Spin-trapping studies of the reaction of the sulfate radical anion with N^1-substituted pyrimidine bases. Comparison with continuous-flow electron paramagnetic resonance experiments

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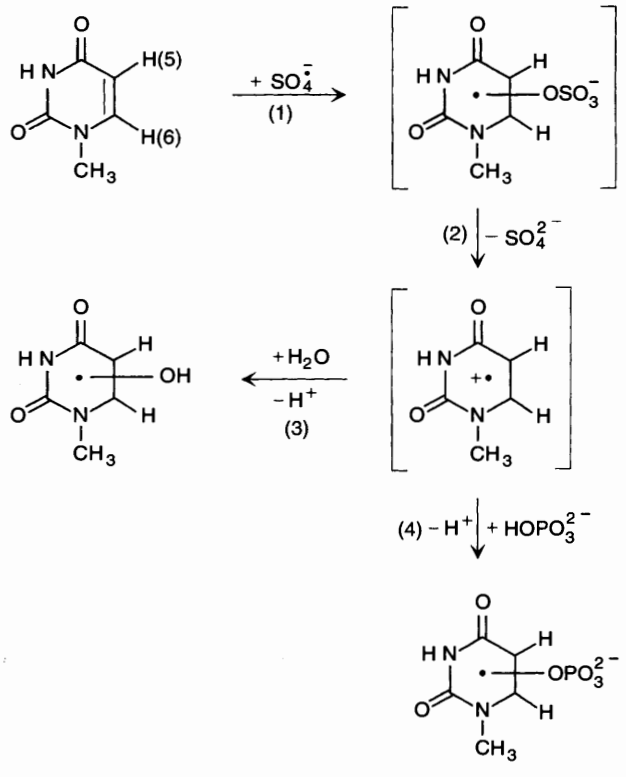
Photolysis of $K_2S_2O_8$-containing solutions of N^1-substituted pyrimidines (1-methyluracil, 1-MU; 1,3-dimethyluracil, 1,3-DMU; 1-methylthymine, 1-MT; 1,3-dimethylthymine, 1,3-DMT; 1-methylcytosine, 1-MC; 2′-deoxycytosine, dC), together with the spin trap 2-methyl-2-nitrosopropane (MNP) resulted in the EPR spectra of persistent nitroxyl radicals. They were due to spin adducts of 5-hydroxy-5,6-dihydropyrimidin-6-yl radicals for 1-MU and 1,3-DMU and to 4-hydroxy-5,6-dihydropyrimidin-5-yl radicals for 1-MT and 1,3-DMT. Spectra obtained from 1-MC and dC were tentatively assigned to ring-opened products. Spin-trapped phosphate adduct radicals generated in the presence of HPO_4^{2-} were characterized by β-nitrogen and β-proton hyperfine splittings and/or by signal intensities. These results are consistent with one-electron oxidation of the substrates by $SO_4^{\bullet -}$ and rapid reaction of the ensuing base radical cations with water or phosphate. Secondary reactions such as oxidation of 5-hydroxy-5,6-dihydrouracil-6-yl radicals by $S_2O_8^{2-}$ or rearrangement of 5-phosphato-5,6-dihydrouracil-6-yl radicals complicating the interpretation of previously reported continuous flow EPR spectra (see ref. 22), were not observed in the spin-trapping experiments.

Introduction

Radiation-induced DNA damage in living cells may be caused either by the 'direct effect' (the energy is absorbed by DNA) or by the 'indirect efect' (the energy is absorbed by water molecules surrounding the DNA).[1] One major event in 'direct' absorption of radiation by DNA is the formation of radical cations. In aqueous solution on a model level radical cations of nucleobases can be conveniently generated from their parent molecules either by photoionization [2-5] or by chemical methods using strongly oxidizing species such as Tl^{2+},[6,7] $Br_2^{\bullet -}$ [7-10] and $SO_4^{\bullet -}$.[11-23] In particular, the reaction of $SO_4^{\bullet -}$ with N^1-substituted pyrimidines has been studied in detail by γ-radiolysis,[18,19] pulse radiolysis [19-21] and by continuous-flow EPR spectroscopy.[22,23] Pulse radiolysis experiments showed that $SO_4^{\bullet -}$ reacts with the nucleobases with rate constants that are nearly diffusion-controlled.[20,21] In the first step, very short-lived sulfate adduct radicals are most likely formed. In favourable cases such as 1,3,6-trimethylthymine, 1,3-dimethylthymine and 1-methylthymine, after elimination of sulfate, base radical cations with lifetimes of 20, 2.0 and 2.4 μs were detected by pulse radiolysis.[20] In N^1-substituted uracils the radical cations, if formed at all, have lifetimes [19] < 1 μs.

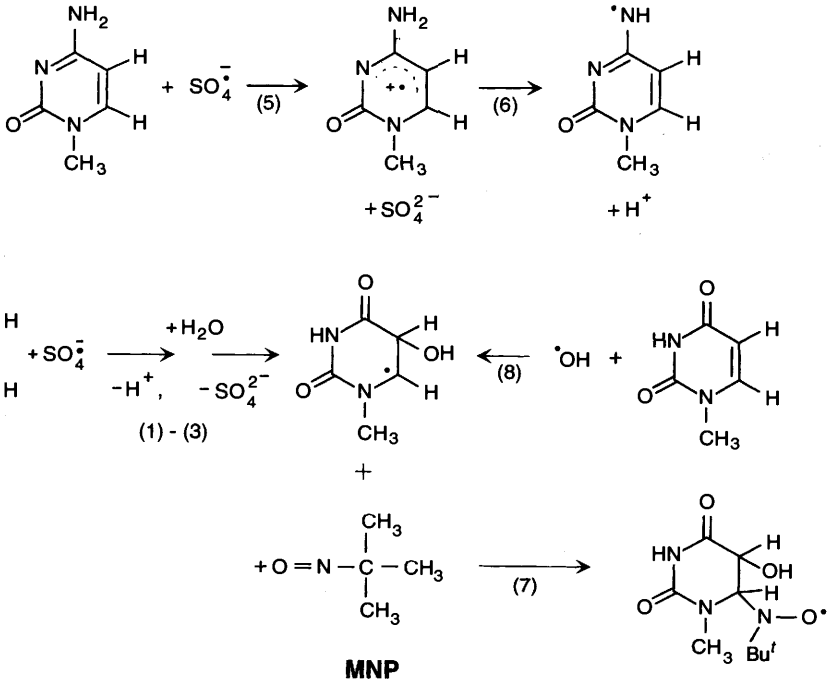
In the EPR experiments $K_2S_2O_8$-containing solutions of the bases were UV-irradiated in the cavity and the resulting radicals were observed immediately after their generation. Under those conditions steady-state concentrations of radicals with lifetimes in the submillisecond range are below the detection limit. Therefore, the EPR signals were usually due to OH-adduct radicals or, in the presence of phosphate anions, to phosphate-adduct radicals [see, for example, reactions (1)–(4) for 1-methyluracil].

One of the problems studied by continuous-flow EPR spectroscopy was the regioselectivity of reaction of nucleophilic agents, *i.e.* water and phosphate, with uracil and thymine radical cations.[22] However, severe difficulties were encountered in the attempt to decide whether the nucleophiles enter the radical cations at C-5 or C-6.



First of all, initial radical concentrations were influenced by reactions on the millisecond time-scale such as oxidation of 5-OH-6-yl† radicals by peroxodisulfate and fast intramolecular rearrangements of 5-phosphato-6-yl† radicals. Secondly, owing to the two nitrogen nuclei and the methyl groups the spectral

† The following abbreviations were used: 5-hydroxy-5,6-dihydropyrimidin-6-yl = 5-OH-6-yl; 6-hydroxy-5,6-dihydropyrimidin-5-yl = 6-OH-5-yl; 5-phosphato-5,6-dihydropyrimidin-6-yl = 5-phosphato-6-yl; 6-phosphato-5,6-dihydropyrimidin-5-yl = 6-phosphato-5-yl.



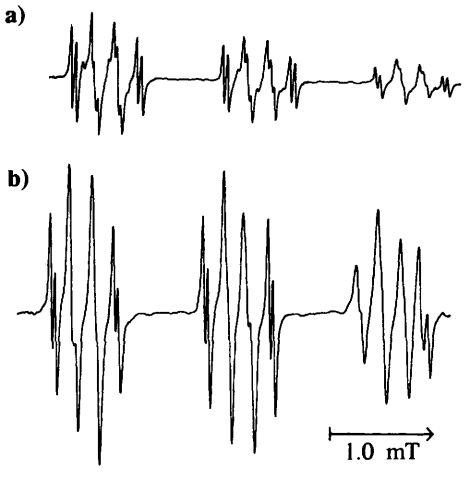


Fig. 1 Spectra obtained by UV-irradiation of solutions of 1-MU, $K_2S_2O_8$, and MNP in D_2O at pD 7.5. (*a*) Spin adduct **1** in the absence of phosphate; (*b*) **3**, in the presence of 4×10^{-2} mol dm^{-3} phosphate.

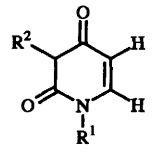
intensity of N^1-methylated pyrimidine radicals was distributed over numerous peaks and the signal:noise ratios of the spectra was rather poor. Moreover, for N^1-substituted uracil-5-yl and uracil-6-yl radicals *g*-factors and hyperfine splittings were similar and unambiguous assignment was difficult.

In pulse radiolysis experiments[17] reaction of $SO_4^{\bullet -}$ with 2'-deoxycytidine [*cf.* reaction (5)] resulted in a radical with oxidizing properties and UV spectra different from those of the species generated with OH radicals. It was proposed that the radical cation deprotonates to yield the N^4-centred neutral anilinyl-type radical [*cf.* reaction (6)].

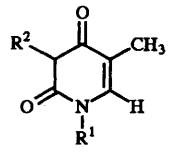
In the EPR experiments[23a] the deprotonated cytosine radical cation was not detected. The spectrum obtained instead could, in principle, be due to a tautomer of the anilinyl-type radical or to a secondary radical with unknown structure.

In order to attack the problems emerging from those EPR studies from a different point of view we applied the spin-trapping technique. In their pioneering work Rustgi and Riesz[12] reported on spin adducts obtained by photolysing solutions of pyrimidine bases or some nucleosides or nucleotides in presence of peroxodisulfate and MNP. The results for uracil, thymine and cytosine were interpreted in terms of deprotonation of base radical cations at N^1 and trapping of the allyl-type radicals at C-5. The results on the nucleosides and nucleotides were ambiguous and trapping of sugar radicals was favoured as an explanation of the spectra.

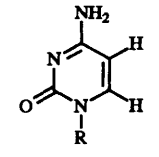
In the present investigation, solutions of N^1-substituted pyrimidine bases (1-MU, 1,3-DMU, 1-MT, 1,3-DMT, 1-MC, dC), $K_2S_2O_8$ and the spin trap 2-methyl-2-nitrosopropane (MNP) were irradiated with UV light in the absence and presence of phosphate. The EPR spectra of the persistent spin-adduct radicals [formed, *e.g.*, in reaction (7)] were relatively simple and allowed unambiguous distinction between trapping of 5-yl and 6-yl radicals. Assignment was supported by comparison with spin adducts generated with OH radicals[24] instead of $SO_4^{\bullet -}$ [see reaction (8) for 1-MU].



$R^1 = CH_3$, $R^2 = H$: 1-methyluracil, 1-MU
$R^1 = R^2 = CH_3$: 1, 3-dimethyluracil, 1,3-DMU



$R^1 = CH_3$, $R^2 = H$: 1-methylthymine, 1-MT
$R^1 = R^2 = CH_3$: 1, 3-dimethylthymine, 1,3-DMT



R = CH_3: 1-methylcytosine, 1-MC
R = 2'-deoxyribose, 2'-deoxycytidine, dC

Results

(a) 1-Methyluracil (1-MU), 1,3-dimethyluracil (1,3-DMU)

The spectrum obtained by photolysis of a solution of 1-MU, $K_2S_2O_8$ and MNP in D_2O [Fig. 1(*a*)] consisted of a triplet (from the nitroxide nitrogen) of 1:2:2:1 quartets of multiplets [Fig. 2(*a*), (*b*)]. The same spectrum was observed in experiments with

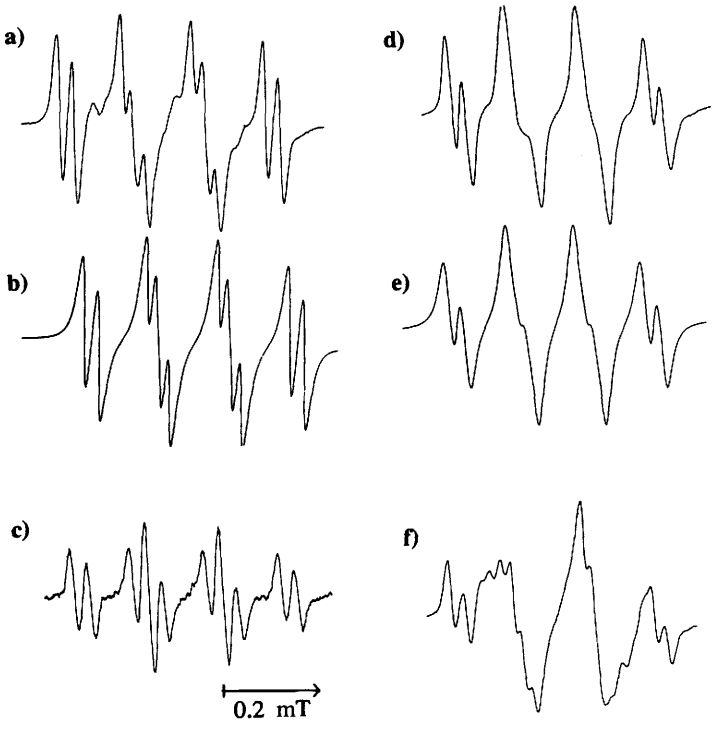
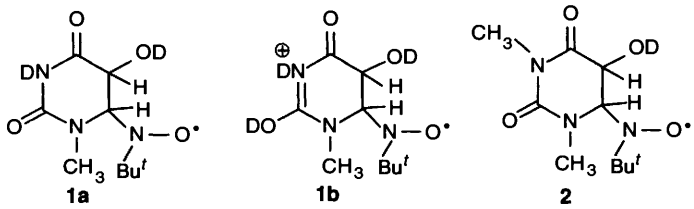


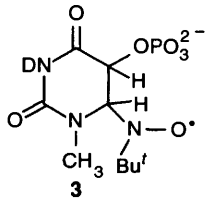
Fig. 2 Expansions of low-field multiplets of spin adducts obtained by UV-irradiation of 1-MU, $K_2S_2O_8$ and MNP in D_2O (*a*) at pD 7.5, in the absence of phosphate; (*b*) simulation with parameters listed in Table 1 for radical **1**, linewidth = 0.025 mT; (*c*) at pD 0.5 in the absence of phosphate; (*d*) pD 7.5 in the presence of 4×10^{-2} mol dm^{-3} phosphate; (*e*) simulation with parameters for radical **3** in Table 1, linewidth = 0.032 G; (*f*) pD 4.9, 4×10^{-2} mol dm^{-3} phosphate.

OH radicals and is characteristic for the spin adduct **1a** of the 5-OH-6-yl radical with values of 0.226 and 0.196 mT for β-N and β-H hyperfine couplings (hyperfine splittings are listed in Table 1). For the spin adduct of the isomeric 6-OH-5-yl radical (not detected) significantly lower values for the secondary nitrogen splitting ($a_{H_\gamma} < 0.06$ mT) and, therefore, a different spectral pattern would be expected.[25] At pD < 1 the values of $a_{N\beta}$ and $a_{H\beta}$ are slightly different from those at pD > 1 due to deuteriation [radical cation **1b**, Fig. 2(*c*)]. In the corresponding radical **2** derived from 1,3-DMU, β-N and β-H splittings were identical ($a_{N\beta} = a_{N\alpha} = 0.218$ mT). Therefore, the three groups of signals consisted of 1:2:2:1 quartets of doublets. Deuteriation of radical **2** was not observed in the pD range ≥0.5.



When the experiment with 1-MU was carried out in the presence of phosphate dianions (pD > 7) the multiplet pattern of the spin adduct was different from that in the absence of phosphate [compare Fig. 2(*d*) with Fig. 2(*a*)]. Simulation [Fig. 2(*e*)] showed that this was due to a decrease of $a_{N\beta}$ from 0.226 to 0.217 mT. The observed change in hyperfine splitting was specific for photolysis in the presence of phosphate. Increase in ionic strength by adding K_2SO_4 instead of phosphate or changes in pD values (1 < pD < 10) in solutions devoid of phosphate had no effect on $a_{N\beta}$ of spin adduct **1**. Therefore, the spectrum in Fig. 2(*d*) was ascribed to spin adduct **3** of the 5-phosphato-6-yl radical with the characteristic 1:2:2:1 multiplets of the three individual groups of signals.

By irradiation of slightly acidic solutions (pD 3–6) of 1-MU, $K_2S_2O_8$, MNP and $H_2PO_4^-$ rather complex spectra were observed [Fig. 2(*f*)] which could not be analysed. At pD < 3 the spin adduct **1** of the C-5-OH-6-yl radical was detected, *i.e.* in strongly acidic solution the presence of phosphate had no influence on the spectra. The spectrum obtained from 1,3-DMU

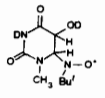
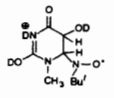
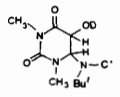
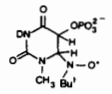
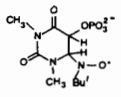
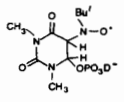
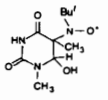
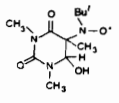


in the presence of phosphate dianions (pD > 7) showed also slightly lower values for $a_{N\beta}$ and $a_{H\beta}$ than did the corresponding spin adduct **2** in the absence of phosphate ($a_{N\beta} = a_{H\beta} = 0.213$ mT compared with 0.218 mT for **2**). The spectral pattern (1:2:2:1 quartets of doublets) did not change, only the expansion of the multiplets decreased from 0.654 to 0.639 mT. As in the case of 1-MU the spectrum was assigned to the 5-phosphato-6-yl radical (spin adduct **4**). A spectral pattern similar to that in Fig. 2(*f*) was observed when photolysis was carried out at pD 3–6 [Fig. 3(*b*)]. In this case simulation showed that signals of a radical with $a_{N\alpha} = 1.49$ mT, $a_{H\beta} = 0.381$ mT and $a_{N\gamma} = 0.033$ mT were superimposed on the signals of the trapped 5-OH-6-yl radical **2** [Fig. 3(*c*)]. These additional signals are assigned to the spin adduct of the 6-phosphato-5-yl radical **5**.

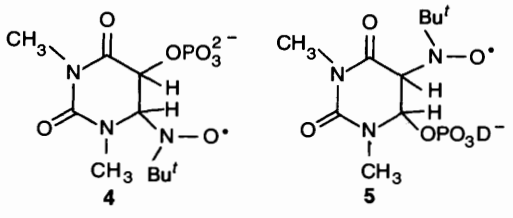
(b) 1-Methylthymine (1-MT), 1,3-dimethylthymine (1,3-DMT)

After short time intervals (*ca.* 30 s) of UV-irradiation 1:1:1

Table 1 Hyperfine splittings[a] (in mT) of spin-adducts obtained from uracil and thymine derivatives

Substrate	$a_{N\alpha}$	$a_{N\beta}$	$a_{H\beta}$	$a_{H\gamma}$	pH/pD	Radical
1-MU	1.482	0.226	0.196	0.050	1–9	 **1a**[b,c]
	1.482	0.232	0.191	0.050	< 1	 **1b**[b]
1,3-DMU	1.48	0.218	0.218	0.052	0.5–9	 **2**[b,c]
1-MU	1.482	0.217	0.194	0.047	> 7	 **3**
1,3-DMU	1.508	0.213	0.213	0.052	> 7	 **4**
	1.49		0.381	0.033 (N_γ)	3–6	 **5**
1-MT[d]	1.60				1–9	 **6**[b,c]
1,3-DMT[d]	1.60				1–9	 **7**[b]

[a] Error limits for **1**–**5**: ± 0.002 mT, for **6** and **7**: ± 0.005 mT. [b] Identical spectra were obtained upon photolysis of solutions containing the substrates, H_2O_2 and MNP. [c] Similar splittings have been reported by Rustgi and Riesz[24] for radicals generated by photolysis of the substrates in the presence of H_2O_2 and MNP. [d] The same spectra with much higher intensity obtained in the presence of HPO_4^{2-} were assigned to radicals **8** and **9**.



triplets of broad lines ($\Delta\nu_{\frac{1}{2}}$ ~ 0.1 mT) with $a_{N\alpha}$ of 1.60 mT appeared in the experiments with 1-MT and 1,3-DMT [Fig. 4(*a*)]. These triplets were identical with those obtained by reaction of •OH radicals (from H_2O_2) with 1-MT and 1,3-DMT and in agreement with ref. 24 they were assigned to spin adducts **6** and **7** of the 6-OH-5-yl radicals. Under the conditions applied these species were not stable. After the UV source had been switched off the signals decreased in intensity on a time-scale of several

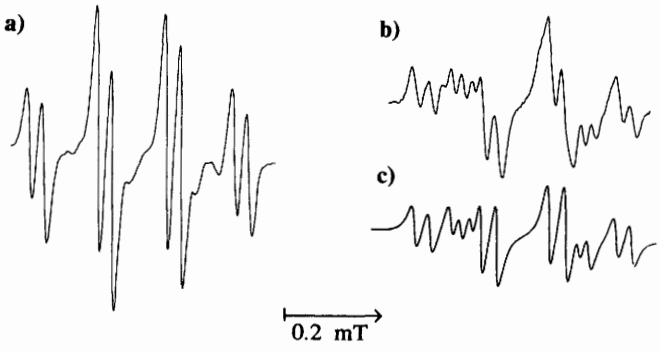


Fig. 3 Expansion of low-field multiplets of spin adducts obtained by UV-irradiation of a solution of 1,3-DMU, $K_2S_2O_8$ and MNP in D_2O, in the presence of 4 × 10^{-2} mol dm^{-3} phosphate, (*a*) at pD 7.5, radical **4**; (*b*) at pD 5.0; (*c*) simulation of a superposition of the spectra of radical **2** (trapped 5-OH-6-yl radical) and **5** (trapped 6-phosphato-5-yl radical) with the parameters listed in Table 1, linewidth = 0.025 mT

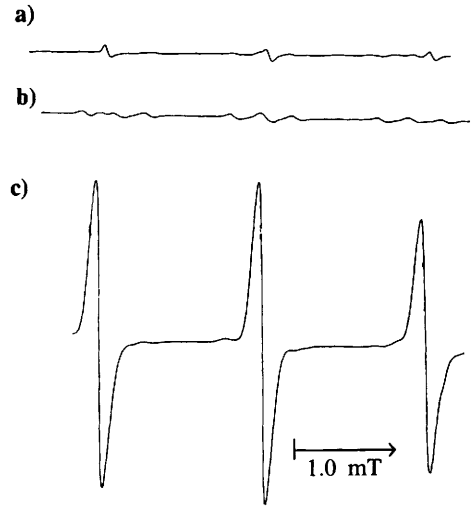
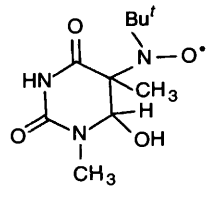


Fig. 4 (a) Spin adducts obtained by UV-irradiation of a solution of 1,3-DMT, $K_2S_2O_8$ and MNP in water, pH 9. (*a*) Spectrum of **7** after irradiation (30 s); (*b*) spectrum after dark reaction (4 min); (*c*) spectrum of **9** obtained in the presence of 4×10^{-2} mol dm^{-3} phosphate, irradiation time 4 min

minutes, and triplets of triplets appeared [Fig. 4(*b*)], probably due to formation of spin adducts of the isomeric 5-OH-6-yl radicals. The reason for this behaviour is not clear. Spin adducts of 5-hydroxythymin-6-yl and 6-hydroxythymin-5-yl radicals generated with $^{\bullet}OH$ radicals by photolysis of solutions containing H_2O_2 were stable for hours.



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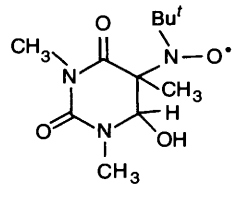

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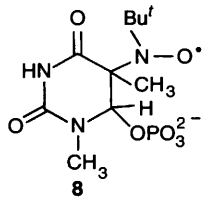
(c) Signal intensities of the trapped phosphate adduct radicals derived from 1-MU, 1,3-DMU, 1-MT and 1,3-DMT

The spectra obtained from 1-MT and 1,3-DMT in the presence of phosphate were triplets with $a_{N\alpha} = 1.60$ mT, *i.e.* with the same values of hyperfine couplings as in the absence of phosphate. Owing to the fact that β-protons are missing and γ-couplings are not resolved, no information on covalent binding of phosphate in the spin adducts can be expected from the hyperfine splittings. However, the drastic increase in signal intensities in the presence *vs.* absence of phosphate of ~ 30:1 for 1,3-DMT [Figs. 4(*b*), 5(*a*)] and ~ 5:1 for 1-MT [Fig. 5(*a*)] is regarded as evidence for structures **8** and **9** with the phosphate group at position 6.



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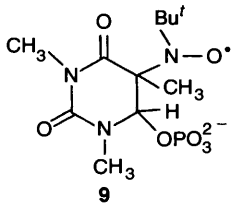

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The same trend of increasing spectral intensities in the presence of phosphate was observed for the uracil derivatives

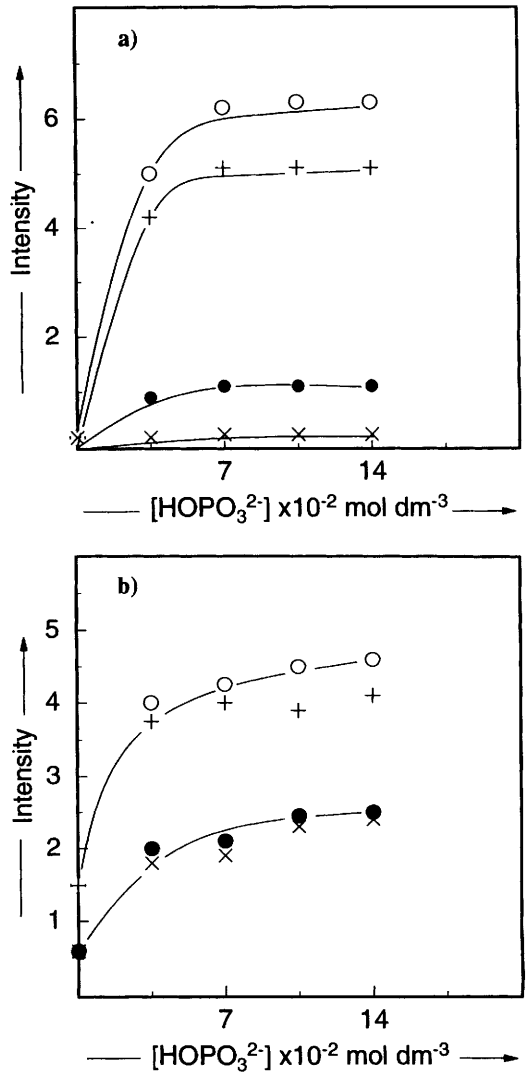


Fig. 5 Plots of intensity of spectra of spin adducts *vs.* phosphate concentrations: (*a*) ● 1-MU, pD 7.4; × 1-MU, pD 9.2; + 1,3-DMU, pD 7.4; ○, 1,3-DMU, pD 9; (*b*) ● 1-MT, pH 7 and 9; ○ 1,3-DMT, pH 9.2; + 1,3-DMT, pH 7.4; × 1,3-DMT and 1-MT, pH 4.8

[Fig. 5(*b*)]. These intensity effects are thought to be due to the slower rate of termination of the negatively charged phosphate-adduct radicals as compared to the neutral OH-adduct radicals resulting in a higher yield of trapping of the phosphate-adduct radicals in competition with termination.

(d) 1-Methylcytosine (1-MC), 2′-deoxycytosine (dC)

For both compounds photolysis in the presence of $S_2O_8^{2-}$ and MNP resulted in triplets of doublets [Fig. 6(*a*)]. These spectra are different from the triplets of quartets of the OH-adduct radicals obtained by photolysis in the presence of H_2O_2 (see also refs. 24 and 26). The doublet splittings in the experiments with $S_2O_8^{2-}$ change at pH ~ 4 (the pK_a-value of dC is 4.4) and are slightly different for 1-MC as compared with dC. This confirms that the spin adducts contain both the amino group and the methyl group (or the sugar residue).

Spin adduct radicals observed for N^1-methylated pyrimidines by loss of a proton from the methyl groups are characterized [24] by β-proton splittings of > 1 mT and can be excluded as an explanation for the result in Fig. 6(*a*). Contribution of sugar radicals to the spectrum of dC as suggested by Rustgi and Riesz [12] is rather unlikely in view of the similarity of the parameters with those of 1-MC (Table 2).

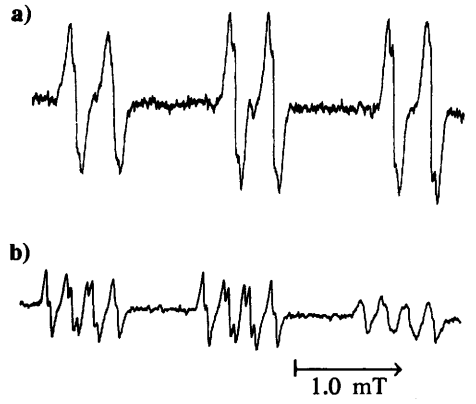
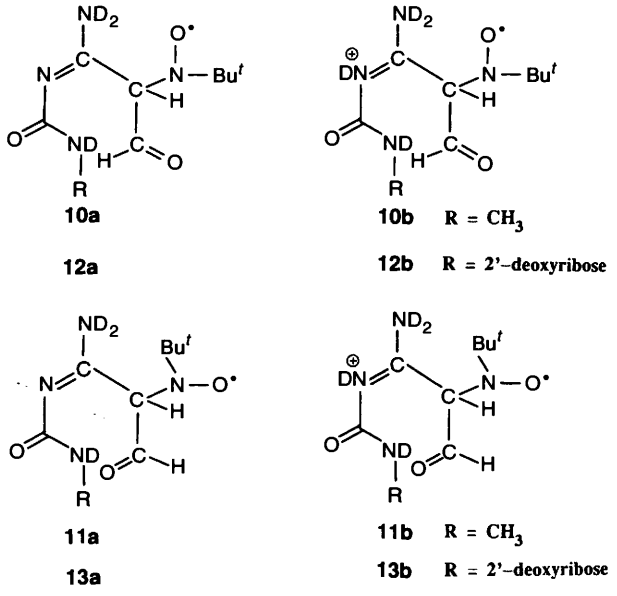


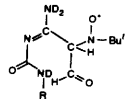
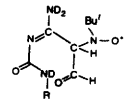
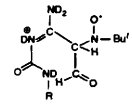
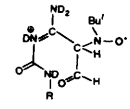
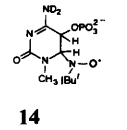
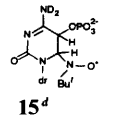
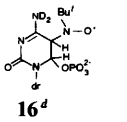
Fig. 6 Spectrum of spin adducts obtained by UV-irradiation of a solution containing 1-MC (1.5×10^{-2} mol dm^{-3}), $K_2S_2O_8$ (1.40×10^{-1} mol dm^{-3}) and MNP (10^{-2} mol dm^{-3}) in D_2O, (*a*) in the absence of phosphate, pD 7.2; (*b*) in the presence of phosphate (0.25 mol dm^{-3}), pD 7.2, 15 min after switching off the light source and after decay of DTBN



The individual doublet signals for 1-MC and dC in D_2O show fine structures (Fig. 7) which can best be interpreted in terms of the contribution of two isomers, one of them with an $a_{H\gamma}$ of 0.08–0.1 mT (**10** and **12**) and the other one with $a_{H\gamma}$ close to zero (**11** and **13**). The concentration ratios of the isomers are ~ 1:1 at pD > 4 and ~ 1:0.75 at pD < 4 for 1-MC and ~ 1:0.5 for dC independent of pD-values (see Table 2). The assignment is not clear. It is tentatively assumed that the spectra are due to trapped open-chain structures originating from rapid hydrolysis of the N-1–C-6 bond and deprotonation of the OH group of the ensuing open-chain radical cation.

In this model the situation is characterized by radicals **10** and **11** (for 1-MC) or **12** and **13** (for dC). Rotation of the aldehyde group around the C-5–C-6 bond hindered by intramolecular hydrogen bonding could be responsible for the postulated equilibrium of two isomeric forms. Strong angular dependence observed for γ-proton hyperfine splittings in aliphatic α-formyloxy radicals is in support of this model.[27] In this context it should be mentioned that ring opening by UV-irradiation of cytosine in alcoholic solution and of 2-alkoxycytosines in phosphate buffer are well known.[28] The underlying mechanisms are not understood, however.

Table 2 Hyperfine splittings[a] (in mT) of spin adducts obtained from 1-MC or dC

Substrate	$a_{N\alpha}$	$a_{N\beta}$	$a_{H\beta}$	$a_{H\gamma}$	pD	Radical
1-MC	1.508		0.366	0.103	> 4	 **10a** (1)[b,c]
	1.508		0.366	< 0.005	> 4	 **11a** (1)[b,c]
	1.480		0.426	0.093	< 4	 **10b** (1)[b,c]
	1.480		0.422	< 0.005	< 4	 **11b** (0.75)[b,c]
dC	1.50		0.38	0.09	> 4	**12a** (1)[b]
	1.50		0.38	< 0.005	> 4	**13a** (0.5)[b]
	1.46		0.42	0.09	< 4	**12b** (1)[b]
	1.46		0.42	< 0.005	< 4	**13b** (0.5)[b]
1-MC	1.495	0.195	0.253	0.052	> 7	 **14**
dC	1.50		0.70		> 7	 **15**[d]
	1.50		0.40		> 7	 **16**[d]

[a] Error limits: ± 0.005 mT for **10**–**14**, ± 0.01 mT for **15** and **16**. [b] Values in parentheses: relative intensities. [c] R = CH_3. [d] R = 2'-deoxyribosyl.

In the presence of phosphate at pD > 7 from 1-MC after UV-irradiation (4 min) a spectrum was obtained which consisted of large signals of di-*tert*-butyl nitroxide, DTBN, a photoproduct of MNP (1:1:1 triplet, $a_{N\alpha}$ = 1.71 mT, see ref. 29) and a triplet of multiplets. About 15 min after the UV source had been switched off the DTBN triplet had decayed and the spectrum shown in Fig. 6(*b*) was left. It is similar to that of the spin adduct of the 5-OH-6-yl radical of 1-MC (spectrum not shown; see also ref. 24). However, by comparison of the values of $a_{N\beta}$ = 0.195 mT and $a_{H\beta}$ = 0.253 mT (see Table 2) with those of the trapped 5-OH-6-yl adduct radical ($a_{N\beta}$ = 0.210 mT, $a_{H\beta}$ = 0.248 mT; see also ref. 24) it was concluded that the spectrum is due to the spin adduct of the 5-phosphato-6-yl radical **14**. At pD < 7 addition of phosphate had no effect on the results, *i.e.* the spectrum was due to a mixture of radicals **10** and **11**.

Similar results were encountered for dC. The spectrum at pD > 7 in the presence of phsophate resembled that of the mixture of spin adducts of the pyrimidinyl 5-OH-6-yl and the 6-OH-5-yl radicals (see also ref. 30 for trapped OH-adduct

radicals of cytidine) whereas at pD < 7 the triplet of doublets of radicals **12** and **13** was detected. In analogy to the results on 1-MC the spectrum at pD > 7 was assigned to a mixture of trapped phosphate adducts **15** and **16**. The small differences in β-proton splittings expected between the trapped phosphate- and OH-adducts are probably obscured by the rather large linewidths.

Discussion

(a) 1-Methyluracil (1-MU), 1,3-dimethyluracil (1,3-DMU)

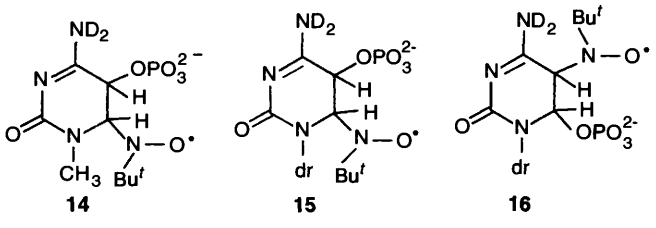
The spectra obtained recently from 1-MU and 1,3-DMU by the continuous-flow EPR technique[22] were assigned to 6-OH-5-yl radicals whereas from pulse radiolysis studies[19] it was concluded that on the μs scale the 5-OH-6-yl radicals were generated. The explanation for this disagreement was based upon γ-radiolysis studies.[19] One has to assume that besides the 5-OH-6-yl radical minor amounts of the 6-OH-5-yl radical are generated by reaction of the radical cation with water [reaction (3)]. In the course of a chain reaction[19] the main radical (6-yl) is oxidized by $K_2S_2O_8$ and the 5-yl radicals accumulate in the soluions on the millisecond time-scale of the EPR experiments. This interpretation was supported by the fact that with a comparatively low $K_2S_2O_8$ concentration (3×10^{-3} mol dm^{-3}) from 1-MU a spectrum of rather poor intensity was generated[22] which differed slightly in *g*-factors and hyperfine splittings from the spectrum obtained with higher $K_2S_2O_8$ concentration (3×10^{-2} mol dm^{-3}) and which was assigned to the 5-OH-6-yl radical.[22] Owing to intensity problems EPR spectra obtained from 1,3-DMU with less than 3×10^{-2} mol dm^{-3} peroxodisulfate could not be analysed.

These observations suggested that the disagreement between pulse radiolysis and continuous-flow EPR experiments was due to differences in the time-scales of the experiments. On the millisecond time-scale the relative concentrations of 5-OH-6-yl and 6-OH-5-yl radicals changed drastically under the influence of the oxidizing peroxodisulfate.

Similar behaviour was observed for the phosphate-adduct radicals identified by ^{31}P hyperfine splittings of ~0.13 mT. Phosphate-adduct radicals were detected only in the experiments with $SO_4^{\bullet -}$ and not with $^{\bullet}OH$ (from H_2O_2) as the radical-inducing agent.[22] It is reasonable to assume that the phosphate dianion also enters the uracilyl radical cation at C-5. However, for the reasons discussed in ref. 22 the spectra were assigned to 6-phosphato-5-yl radicals. As an explanation for these results 1,2-phosphate shift on the millisecond time-scale was favoured although the above-mentioned chain reaction could not be excluded. From the spin-trapping results on 1-MU and 1,3-DMU (Figs. 1–3 and Table 1) it is clear that the uracil-6-yl radicals were scavenged by MNP. Assuming a typical value[31] of 10^7 dm^3 mol^{-1} s^{-1} for the rate constant k of the reaction of MNP with carbon-centred radicals a half-life $t_{\frac{1}{2}} = \ln 2/k$ [MNP] for the radicals of 14 μs is estimated (with [MNP], the concentration of the spin-trap = 5×10^{-3} mol dm^{-3}). This means that scavenging of the uracil radical is fast enough to avoid the secondary reactions observed in the continuous-flow experiments.

In general, in the continous-flow experiments phosphate-adduct radicals of uracil and thymine derivatives were detected in their dianionic form only (the pK_a-value of the β-phosphate group is ~6.5). The stability[32] of the monoanionic and of the neutral phosphate adduct radicals (pK_a ~ 2) is obviously too low for detection [see equilibria (9)/(10) and (12)/(13)]. However, as shown in Figs. 2(*f*) and 3(*b*) for 1-MU and 1,3-DMU, trapping by MNP was fast enough to yield the spin adducts of the monoanion radicals at pH 3–6.

At pH > 6.5 transformation of the 5-phosphato-6-yl radical



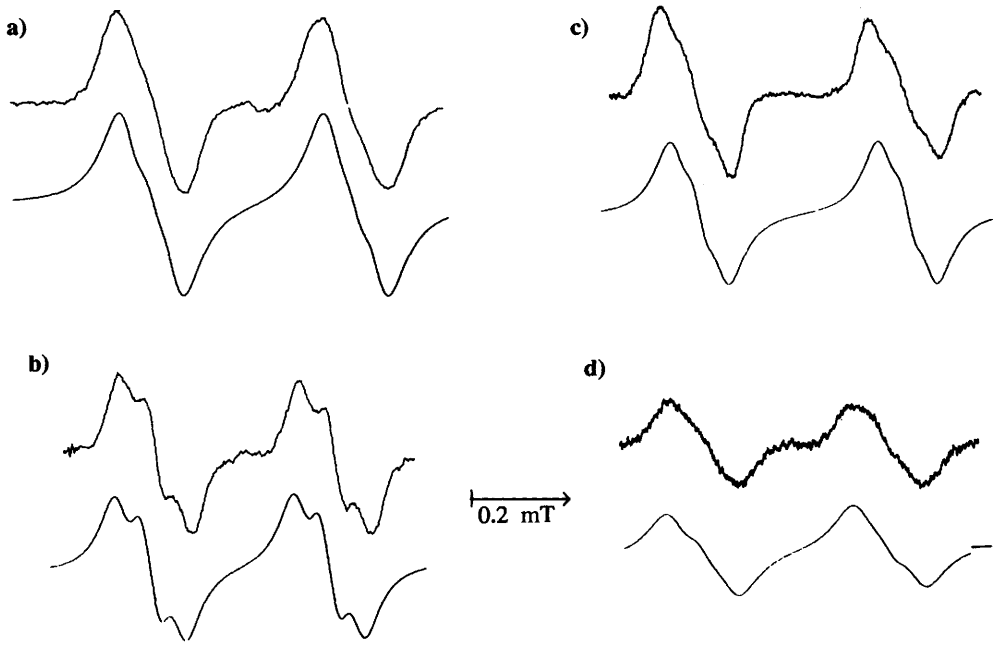
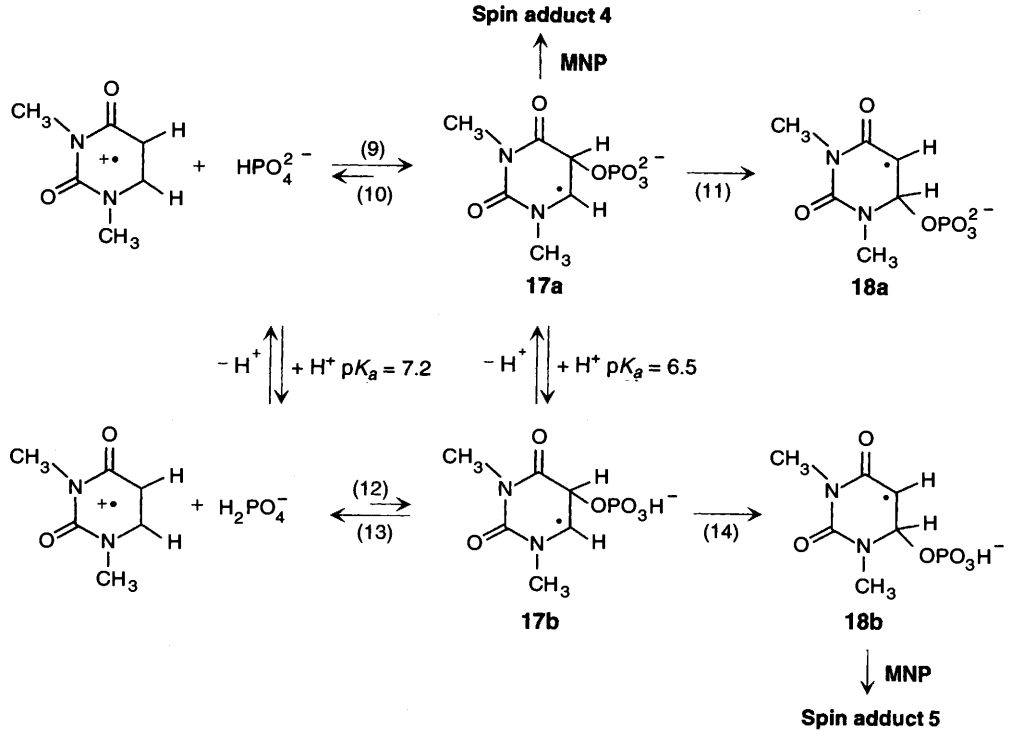


Fig. 7 Expansions of the low-field doublets of the spin adducts of 1-MC (*a*) at pD 2.5; (*b*) at pD 7.4; and of dC (*c*) at pD 1.6; and (*d*) at pD 7.4. Upper traces: experimental spectra, lower traces: simulations of overlaps of spectra for **10** and **11** for 1-MC and of **12** and **13** for dC with the parameters in Table 2, linewidth: 0.05 mT.



17a into the isomeric 5-yl radical **18a** [reaction (11)] is slower than trapping of **17a** by MNP and therefore spin adduct **4** (MNP bound to C-6) was detected. At pH < 6.5 rearrangement [reaction (14)] of the monoprotonated 5-phosphato-6-yl adduct **17b** was faster than reaction with MNP and consequently spin adduct **5** of the isomeric 6-phosphato-5-yl radical **18b** was generated. This increase in the rate of rearrangement upon protonation [reaction (14)] is a consequence of the lower stability of the C-5–phosphate bond in **17b** as compared with **17a**. Whereas the continuous-flow experiments yielded only circumstantial evidence for 1,2-shift of the phosphate group, both phosphate adducts, the 6-yl radical **17a** (at pH > 6.5) and the 5-yl radical **17b** (after rearrangement at pH < 6.5) were trapped by MNP.

(b) 1-Methylthymine (1-MT), 1,3-dimethylthymine (1,3-DMT)

$SO_4^{\bullet -}$-induced continuous-flow EPR signals[22] of N^1-substituted thymines had been unambiguously assigned to 6-OH-5-yl and 6-phosphato-5-yl radicals because of the large quartet coupling of 2.2 mT. In agreement with pulse radiolysis data[20] the isomeric C-5-OH-6-yl radicals had not been detected. Spin adducts **3** and **4** observed immediately after starting photolysis [Fig. 4(*a*)] and the intense signals of **5** and **6** [Fig. 4(*c*)] are in agreement with those observations.

(c) 1-Methylcytosine (1-MC), 2'-deoxycytidine (dC)

As was already mentioned in the EPR experiments there was no evidence for the anilinyl-type radical. If formed at all it probably disappeared in the time-scale of the experiment in secondary reactions.

Spin adducts from reaction of nitrogen-centred radicals with MNP have not been observed so far.[29] Accordingly, no EPR signals were obtained by photolysis of a solution of *p*-aminobenzoic acid, $K_2S_2O_8$ and MNP although in continuous-flow EPR experiments the spectrum of the anilinyl radical was detected. (The spectrum obtained by photolysis of *p*-aminobenzoic acid and $K_2S_2O_8$ in the continuous-flow system was identical with that generated by Neta and Fessenden[33] in radiolysis experiments with $^{\bullet}OH$.) Therefore, if deprotonation were the only pathway of deactivation of the radical cations of 1-MC and dC a complete lack of spin adducts would be expected. However, detection of spin adducts **10**–**13** indicates that there are reactions of the radical cation besides deprotonation. This observation agrees with the contribution of 13% of reducing radicals detected in pulse radiolysis[17] in addition to the oxidizing anilinyl radical.

It is interesting to note that there was no evidence from the continuous-flow EPR experiments for covalent binding of the phosphate group to the cytosine ring. The stability of the phosphate-adduct radical of 1-MC is obviously too low for direct detection. The observation of phosphate-adduct radicals in spin-trapping experiments with 1-MC and dC completes the overall scheme of reaction of pyrimidine radical cations with nucleophiles.

(d) Correlation of the relative yields of spin adducts at C-5 and C-6 with the initial concentrations of the corresponding radicals

In the present study from EPR spectra of spin adducts conclusions have been drawn as to the initial concentrations of C-5- and C-6-centred pyrimidine radicals. For example, the failure to detect the spin adduct of the 6-hydroxyuracil-5-yl radical has been interpreted in terms of selective reaction of water with position 5 of the base radical cation yielding almost exclusively the 5-OH-6-yl radical. This interpretation requires similar rates of reaction of MNP with pyrimidin-5-yl and -6-yl radicals. From the comparison of yields of pyrimidine OH-adduct radicals from pulse radiolysis[34] and spin-trapping experiments (Table 3) it seems that this assumption is justified for N^1-methylated pyrimidines. In contrast, in nucleoside radicals (*e.g.*, dC) trapping at C-6 is hindered, probably for steric reasons.

(e) Comments on the reaction mechanism

For the interpretation of the results a reaction mechanism *via*

Table 3 Site of 'OH attack of pyrimidine derivatives. Comparison of yields of 5-OH-6-yl and 6-OH-5-yl radicals (in %) obtained by pulse radiolysis and by spin-trapping with MNP

	Pulse radiolysis		Spin-trapping	
System	5-OH-6-yl	6-OH-5-yl	5-OH-6-yl	6-OH-5-yl
1-MU[a]	82[b]	18	100[d]	0
Cytosine	87[c]	13		
1-MC	87[c]	13	100[d]	0
dC	88[c]	12	43[e]	57
Thymine[a]	60[b]	30	75[d]	25

[a] Pulse radiolysis data available for unsubstituted bases only; substitution at N-1 has no influence on the relative yields 5-yl/6-yl (see cytosine system). [b] Ref. 34*a*. [c] Ref. 34*b*. [d] Own measurements and ref. 24. [e] Ref. 35.

$SO_4^{\bullet -}$-induced formation of base radical cations has been proposed [see, *e.g.*, reactions (1)–(4) for 1-MU]. Other pathways are of minor importance as judged by the following observations:

(a) $S_2O_8^{2-}$ was essential for generation of the spin adducts. This means that direct phototransformations of the pyrimidine bases as well as photoionization of bases or of MNP are not involved in the reactions leading to the spin adducts.

(b) The rate constants[19–21] for reaction of $SO_4^{\bullet -}$ with N^1-substituted uracil and thymine are $\sim 5 \times 10^9$ mol dm^{-3} s^{-1}, for reaction of $SO_4^{\bullet -}$ with dC a value of 2.5×10^8 dm^3 mol^{-1} s^{-1} was reported.[17] MNP and pyrimidine bases were normally present in equal amounts in the photolysis solutions. However, in some experiments the concentration ratios of base:MNP were increased to 10:1 and 25:1 without there being any observable changes in spectral patterns of the spin adducts. It is concluded, therefore, that a pathway *via* reaction of $SO_4^{\bullet -}$ with MNP and of the ensuing $MNP^{\bullet +}$ with the pyrimidine bases does not contribute to the results.

(c) Trapping of base radical cations by MNP was excluded for the following reasons: in order to compete with water for the radical cations MNP would have to react with a pseudo-monomolecular rate constant k_{MNP}[MNP] of the order of magnitude of the reciprocal lifetime[20] of the radical cations, *i.e.* $\sim 10^6$ s^{-1}. Spin adducts were obtained with MNP concentrations [MNP] $\sim 1 \times 10^{-3}$ mol dm^{-3}, *i.e.* a value of the bimolecular rate constant k_{MNP} of $\sim 10^9$ mol dm^{-3} s^{-1} would be required for trapping of the radical cations. For competition with phosphate anions[22] ($k_{ph} \sim 5 \times 10^8$ dm^3 mol^{-1} s^{-1}, [ph] = 10^{-1}, [MNP] = 10^{-3} mol dm^{-3}) a hypothetical value of $k_{MNP} \sim 5 \times 10^{10}$ mol dm^{-3} s^{-1} is calculated. These rate constants are far beyond the range observed for reactions of MNP with carbon-centred radicals.[31]

Conclusions

The structures of the spin adducts obtained by reaction of $SO_4^{\bullet -}$ with N^1-substituted pyrimidines in the presence of MNP are consistent with intermediate formation of base radical cations, which in the case of uracil and thymine derivatives either react with water or are scavenged by phosphate dianions. From the hyperfine nitrogen and proton splittings of the trapped OH- and phosphate-adduct radicals unambiguous information on the regioselectivity of reaction of water and phosphate with the C-5–C-6 bond of the pyrimidine radical cations has been obtained. In this respect the spin-trapping EPR technique proves superior to the continuous-flow method previously[22] applied to the study of reactions of pyrimidine radical cations. On the millisecond time-scale of the continuous-flow EPR experiments the ratio of concentrations of uracil-5-yl and -6-yl radicals was strongly influenced by secondary reactions (*e.g.*, oxidation of reducing OH-adduct radicals by peroxodisulfate, rearrangement of phosphate adduct radicals). These complications are circumvented by rapid trapping of the pyrimidine OH- and phosphate-adduct radicals by MNP. A remarkable difference between the two EPR methods is also observed for N^1-substituted cytosine. Whereas the steady-state concentrations of cytidyl phosphate-adduct radicals was too low for direct detection the spin-trapping technique was fast and sensitive enough to indicate reaction of HPO_4^{2-} with the cytidyl radical cations.

Experimental

Experiments were carried out at 20 °C on a Varian X-band spectrometer, using an aqueous sample cell. Solutions were prepared by dissolution of the pyrimidine derivatives and $K_2S_2O_8$ or H_2O_2 in water or D_2O (Merck, Darmstadt) and adding MNP from a stock solution (5×10^{-2} mol dm^{-3}). Unless otherwise noted the concentrations were 5×10^{-3} mol dm^{-3} for pyrimidine bases and MNP and 4×10^{-2} mol dm^{-3} for $K_2S_2O_8$ or H_2O_2. pH-Values were adjusted with $HClO_4$ or NaOH. The solutions were bubbled for 20 min with N_2, transferred to an aqueous solution quartz cell, and irradiated in the EPR cavity with the unfiltered, focussed light of a LX300UV Cermax lamp, ICL Technology, Sunnyvale, CA. The concentrations of spin adducts increased with time and reached a maximum after *ca.* 4 min of irradiation. The lifetime of the spin adducts was in the range from 10 min to 24 h. Changes in pH or pD during photoirradiation of unbuffered solutions were less than 1 unit. Linewidths of trapped phosphate adducts were identical with those of trapped OH adducts. Therefore, intensities of spectra in the absence and presence of phosphate were determined by comparing the heights of corresponding signals. From repeated measurements, error limits < 25% for relative intensities were estimated. All chemicals were commercial samples of high purity and used without further purification.

References

1 C. von Sonntag, *The Chemical Basis of Radiation Biology*, Taylor and Francis, London, 1987.
2 M. D. Sevilla, *J. Phys. Chem.*, 1971, **75**, 626; M. D. Sevilla, C. van Paemel and C. Nichols, *J. Phys. Chem.*, 1972, **76**, 3571; M. D. Sevilla and M. L. Engelhardt, *Faraday Discuss. Chem. Soc.*, 1978, **63**, 255; M. D. Sevilla, J. B. D'Arcy, K. M. Morehouse and M. L. Engelhardt, *Photochem. Photobiol.*, 1979, **29**, 37.
3 L. B. Rubin, T. N. Menshonkova, N. A. Simukova and E. I. Budovsky, *Photochem. Photobiol.*, 1981, **34**, 339.
4 M. Wala, E. Bothe, H. Görner and D. Schulte-Frohlinde, *J. Photochem. Photobiol. A: Chem.*, 1990, **53**, 78 and references therein.
5 H. Görner, *Photochem. Photobiol.*, 1990, **52**, 935.
6 M. G. Simic and S. V. Jovanovic, in *Mechanisms of DNA Damage and Repair*, ed. M. G. Simic, L. Grossman and A. D. Upton, Plenum, New York, 1986, p. 39.
7 L. P. Candeias and S. Steenken, *J. Am. Chem. Soc.*, 1989, **111**, 1094.
8 R. L. Willson, P. Wardman and K.-D. Asmus, *Nature (London)*, 1974, **252**, 323.
9 P. O'Neill and P. W. Chapman, *Int. J. Radiat. Biol.*, 1985, **47**, 71.
10 S. V. Jovanovic and M. G. Simic, *J. Phys. Chem.*, 1986, **90**, 974.
11 K. M. Bansal and R. W. Fessenden, *Radiat. Res.*, 1978, **75**, 497.
12 S. N. Rustgi and P. Riesz, *Int. J. Radiat. Biol.*, 1978, **34**, 301.
13 M. D. Sevilla, D. Suryanarayana and K. M. Morehouse, *J. Phys. Chem.*, 1981, **85**, 1027.
14 H. Riederer and J. Hüttermann, *J. Phys. Chem.*, 1982, **86**, 3454.
15 A. J. S. C. Vieira and S. Steenken, *J. Am. Chem. Soc.*, 1987, **109**, 7441; *J. Phys. Chem.*, 1987, **91**, 4138.
16 S.-I. Fujita and Y. Nagata, *Radiat. Res.*, 1987, **114**, 207; S.-I. Fujita, Y. Nagata and T. Dohmaru, *Int. J. Radiat. Biol.*, 1988, **54**, 417.
17 P. O'Neill and S. E. Davies, *Int. J. Radiat. Biol.*, 1987, **52**, 577.
18 R. Rashid, F. Mark, H.-P. Schuchmann and C. von Sonntag, *Int. J. Radiat. Biol.*, 1991, **59**, 1081.

19 H.-P. Schuchmann, D. J. Deeble, G. Olbrich and C. von Sonntag, *Int. J. Radiat. Biol.*, 1987, **51**, 441.
20 D. J. Deeble, M. N. Schuchmann, S. Steenken and C. von Sonntag, *J. Phys. Chem.*, 1990, **94**, 8186.
21 E. Bothe, D. J. Deeble, D. G. E. Lemaire, R. Rashid, M. N. Schuchmann, H.-P. Schuchmann, D. Schulte-Frohlinde, S. Steenken and C. von Sonntag, *Radiat. Phys. Chem.*, 1990, **36**, 149.
22 G. Behrens, K. Hildenbrand, D. Schulte-Frohlinde and J. N. Herak, *J. Chem. Soc., Perkin Trans. 2*, 1988, 305.
23 (*a*) K. Hildenbrand, G. Behrens, D. Schulte-Frohlinde and J. N. Herak, *J. Chem. Soc., Perkin Trans. 2*, 1989, 283; (*b*) D. Schulte-Frohlinde and K. Hildenbrand, in *Free Radicals in Synthesis and Biology*, ed. F. Minisci, NATO ASI Series C, no. 260, Kluwer Academic, Dordrecht, 1989, p. 335; (*c*) K. Hildenbrand, *Z. Naturforsch., Teil C*, 1990, **45**, 47.
24 S. Rustgi and P. Riesz, *Int. J. Radiat. Biol.*, 1978, **33**, 21.
25 P. Riesz and S. Rustgi, *Radiat. Phys. Chem.*, 1979, **13**, 21.
26 A. Joshi, S. Rustgi and P. Riesz, *Int. J. Radiat. Biol.*, 1976, **30**, 151.
27 P. Smith, K. K. Karukstis and S. M. Denning, *J. Magn. Reson.*, 1980, **41**, 91; P. Smith and K. K. Karukstis, *J. Magn. Reson.*, 1981, **42**, 208.
28 A. A. Shaw and M. D. Shetlar, *Photochem. Photobiol.*, 1989, **49**, 267, 273.
29 G. R. Buettner, *Free Radical Biol. Med.*, 1987, **3**, 259.
30 H. Catterall, M. J. Davies, B. C. Gilbert and N. P. Polack, *J. Chem. Soc., Perkin Trans. 2*, 1993, 2039.
31 Y. Maeda, P. Schmid, D. Griller and K. U. Ingold, *J. Chem. Soc., Chem. Commun.*, 1978, 525.
32 G. Behrens, G. Koltzenburg, A. Ritter and D. Schulte-Frohlinde, *Int. J. Radiat. Biol.*, 1978, **33**, 163.
33 P. Neta and R. W. Fessenden, *J. Phys. Chem.*, 1974, **78**, 523.
34 (*a*) S. Fujita and S. Steenken, *J. Am. Chem. Soc.*, 1981, **103**, 2540; (*b*) D. K. Hazra and S. Steenken, *J. Am. Chem. Soc.*, 1983, **105**, 4380.
35 M. J. Davies, B. C. Gilbert, C. Hazlewood and N. P. Polack, *J. Chem. Soc., Perkin Trans. 2*, 1995, 13.

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