Electron paramagnetic resonance studies of the reaction of aryl radicals with nucleic acids and their components

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Aryl radicals may be responsible for the DNA damage observed in both cellular systems and isolated DNA exposed to a number of systems (such as benzoyl peroxide or arenediazonium ion/metal ion couples) which are believed to be capable of generating such species, though it is unclear how this damage arises. EPR spectroscopy in conjunction with spin trapping [using 2-methyl-2-nitrosopropane (MNP)] has therefore been utilised to study the mechanism and sites of attack of aryl radicals (generated by treatment of the corresponding diazonium ions with Fe^{2+}–EDTA or Ti^{3+}) on DNA, RNA and their components. The results obtained suggest that, for the pyrimidine nucleobases, nucleosides and nucleotides, the major mode of reaction is addition to the alkenic C^5–C^6 double bond of the base moiety, though significant yields of other radicals, believed to arise from abstraction of hydrogen at the sugar moiety, are also observed with some of the nucleosides and nucleotides. Radicals arising from attack on adenosine 5′-triphosphate have also been detected. The increased yield of sugar-derived radicals in these reactions, when compared with those previously reported for (electrophilic) HO• and alkoxyl radicals, is in accord with the known nucleophilic nature of aryl radicals.

Studies with the polyU, polyA•polyU, polyC, RNA and DNA suggest that aryl radicals also damage these macromolecules, though the broad nature of the spectral lines and interference from the signal of the aryl-radical adduct to the spin trap prevent detailed identification of the site(s) of attack. For DNA and RNA the signals obtained are pH dependent. At pH 7.4 both slowly tumbling and rapidly tumbling spin adducts are observed with tRNA, which is consistent with the spin trapping of both large, substrate-derived, radicals and low-molecular-mass fragments, possibly from the sugar moieties. With DNA only spectra from rapidly tumbling species are seen at pH 7.4; these are again believed to be due to the presence of low-molecular-mass material. The formation of these small fragments suggests that aryl radicals are capable of generating strand breaks in nucleic acids, and therefore that such species may be responsible for the genetic damage observed in cells exposed to aryl-radical-generating systems.

Introduction

Considerable attention has recently been focused on the mechanism(s) through which cellular damage is induced by a large number of organic peroxides (such as benzoyl peroxide) and related compounds, which are used extensively in the chemical, pharmaceutical, and cosmetic industries. A number of these materials have 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.[1–5] The mechanism of this promotion activity is poorly understood, though it is believed to involve clonal expansion and/or selection of an altered phenotype; this may either involve genetic damage or occur *via* changes in epigenetic processes.[1–8] Evidence has been provided to support the hypothesis that these effects are mediated through the generation of free radicals from these compounds.[1–9]

It is known that benzoyl peroxide can be metabolised by cells by one-electron reduction, to give free radicals which can subsequently damage DNA.[7,10–14] The generation of radicals from this material has been demonstrated, principally by electron paramagnetic resonance (EPR) spectroscopy and spin trapping, in a number of cells and sub-cellular fractions including rat liver microsomes,[14] epidermal homogenates,[11] and isolated murine keratinocytes;[11] the catalytic source of these radicals has not been unequivocally demonstrated though evidence has been presented for the involvement of the cytochrome *P*450 enzyme family.[14] The nature of the species produced has been studied in some depth and it has been suggested, on the basis of both EPR data[11,12,14] and product studies,[11,15] that the initially generated species is the benzoyloxyl radical [PhC(O)O•] and that this radical rapidly decarboxylates to yield phenyl (Ph•) radicals; it is unclear which of these species is the more important, biologically damaging, agent, though there is considerable evidence that the latter species may play a significant role in DNA-strand breakage.

Exposure of plasmid DNA to phenyl-radical-generating systems consisting of arenediazonium ions plus either Cu^I or NaI[16] results in the generation of DNA strand breaks, and treatment of mice with *p*-(hydroxymethyl)benzenediazonium salts, isolated from cultivated mushrooms (*Agaricus bisporus*), gives rise to high yields (up to 32% after a single dose) of stomach tumours (adenomas and adenocarcinomas).[17,18] DNA strand breaks have also been observed upon incubation of *o*- and *p*-diazoquinones with isolated DNA.[19,20] In the latter case, the formation of strand breaks was found to be inhibited by the radical scavengers butylated hydroxyanisole (BHA), EtOH, cysteine, 2-sulfanylethanol and 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) but not by catalase, superoxide dismutase (SOD) or 2,5-dimethylfuran, indicating that carbon-centred radicals (presumably aryl species) and not H_2O_2, $O_2^{\bullet-}$ or $^1O_2$ were involved. When the spin traps DMPO and *N*-(*tert*-butyl)-α-phenylnitrone (PBN) were included, signals assigned to the *o*- and *p*-hydroxyphenyl radicals adducts were also observed. The metabolism of carcinogenic diazo dyes by peroxidases has also been postulated to involve the formation of aryl radicals.[21]

Furthermore, the active form of the enediyne family of

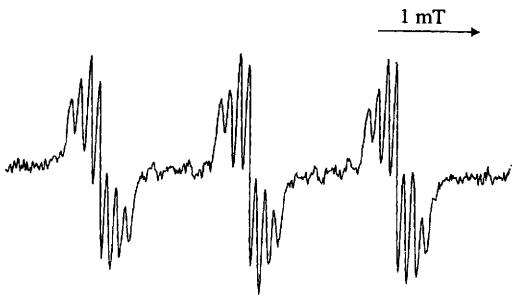


Fig. 1 EPR spectrum of the spin-trapped 4-methoxyphenyl radical at pH 7.4, obtained from the reaction of $ArN_2^+BF_4^-$ (2.3×10^{-3} mol dm^{-3}) and Fe^{II}–EDTA (1.1×10^{-3} mol dm^{-3}) in the presence of $\sim 1 \times 10^{-3}$ mol dm^{-3} MNP

antitumour antibiotics, which includes calicheamicin, neocarzinostatin and the esperamicins, is believed to be an aromatic diradical, formed by thiol-triggered aromatisation of the enediyne moiety. The diradical has been postulated to abstract hydrogen atoms from the 2-deoxyribose moieties of the DNA duplex, to which the drug molecule is bound in a site-specific manner, resulting in damage to DNA and cell death.[22,23]

However, though there is now a strong causative link between tumour promotion and the generation of aryl radicals (and possibly other intermediates) from these compounds, relatively little is known about the biological effects of these radicals and the key cellular targets of these species. In particular, the site of phenyl-radical attack on DNA (*i.e.*, attack on the base or the sugar) is not clearly established, and it is not obvious whether strand breaks arise *via* direct abstraction of hydrogen from the sugar moiety or whether this arises from a secondary process, such as transfer of damage from the base to the sugar backbone (*cf.* previous studies which have suggested that this is an important route in HO•-induced strand breakage[24,25]). Similarly it is not known whether strand-breakage is the major form of DNA damage, or whether, like HO•- and 'BuO•-induced damage,[24–28] attack at (and hence modification of) the base moiety is also important; previous studies on the reactivity of aryl radicals suggest that such species might be expected, on the basis of their nucleophilic character, to attack both the sugar and the base moieties.[29–31]

In order to elucidate further the mechanism of DNA damage induced by radicals derived from these tumour promoters and carcinogens, we have carried out a detailed study of the reactions of aryl radicals (and especially 4-methoxyphenyl) with isolated DNA, RNA and their components. These species were generated by use of a diazonium ion/Ti^{III} or diazonium ion/Fe^{II}–ethylenediaminetetraacetic acid (EDTA) couple,[30,31] and its reactions examined by use of EPR spin trapping using the spin trap 2-methyl-2-nitrosopropane (Me_3CNO; MNP), with the aim of characterising 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.

Results and discussion

(a) Generation and trapping of 4-methoxyphenyl radicals

Most of our experiments were carried out with the 4-methoxyphenyl radical generated by reduction of 4-methoxybenzenediazonium tetrafluoroborate with Fe^{II}–EDTA or Ti^{III}. In the presence of the spin trap MNP, a spectrum analysed in terms of a triplet of septets was observed (Fig. 1); this spectrum is believed to arise from the trapping of the 4-methoxyphenyl radical, with the hyperfine structure arising from coupling to the nitroxide nitrogen [a(N) 1.50 mT] and pairs of *ortho*- and *meta*-hydrogens [a(2H-*ortho*) 0.194 mT, a(2H-*meta*) 0.097 mT]. In some reactions we also used other aryl radicals (phenyl itself and 4-methylphenyl) which behaved in essentially the same manner as did the 4-methoxyphenyl radical.

(b) Reactions of the 4-methoxyphenyl radical with pyrimidine nucleobases

Inclusion of either uracil or thymine in the reaction of 4-methoxybenzenediazonium ion with Fe^{II}–EDTA and MNP at pH 7.4 resulted in the detection of additional, weak, signals from what are believed to be (one or more) base-derived spin adducts, in addition to residual features from the adduct of the aryl radical. In the case of uracil this spectrum consists of a triplet of triplets of doublets; these signals are, however, partially overlapped by the aryl-adduct signals so that splittings from this base-derived species cannot be measured accurately, though they are approximately a(N) 1.53, a(N) 0.31, a(H) 0.18 mT. The nature of this spectrum and these approximate parameters are similar to those observed following attack of HO• and 'BuO• on uracil at this same pH and which have been previously assigned[26,28] (on the basis of the β-N splitting) to the C^5 adducts trapped through the C^6 position (*i.e.*, C^5-OR C^6-yl spin adducts with R = H or Bu^t); the signals observed in this case are therefore assigned to the species formed by addition of the aryl radical at the C^5 end of the C^5–C^6 double bond of the base and subsequent spin-trapping *via* the C^6 position (*i.e.*, the radical adduct of **1**, see Table 1).

In the corresponding experiments with thymine two additional species were also observed and these have been assigned, by comparison with the parameters of the corresponding HO• and 'BuO• adducts to thymine,[26–28] to the trapped C^5-Ar C^6-yl **2** and C^6-Ar C^5-yl radicals **3**, the former with a characteristic second nitrogen splitting, the latter with no additional splittings apart from the nitroxide nitrogen [see Fig. 2(*a*) and Table 1]. The ratio of these two trapped radicals was estimated by spectral simulation as 0.3 : 1 (for Ar = 4-methoxyphenyl), though it should be noted that this ratio may not be an accurate measure of the initial aryl-radical adducts to the base, as the concentration of the spin-trapped species detected is also dependent on the rate of trapping of the initial adducts; these rate constants are not known. This ratio is, however, in marked contrast to those determined in the corresponding experiments with (the highly electrophilic) HO• and 'BuO• radicals where a much higher proportion of the trapped C^5-OR C^6-yl radicals are observed,[26–28] and this is in agreement with the nucleophilic nature of the aryl species.[29–31]

Repetition of the above experiments at low pH produced somewhat different behaviour in that, with uracil at pH < 2, a triplet of doublet signals was observed (for parameters see Table 1) which is assigned to the alternative adduct where the initial addition of the aryl radical has occurred at the C^6 position (*i.e.*, the spin-trapped C^6-Ar C^5-yl radical, **4**). With thymine the same species observed at pH 7.4 are believed to be formed at pH 2, but, at this pH, the hyperfine couplings are slightly different and the ratio of spin-trapped radicals is 0.85 : 1. The reason for these changes (in both regioselectivity of attack and parameters) at very low pH is not known, though it may be due to protonation at one of the sites on the ring; further speculation as to the reasons for this behaviour does not appear to be warranted.

In contrast to the uracil and thymine systems, no base-derived signals could be obtained from cytosine at pH 7.4. However, at low pH, where the ring amino group is protonated ($pK \sim 4.5$[32]) which would be expected to make the ring more susceptible to attack by the nucleophilic aryl radical, a triplet of doublets was observed (for parameters see Table 1); as with uracil at this pH, this signal is assigned to the spin-trapped C^6-Ar C^5-yl radical **5**.

Table 1 EPR parameters of radicals generated from reaction of 4-methoxyphenyl radicals with nucleobases, nucleosides, nucleotides, RNA and DNA in aqueous solution in the presence of the spin trap MNP

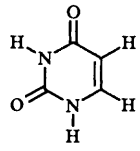
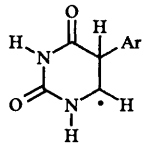
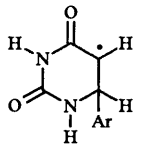
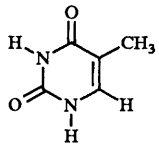
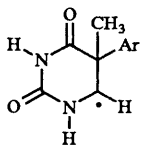
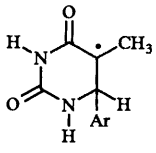
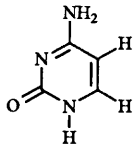
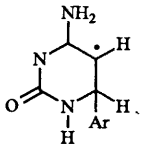
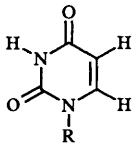
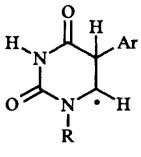
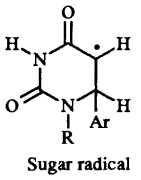
Substrate[a]	Radicals trapped	pH	aN[b]	a(other)[b]
	 **1**	7.4	1.53	0.31 (β-N) 0.18 (β-H)
	 **4**	2	1.50	0.25 (β-H)
	 **2**	7.4 2	1.51 1.50	0.35 (β-N) 0.35 (β-N)
	 **3**	7.4 2	1.56 1.54	
	 **5**	2.5	1.48	0.50 (β-H)
D-Ribose	Sugar radicals,[c]	7.4	1.51 1.51	0.10 (β-H) 0.29 (β-H)
2-Deoxyribose	Sugar radicals,[c]	7.4	1.51 1.53	 0.11 (β-H)
Ribose 5-phosphate	Sugar radicals,[c]	7.4	1.51 1.51 1.49	 0.22 (β-H) 0.36 (β-H)
		7.4	1.51	0.35 (β-H)
		7.4	1.50	0.61 (β-H)
	Sugar radical	7.4	1.48	0.09 (β-H)

Table 1 (*continued*)

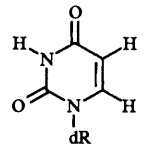
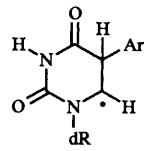
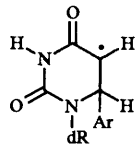
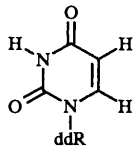
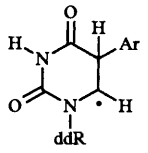
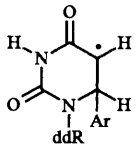
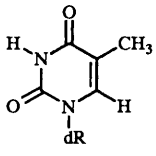
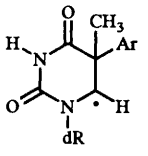
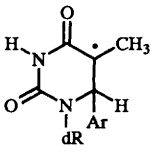
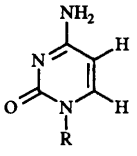
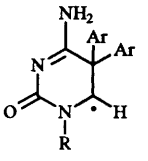
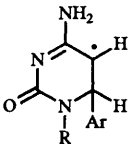
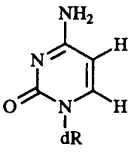
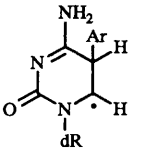
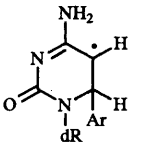
Substrate[a]	Radicals trapped	pH	aN^b	a(other)[b]
		7.4	1.51	0.34 (β-H)
		7.4	1.50	0.61 (β-H)
	Sugar radical	7.4	1.50	
		7.4	1.54	0.34 (β-H)
		7.4	1.50	0.64 (β-H)
	Sugar radical	7.4	1.54	0.15 (β-H)
		7.4	1.46	0.46 (β-N)
		7.4	1.60	
		7.4	1.52	0.63 (β-H)
		7.4	1.50	0.38 (β-H)
	Sugar radical	7.4	1.47	
		7.4	1.49	0.51 (β-H)
		7.4	1.47	0.35 (β-H)
	Sugar radical	7.4	1.49	

Table 1 (*continued*)

Substrate[a]	Radicals trapped	pH	aN^b	a(other)[b]
ATP	*c*	3	1.54	0.14 (β-H)
tRNA	*c*	7.4	1.60	0.25 (β-H)
			$a_{\|\|} \sim 2.7$	
		3	1.53[d]	
			$a_{\|\|} \sim 2.9$	
Calf-thymus DNA	*c*	7.4	1.60	0.25 (β-H)
			~1.6[d]	
Herring testes DNA	*c*	7.4	~1.6[d]	
		2	~1.58[d]	
Yeast RNA	*c*	7.4	~1.53[d]	
		3	~1.5[d]	
polyU	*c*	7.4, 4	~1.5[d]	
polyC	*c*	7.4	~1.5[d]	
			~1.53[d]	~0.4 (β-H)[d]
polyA·polyU	*c*	7.4, 4	~1.5[d]	

[a] Abbreviations used: R = ribose, dR = 2′-deoxyribose, ddR = 2′,3′-dideoxyribose. [b] Typically ±0.005 mT, except where otherwise stated. [c] For discussion of possible assignments, see text. [d] High-field line broadened by slow molecular motion; estimated parameters.

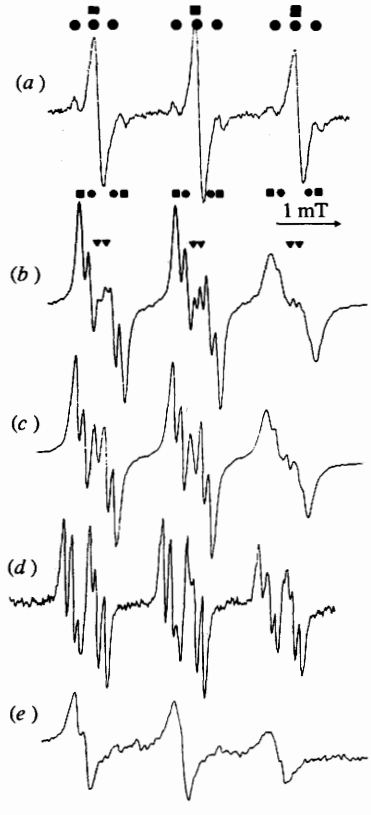


Fig. 2 (*a*) EPR spectra of the spin-trapped C^6-Ar-C^5-yl (■) and C^5-Ar-C^6-yl (●) radicals obtained from reaction of the 4-methoxyphenyl radical with a saturated solution of thymine at pH 7.4, with $[ArN_2^+BF_4^-]$ 4.7×10^{-3} mol dm^{-3}, $[Fe^{II}$–EDTA] 2.3×10^{-3} mol dm^{-3} and [MNP] $\sim 0.7 \times 10^{-3}$ mol dm^{-3}. (*b*) As (*a*) except with uridine; spectra assigned to spin trapped C^6-Ar-C^5-yl (■), C^5-Ar-C^6-yl (●) and sugar radicals (▼), with [uridine] 3 mol dm^{-3}, $[ArN_2^+BF_4^-]$ 1.5×10^{-3} mol dm^{-3}, $[Fe^{II}$–EDTA] 0.9×10^{-3} mol dm^{-3} and [MNP] 5×10^{-3} mol dm^{-3}. (*c*) As (*b*) except with 2′-deoxyuridine, with [2′-deoxyuridine] 3.4 mol dm^{-3}. (*d*) As (*b*) except with 2′,3′-dideoxyuridine, with [2′,3′-dideoxyuridine] 0.09 mol dm^{-3}, $[ArN_2^+BF_4^-]$ 2.4×10^{-3} mol dm^{-3}, $[Fe^{II}$–EDTA] 1.1×10^{-3} mol dm^{-3} and [MNP] $\sim 1 \times 10^{-3}$ mol dm^{-3}. (*e*) EPR spectrum observed on reaction of ATP with 4-methoxyphenyl radicals at pH 3 with [ATP] 0.5 mol dm^{-3}, $[ArN_2^+BF_4^-]$ 2.5×10^{-3} mol dm^{-3}, $[Fe^{II}$–EDTA] 2.0×10^{-3} mol dm^{-3}, and [MNP] $\sim 3 \times 10^{-3}$ mol dm^{-3}; for assignment of signals see text.

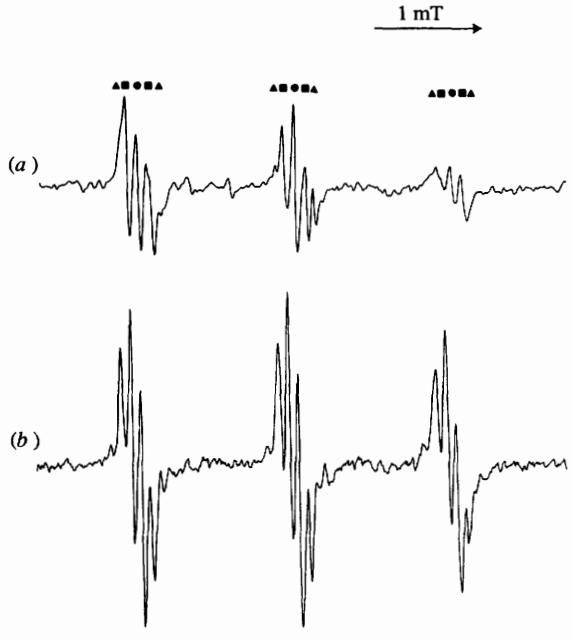


Fig. 3 (*a*) EPR spectra of spin-trapped sugar radicals obtained in the reaction of the 4-methoxyphenyl radical with ribose 5-phosphate, with [ribose 5-phosphate] 0.31 mol dm^{-3}, $[Fe^{II}$–EDTA] 1.1×10^{-3} mol dm^{-3} and [MNP] 1.1×10^{-3} mol dm^{-3}. Signals assigned to spin-trapped $C^{4'}$ or $C^{2'}$ radicals (●) and $C^{5'}$ and/or rearranged $C^{3'}$ and $C^{2'}$ (■, ▲). (*b*) As (*a*) but with ribose at pH 7.4, with [ribose] 1.2 mol dm^{-3}, $[ArN_2^+BF_4^-]$ 4.4×10^{-3} mol dm^{-3}, $[Ti^{III}]$ 1.1×10^{-3} mol dm^{-3} and [MNP] $\sim 1.4 \times 10^{-3}$ mol dm^{-3}.

(c) Reactions of the 4-methoxyphenyl radical with purine nucleobases

Inclusion of either adenine or guanine in the reaction system outlined above did not yield any signals which could be ascribed to spin-trapped base-derived species in the pH range 1–7.4, although the presence of these bases did cause a reduction in the concentration of trapped aryl radicals. This suggests that this radical does react with these materials, but does not give rise to detectable adducts; this may be due to either steric or electronic (delocalisation) factors.

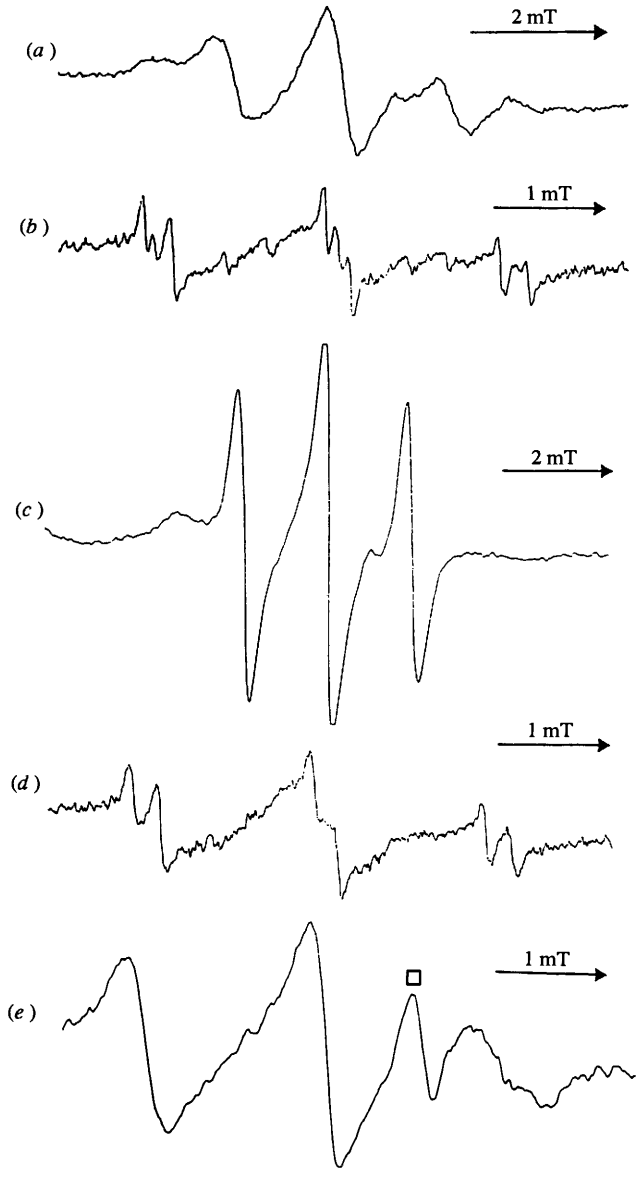
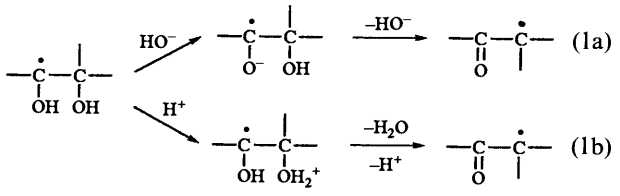


Fig. 4 (*a*) EPR spectra obtained on reaction of the 4-methoxyphenyl radical with tRNA at pH 7.4, with [tRNA] 90 mg cm^{-3}, [$ArN_2^+BF_4^-$] 2.3 × 10^{-3} mol dm^{-3}, [Fe^{II}–EDTA] 1.1 × 10^{-3} mol dm^{-3} and [MNP] ~1 × 10^{-3} mol dm^{-3}, under conditions of high modulation amplitude and high power. (*b*) As (*a*) except with low modulation amplitude and decreased field scan. (*c*) As (*a*) except at pH 3 and with lower modulation amplitude and power. (*d*) As (*b*) except with highly polymerised calf-thymus DNA at pH 7.4, with [DNA] 45 mg cm^{-3}. (*e*) As (*b*) but with partially degraded herring testes DNA, with [DNA] 31 mg cm^{-3}. For discussion of possible assignments of all signals see text. The signal marked (□) in some of these spectra is due to a paramagnetic impurity in the glass of the EPR cell.

(d) Reaction of the 4-methoxyphenyl radical with sugars

Reaction of the sugars D-ribose, 2-deoxyribose and the corresponding 5-phosphates with the 4-methoxyphenyl radical in the presence of MNP led to a decrease in the concentration of trapped aryl radicals and, except in the case of 2-deoxyribose 5-phosphate, the replacement of these signals by new features which are believed to be due to two, or more, trapped sugar radicals produced by abstraction of hydrogen by the aryl radical (see Fig. 3). The parameters of these sugar-derived radicals are identical with those obtained in a previous study on HO• attack on the same sugars,[27] though the relative concentrations of the trapped species are somewhat different; this is as expected in view of the different characters of these attacking species. For ribose 5-phosphate the signal comprises a triplet of singlets and two triplets of doublets. The former is believed to be from the trapping of radicals formed at $C^{4'}$ and/or $C^{2'}$, and the two doublets are attributed to the trapping of radicals at $C^{5'}$ and/or carbonyl-conjugated species derived from 3-OH and 2-OH by loss of H_2O [*via* reaction (1)].[27]



(e) Reaction of the 4-methoxyphenyl and other aryl radicals with pyrimidine nucleosides, 2'-deoxynucleosides and 2',3'-dideoxynucleosides

Reaction of a number of pyrimidine nucleosides, 2'-deoxynucleosides and 2',3'-dideoxynucleosides with the 4-methoxyphenyl and other aryl radicals resulted in the detection of spin adducts which can be assigned to trapped base- and sugar-derived radicals [see Fig. 2(*b*)–(*d*) and Table 1]. In each case the observed species have been assigned by comparison with data previously reported for the corresponding HO• and 'BuO• systems,[26–28] and these assignments are consistent with the aryl radical undergoing predominantly addition at the C^5–C^6 double bond on the base to give species which have spectra consisting of triplets of doublets, with the latter splitting relatively large. The narrow triplets of doublets observed in some of these cases are believed to arise from the trapping of sugar-derived species. The latter radicals are believed to arise *via* direct abstraction of hydrogen from the sugar ring, rather than through transfer of damage from the base to the sugar moiety. In contrast to the behaviour of the species seen in the corresponding HO• system, the yield of these radicals does not vary dramatically with the pH of the reaction mixture where the formation of sugar-derived species results from transfer of damage to the sugar *via* an acid-catalysed process.[25] Comparison of these data with those obtained in the corresponding studies with HO• and 'BuO• suggests that the yield of sugar radicals (compared with base-derived species) is much greater with the aryl system compared with these other radicals.[26–28] Thus the ratios of trapped base radical: trapped sugar radical following attack by HO• and 'BuO• and 4-methoxyphenyl with uridine at pH 7.4 have been estimated from spectral simulations as ~13:1, 5:1 and 4:1 respectively.

Similar sugar-derived species may also be generated with some (or all) of the other substrates tested, but the low concentrations and/or nature of the signals from these species might result in them not being readily detected; this is likely to be of particular importance in some cases, such as thymidine, where there are strong absorptions from base-derived species at the magnetic-field values where the sugar-derived adduct signals would be expected. In all cases, the major species trapped is believed to be the C^5-yl radical, formed by attack of Ar• at the C-6 position.

(f) Reaction of the 4-methoxyphenyl radical with purine nucleosides and nucleotides

Inclusion of adenine, adenosine, 2'-deoxyadenosine, adenosine monophosphate (AMP), guanine or guanosine in the above aryl-radical-generating system did not give rise to any substrate-derived radicals, although the signals from the aryl-radical adduct to the spin trap were either completely or partially lost,

indicating that reaction between the aryl radicals and these substrates does occur. In contrast, adenosine triphosphate (ATP) gave rise to a triplet of doublets signal with a(N) 1.54 and a(H) 0.14 mT [Fig. 2(*e*)]. This signal cannot be unambiguously assigned, as several base- or sugar-derived radicals could, in principle, give rise to this type of spectrum.

(g) Reaction of the 4-methoxyphenyl radical with RNA, DNA and polynucleotides

We have extended our investigations to explore the reaction of aryl radicals with RNA, DNA and some model compounds (polyU, polyC and double-stranded polyA·polyU) to gain further evidence for the nature and sites of radical attack and, if possible, for the radical-induced cleavage of the nucleic acid chains which has previously been postulated.[17,18]

In a series of experiments at pH 7.4 transfer RNA (tRNA) reacted to give a relatively intense signal which, especially when recorded at conditions of high modulation amplitude and power, showed the characteristic spectrum of a slowly tumbling species (with $2a_{\|}$ 5.4 mT) typical of the trapping of a relatively intact macromolecule radical following initial damage [see Fig. 4(*a*)]. This spectrum is similar in shape and overall width to that obtained on reaction of tRNA molecules with HO$^{\bullet}$;[26] this slow motion is perhaps not surprising in view of the partially double-stranded nature of tRNA molecules. At pH 7.4 and under conditions of low modulation amplitude, relatively weak spectra, consisting of triplets of doublets, from rapidly tumbling species could also be detected [Fig. 4(*b*) and Table 1] which indicates that some radical-induced fragmentation has occurred. Whilst these spectra do not match exactly those detected earlier with model compounds [in particular the larger a(N)-value from the tRNA adducts] it is thought that the species giving rise to these signals is either a base-derived species with dramatically altered parameters (possibly as a result of steric factors), or a sugar-derived species; the latter assignment is supported by the recent detection of similar spin adducts [with high a(N)-values] arising from the trapping of $C^{5'}$ sugar radicals derived from nucleosides with MNP during the photolytic degradation of coenzyme B_{12} analogues.[33]

At lower pH (~3) we detected a relatively strong spectrum, essentially isotropic but with high-field broadening, characteristic of a nitroxide with some restriction of motion [Fig. 4(*c*)]; weak features from slowly tumbling species similar to those seen at pH 7.4 were also detected. The mobility of the former species is believed to reflect the occurrence of radical-induced cleavage which might be expected to be H^+ dependent [*cf.* reaction (1b)]; it could also, however, reflect structural changes in the parent tRNA molecule at this acidic pH, though the similarity of the $2a_{\|}$-values for the slowly tumbling species seen in both cases suggests that this may not be the reason.

With highly polymerised DNA (obtained from calf thymus) only weak signals could be obtained at pH 7.4, with no evidence for slowly tumbling species; *cf.* those obtained with tRNA. The signals observed, which were isotropic in nature [see Table 1 and Fig. 4(*d*)] and resemble those seen with tRNA at this pH, are again believed to be indicative of the presence of low-molecular-mass materials produced by fragmentation of the parent molecule. These signals were not observed at lower pH-values, suggesting that the species observed at pH 7.4 may arise from a base-catalysed fragmentation [*cf.* reaction (1a)].

In contrast strong spectra from rapidly tumbling species were obtained from partially degraded DNA (herring testes) and yeast RNA which showed high-field broadening [see, *e.g.*, Fig. 4(*e*)]. This may reflect fragmentation of these molecules, or attack on less ordered substrates, with relatively great freedom of motion. Similar, but relatively weak, spectra were obtained from the polyU, polyC and polyA·polyU samples, these being partially obscured by residual signals from the aryl radical adduct to the trap.

Conclusions

The spin adducts detected in this study by EPR spectroscopy are consistent with the reaction of aryl radicals with pyrimidine nucleobases by addition at the C^5–C^6 double bond. The selectivity of attack at this double bond is, however, somewhat different to that observed with (electrophilic) HO$^{\bullet}$ and ^{t}BuO$^{\bullet}$ radicals,[26–28] in that with this (nucleophilic) carbon-centred radical there is a greater yield of radical adducts arising from addition at C^6, presumably reflecting a higher percentage of attack at this site; this is as expected as previous studies have demonstrated that the C^5 position is the more favoured site of attack for electrophilic radicals.[24] With the nucleosides and nucleotides addition at C^6 appears to predominate in all cases and, although there are also significant yields of sugar-derived radicals present in most cases, addition still appears to be preferred to abstraction of hydrogen, though to a much lesser extent than with HO$^{\bullet}$ and ^{t}BuO$^{\bullet}$;[26–28] this is consistent with a greater preference for attack by the nucleophilic aryl radical at relatively electron-poor sites. These sugar radicals are believed to be generated *via direct* abstraction of hydrogen rather than *via* transfer of damage from the base to the sugar, as the yield of these radicals appears to be largely unaffected by changes in the pH of the reaction system (thus appearing to rule out acid- or base-catalysed transfer processes; *cf.* the evidence for acid-catalysed transfer reactions in the case of HO$^{\bullet}$-induced damage to these materials[25]) or by the absence of oxygen (thus ruling out abstraction of hydrogen by base-derived peroxyl radicals).

The results obtained with model sugar compounds confirmed that aryl radicals could react at multiple sites to give species analogous to those observed with the hydroxyl radical though with somewhat different selectivity; the complexity of these spectra and the similarity in structure of many of the possible sugar radicals formed has meant that these signals cannot be unambiguously assigned.

Little information has been obtained about the selectivity of aryl-radical attack on purine bases or derivatives, though the loss of the aryl-radical adduct signal on inclusion of these substrates confirmed previous product studies which have demonstrated that 8-aryl adducts to adenine and guanine derivatives are formed in the reaction of these materials with some diazonium ions;[34,35] the radical-adduct species observed with ATP in the present study may be due to the trapping of this type of species.

The signals observed with RNA, DNA and polynucleosides confirmed that aryl radicals are capable of inducing genetic damage, though the exact identity of many of the species formed in these cases is unclear at this stage; extrapolation from the behaviour seen with the isolated nucleosides and nucleotides would suggest that there are mixtures of species present with attack occurring at both the base and the sugar moieties. Evidence has also been obtained for the induction of strand breaks by aryl species, in that pH-dependent changes were detected in the spectra with both tRNA and highly polymerised DNA with, at pH 7.4, features present which are believed to be due to the formation of low-molecular-mass fragments arising from rearrangement reactions of the initial DNA- and RNA-derived radicals. The parameters of these signals do not match those observed with sugars, bases, nucleosides or nucleotides and it is therefore suggested that these may be rearranged or fragmented materials (possibly from the sugar moieties) arising from the degradation of the macromolecule. It is therefore concluded that, as with HO$^{\bullet}$ and ^{t}BuO$^{\bullet}$,[26–28] aryl radicals can generate both altered nucleobases and induce strand breaks in DNA and RNA, and this may account for the known carcinogenic or anti-tumour action of a number of systems

which generate aryl radicals. It remains to be determined whether in cases such as benzoyl peroxide, where both aryl and aryloxyl radicals are believed to be formed,[11,12,14,15] these are the sole or ultimate carcinogens.

Experimental

Experiments were carried out on a JEOL RE1X EPR spectrometer, using aqueous solution sample cells. Samples were prepared in de-ionised water, by mixing aqueous solutions of the substrate, arenediazonium tetrafluoroborate, MNP and $TiCl_3$ or $FeSO_4$–EDTA (prepared in nitrogen-purged water), in this order, to give typical final concentrations of 2.2×10^{-3} mol dm^{-3} MNP, 4.7×10^{-3} mol dm^{-3} arenediazonium tetrafluoroborate and 1.1×10^{-3} mol dm^{-3} Ti^{III} or Fe^{II}–EDTA; the solution contained 6.3% v/v acetonitrile to aid dissolution of MNP. 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 either HCl or H_2SO_4. All chemicals were commercial samples of high purity and were used without further purification except for the arenediazonium tetrafluoroborates which were prepared as described previously.[29]

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 Dept. of Chemistry, University of York) to run on an IBM-compatible PC.

Acknowledgements

We are grateful to the Association for International Cancer Research, the Yorkshire Cancer Research Campaign, and the SERC for financial support, and Dr Adrian Whitwood and Dr Graham Timmins for helpful discussions.

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Paper 5/02250I
Received 7*th April* 1995
Accepted 10*th May* 1995