mol dm^{-3}. For assignment of substrate-derived signals see text; the feature marked (■) is due to a paramagnetic impurity in the sample cell.

induced damage.[32,33] In order to investigate this possibility, and to examine the role of three-dimensional structure of the polymers in these reactions, further experiments were carried out with a number of the homopolymers (poly-U, poly-C, poly-A, poly-A-poly-U) as well as RNA and DNA. With all the homopolymers tested with the exception of poly-A, reaction with $Bu^tO^•$ in the presence of MNP at pH 7.4 resulted in the detection of spectra which were partially anisotropic in nature; this is believed to be due to the presence of (at least one) large, slowly-tumbling, spin adduct species. Due to the broad nature of these spectra exact determination of the nitrogen hyperfine coupling constants [*a*(N)] proved difficult though an approximate value of 1.50 mT could be obtained. In the case of both poly-U and poly-A-poly-U further splittings could also be discerned with a clearly resolved doublet splitting from a single hydrogen (of *ca.* 0.4 mT). No further splittings could be clearly resolved in the case of poly-C though the shape of the spectral lines suggest that there are either further unresolved splittings present or additional species. No polynucleotide-derived radicals were observed in the case of poly-A. The signals observed with both poly-U and poly-A-poly-U are somewhat akin to, and have similar hyperfine coupling constants to, the C_5-yl C_6-OBu^t base-derived adduct seen with uridine; low concentrations of the C_5-OBu^t C_6-yl base adduct from uridine may also be present. No signals with parameters similar to those detected with the adenosine derivatives could be discerned, though this does not preclude their presence or formation, due to the complex and broad nature of these spectra.

Similar, partially anisotropic, spectra to those seen with poly-A-poly-U, with a clear triplet of doublets pattern [with *a*(N) 1.50, *a*(H) 0.36 mT], were obtained with yeast RNA at both pH 7.4 and at pH 2. These spectra are believed to arise from the superposition of signals from several adducts; there is marked resemblance between these spectra and those of the C_5-yl C_6-OBu^t base-derived adducts detected with uridine and cytidine, suggesting that such species may also be generated in high yields with these samples of RNA; the presence of low concentrations of other adduct species, such as the C_5-OBu^t C_6-yl base adducts from these two species, cannot however be excluded. The partially anisotropic nature of these spectra is believed to be due to either the molecular weight of this material and/or its partially double-stranded nature; the latter would be expected to decrease any putative increase in mobility arising from the generation of strand breaks. In contrast, when similar experiments were carried out with DNA at either pH 7.4 or pH *ca.* 3.5, anisotropic triplet spectra were observed which are consistent with the presence of high molecular weight radical adducts and a relatively low yield of low molecular weight fragments, though as with RNA this does not preclude the formation of single strand breaks as the double stranded nature of this material might be expected to minimize potential increases in mobility of the damaged chains. The nitrogen hyperfine coupling constants of the adduct(s) giving rise to these spectra is difficult to determine accurately due to the broad nature of the lines, but is of the order of 1.6 mT; unlike the RNA and polynucleotide systems no further splittings could be resolved, making the exact analysis of these spectra difficult. It should however be noted that these spectra have a similar nitrogen coupling constant (and lack of further hyperfine splittings) to the C_5-yl C_6-OBu^t adduct observed with thymidine, suggesting that a similar species might also be being generated in the case of DNA. It is clear, however, whatever the exact identities of these adducts are, that $Bu^tO^•$ radicals are capable of inducing damage to both RNA and DNA.

Conclusions

The EPR spin trapping results reported here are consistent with reaction of alkoxyl (particularly $Bu^tO^•$) radicals with pyrimidine nucleobases, nucleosides and nucleotides, primarily *via* addition to the C_5–C_6 double bond of the base. The selectivity of attack is shown, in some cases, to be different to that previously seen with hydroxyl radicals;[32,33] the proportion of attack at either end of this double bond appears to depend on more than one factor, with both electronic factors (*cf.* the preponderence of attack at C_5 with uracil at pH 7.4) and steric constraints playing a role (*cf.* the ratios of attack at C_5 relative to C_6 on going from uracil to thymine, and on varying the attacking radical with thymine). It is, however, impossible to rule out the effect of steric factors on the rate of trapping of the substrate-derived radicals as the cause of these changes in some cases. Evidence has also been obtained for attack of alkoxyl radicals on the purine analogues, though signals have only been obtained in a small number of cases and the exact identity of these adduct species is not known. Damage to the sugar moieties of the pyrimidine nucleosides has also been detected, though it is not entirely clear whether this is due to direct hydrogen abstraction or transfer of damage from an initially

generated base adduct; experiments with equimolar mixtures of the nucleobase and sugar suggest that the latter process may be the more likely source. The mechanism of such a transfer process (if this is indeed occurring) would appear not to be the same as that previously suggested for the corresponding hydroxyl radical system at low pH, where radical cation intermediates have been invoked,[35] as acid-catalysed formation of such species from the Bu^{t}O–base adducts would be expected to be less ready, and no evidence has been obtained for a marked alteration in the yield of the sugar-derived species with changes in pH.

Damage to polynucleosides, RNA and DNA has also been detected in these experiments and it is suggested that addition of the alkoxyl radical to the base moieties is the major mode of attack. The generation of what appear to be base-adducts with these substrates is in contrast to the strand-breaks previously shown to be generated by these hydroperoxides in isolated cells (though the latter may be arising from initial attack at the base),[36–38] and suggests that altered bases (either adducts or degradation products) may be a major component of the genetic damage induced by these tumour promoters.

Experimental

Experiments were carried out using either a Bruker ESP300 or JEOL RE1X EPR spectrometer, with an aqueous solution sample cell. Samples were prepared in deionised water, by mixing aqueous solutions of the substrate, Bu^{t}O$_2$H, MNP and $TiCl_3$ (prepared in nitrogen-purged water), in this order, to give typical final concentrations of 0.2–3.8 × 10^{-3} mol dm^{-3} MNP, 8.3 × 10^{-3} mol dm^{-3} Bu^{t}O$_2$H and 0.5–2 × 10^{-3} mol dm^{-3} Ti^{III}; the MNP solution contained 3–17% v/v acetonitrile to aid dissolution of this material. pH control was achieved by use of 50 mmol dm^{-3} phosphate buffer for experiments carried out at pH 7.4, and at other pH values by addition of HCl. All chemicals were commercial samples of high purity and used without further purification.

Computer simulations of experimental spectra were carried out using a program written by Dr M. F. Chiu and adapted by Dr A. C. Whitwood (both Department of Chemistry, University of York) to run on an IBM-compatible PC.

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References

1 T. J. Slaga, A. J. P. Klein-Szanto, L. L. Triplett, L. P. Yolti and J. E. Trosko, *Science*, 1981, **213**, 1023.
2 M. Athar, J. R. Lloyd, D. R. Bickers and H. Mukhtar, *Carcinogenesis*, 1989, **10**, 1841.
3 R. M. Hicks, *Carcinogenesis*, 1983, **4**, 1209.
4 J. A. Hartley, N. W. Gibson, A. Kilkenny and S. H. Yuspa, *Carcinogenesis*, 1987, **8**, 1827.
5 B. E. Butterworth and T. L. Goldsworthy, *Proc. Soc. Exp. Biol. Med.*, 1991, **198**, 683.
6 J. P. Perchellet and E. M. Perchellet, *Free Rad. Biol. Med.*, 1989, **7**, 377.
7 T. W. Kensler and B. G. Taffe, *Adv. Free Rad. Biol. Med.*, 1986, **2**, 347.
8 G. S. Timmins and M. J. Davies, *Carcinogenesis*, 1993, **14**, 1615.
9 B. Chance, H. Sies and A. Boveris, *Physiol. Rev.*, 1979, **59**, 527.
10 M. J. Davies, *Biochem. J.*, 1989, **257**, 603.
11 T. L. Greenley and M. J. Davies, *Biochim. Biophys. Acta*, 1992, **1116**, 192.
12 C. H. Kennedy, J. M. Dyer, D. F. Church, G. W. Winston and W. A. Pryor, *Biochem. Biophys. Res. Commun.*, 1989, **160**, 1067.
13 T. L. Greenley and M. J. Davies, *Biochim. Biophys. Acta*, 1994, **1226**, 56.
14 M. J. Davies, *Free Rad. Res. Commun.*, 1990, **9**, 251.
15 M. Athar, H. J. Mukhtar, D. R. Bickers, I. U. Khan and B. Kalyanaraman, *Carcinogenesis*, 1989, **10**, 1499.
16 B. G. Taffe, N. Takahashi, T. W. Kensler and R. P. Mason, *J. Biol. Chem.*, 1987, **262**, 12143.
17 S. Bottomley, J. Milner and M. J. Davies, unpublished data.
18 G. S. Timmins and M. J. Davies, *Carcinogenesis*, 1993, **14**, 1499.
19 T. J. Slaga, V. Solanki and M. Logani, in *Radioprotectors and Anticarcinogens*, ed., O. F. Nygaard and M. G. Simic, Academic Press, New York, p. 471.
20 J. A. Hartley, N. W. Gibson, L. A. Zwelling and S. H. Yuspa, *Cancer Res.*, 1985, **45**, 4864.
21 H. C. Birboim, *Carcinogenesis*, 1986, **7**, 1511.
22 J. E. Swauger, P. M. Dolan, J. L. Zweier, P. Kuppusamy and T. W. Kensler, *Chem. Res. Toxicol.*, 1991, **4**, 223.
23 C. von Sonntag, *The Chemical Basis of Radiation Biology*, Taylor and Francis, London, 1987.
24 M. Erben-Russ, C. Michel, W. Bors and M. Saran, *J. Phys. Chem.*, 1987, **91**, 2362.
25 B. C. Gilbert, R. G. G. Holmes, H. A. H. Laue and R. O. C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 1976, 1047.
26 K. Y. Choo and S. W. Benson, *Int. J. Chem. Kinet.*, 1981, **13**, 833.
27 B. C. Gilbert, P. D. R. Marshall, R. O. C. Norman, N. Pineda and P. S. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1981, 1392.
28 P. Neta, M. Dizdaroglu and M. G. Simic, *Isr. J. Chem.*, 1984, **24**, 25.
29 W. Bors, C. Michel and K. Stettmaier, *J. Chem. Soc., Perkin Trans. 2*, 1992, 1513.
30 K. P. Madden and H. Taniguchi, *J. Am. Chem. Soc.*, 1991, **113**, 5541.
31 G. R. Buettner, *Free Rad. Biol. Med.*, 1987, **3**, 259.
32 H. Catterall, M. J. Davies, B. C. Gilbert and N. P. Polack, *J. Chem. Soc., Perkin Trans. 2*, 1993, 2039.
33 M. J. Davies, B. C. Gilbert, C. Hazlewood and N. P. Polack, *J. Chem. Soc., Perkin Trans. 2*, 1995, 13.
34 C. Hazlewood, M. J. Davies, B. C. Gilbert and J. E. Packer, unpublished experimental data.
35 H. Catterall, M. J. Davies and B. C. Gilbert, *J. Chem. Soc., Perkin Trans. 2*, 1992, 1379.
36 B. E. Sandström, *Free Rad. Res. Commun.*, 1991, **15**, 79.
37 B. E. Sandström and S. L. Marklund, *Biochem. J.*, 1990, **271**, 17.
38 T. Ochi and P. A. Cerutti, *Chem.-Biol. Interact.*, 1989, **72**, 335.

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