An EPR Study of Photoionised Thymine and its Derivatives at 77 K

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The effect of high-energy ionising radiation on thymine and a wide range of its derivatives has been widely studied. Liquid-phase studies on aqueous solutions using pulse radiolysis and UV-photoionisation methods have been reported. Our aim was to use low temperature matrices in combination with laser photolysis and EPR spectroscopy in order to learn more about the double quantum event leading to UV-photoionisation. For thymine, electron-loss results in the normal π-radical cation but this rapidly undergoes proton loss, probably from the N^1–H group. The EPR spectrum is well defined and is characteristic of this radical. This species was detected in perchlorate matrices and there was no evidence of electron capture by thymine. However, in pure aqueous systems, $TH^{\cdot}$ radicals, possibly formed by electron capture followed by protonation, were detected.

In contrast, for N^1 substituted species such as TMP, the π-radical cations were not detected by EPR spectroscopy. Instead, there were comparable yields of $TCH_2^{\cdot}$ radicals, in which one of the methyl protons has been lost. These give rise to a characteristic quartet splitting from hyperfine coupling to the two $-CH_2^{\cdot}$ protons, together with the C^6–H proton which gives about the same splitting. Only in the case of alkaline perchlorate glasses, when the N^3–H proton is removed, was the electron lost from the π system to give the neutral π-radical.

When frozen aqueous systems containing $TCH_2^{\cdot}$ radicals were annealed the quartet signals were lost, and quintets grew in. Similar quintets have been previously assigned to $TOH^{\cdot}$ radicals formed by the addition of water to $TCH_2^{\cdot}$. However, use of D_2O did not modify the signals, so we propose that this species is due to a dimer radical and/or a cyclic radical. The former is formed by the addition of the $TCH_2^{\cdot}$ unit to C^6 of another molecule and the latter arises from intramolecular hydrogen-atom transfer from the $C^{5'}$ hydrogen followed by cyclisation.

Use of 5-ethyl-2′-deoxyuridine, systems that gave the $TCH_2^{\cdot}$ radicals for thymine derivatives, gave $U\dot{C}HCH_3$ radicals. Reasons for the formation of these species by biphotonic photoionisation are discussed. It is difficult to understand why the primary π-radical cations of the N^1-substituted thymine derivatives should prefer to lose a proton from the carbon rather than the $>N_3$–H groups, since the latter, but not the former, has an adjacent proton-acceptor. An alternative is considered, in which an excited state of the primary electron-loss centre is initially formed. This state has the local structure $>C=O^{\cdot+}$, the SOMO being the in-plane n-orbital on oxygen. By analogy with, for example, ester radical cations, it is suggested that hydrogen-atom transfer from the adjacent methyl group occurs more rapidly than the switch from this state to the π-ground state, thereby giving the required $TCH_2^{\cdot}$ centre, with a proton on the adjacent carbonyl group.

It is commonly thought that cell death on exposure to ionising radiation arises primarily as a result of DNA damage, and it is therefore important to understand the mechanism of this damage. There are two limiting mechanisms of radiation damage. One arises from the direct interaction between the radiation quanta and DNA giving rise to electron-gain and electron-loss centres in the DNA (direct damage). The other is indirect damage *via* attack by water radicals ($^{\cdot}OH$, $H^{\cdot}$, and e_{aq}^-). Exposure of nucleic acids in dilute aqueous solution to electron and γ-radiations generates numerous base and sugar lesions.[1–4] These are mostly accounted for by the indirect mechanism whilst the direct ionisation pathway represents a minor component of the total damage. The study of the direct damage mechanism by conventional pulse radiolysis techniques is not easy because of the overriding influence of the indirect mechanism. However, photoionisation models the so-called direct effect and provides a means selectively to generate the radical cations of the nucleic acids and their derivatives.[5–7] Photolysis of the nucleic acids with high intensity nanosecond and picosecond UV radiation (248 nm) results in the successive absorption of two quanta of light and excitation of the bases to an excited energy state of about 9–10 eV. This energy is much higher than the threshold energy required for ionisation of the bases (5.4–6 eV)[6] and hence results in photoionisation of the bases, and formation of a radical cation and an electron. Consequently, the study of strand-break formation in DNA and polynucleotides is of increasing interest as a means of mimicking direct radiation damage to DNA since electron-loss is only from the DNA.[6–11]

$$\mathrm{DNA} \xrightarrow[248\,\mathrm{nm}]{2h\nu} \mathrm{DNA}^{\cdot+} + e^- \qquad (1)$$

We and others have studied the effects of ^{60}Co γ-radiation damage to DNA between 77 K and 273 K using EPR spectroscopy.[12–25] Our approach has been to study frozen aqueous DNA calling on previous results from the individual DNA constituents namely the four DNA bases, and their nucleosides and nucleotides, to support our arguments.[12–17] Frozen aqueous DNA forms two phases on freezing, a pure ice phase, and an aqueous DNA phase which is probably glassy. We have probed this glassy phase by a variety of techniques and conclude that there are roughly 180 water molecules per base-pair which are distinguishable from normal ice water.[26] These become sufficiently mobile at temperatures of *ca.* 170 K to allow radical–radical reactions to occur despite the fact that the systems appear to be completely rigid.

Under frozen aqueous conditions the DNA bases act both as electron-donors and electron-acceptors. The EPR results suggest that the major electron loss centre is $G^{\bullet +}$, and the major electron gain centres are $T^{\bullet -}$ and $C^{\bullet -}$. From the EPR results of single crystals,[27,28] low-temperature aqueous glasses of the DNA bases,[17,29,30] and pulse radiolysis studies,[31] it seems likely that $G^{\bullet +}$ loses a proton whilst the two radical anions gain protons. Nevertheless, it is customary to use these simple radical ion representations which indicate that G has lost a π-electron, whilst C and T gain π-electrons.

Hydroxyl radicals are also detected in abundance by EPR spectroscopy at 77 K. These have parameters of $^{\bullet}OH$ radicals trapped in ice crystallites.[32] They are lost irreversibly at 130 K (exactly the same temperature of loss as in pure ice), and we suggest that these radicals are trapped in phase-separated ice crystals and are not available to react with the DNA. The product H_2O_2, does not react with the DNA in fluid solution in the absence of transition-metal-ion catalysts, and can therefore be ignored for our purposes.

Strand-break studies of annealed samples after exposure to γ-radiation at 77 K indicated that both types of radical centre yield up to *ca.* 20% single-strand breaks (SSB) under our conditions.[12–16] Furthermore, the relative number of double-strand breaks (DSB) seemed to be unusually high and we suggested that they stemmed from radical cation and radical anion centres trapped in fairly close proximity on opposite strands.

This simple model has been seriously thrown into question by recent studies using, for example, high powered lasers which are able to photoionise the DNA bases directly.[5–11] So far only liquid-phase studies have been reported and have led to the conclusion that guanine is the main electron loss site,[8,9] but does not lead on to a significant number of strand breaks.[6,10,11] In fact the highest values for the quantum yields of single-strand break formation in single- and double-stranded DNA were 0.05 and 0.09, respectively.[10,11] Furthermore, the ejected electrons become solvated and do not appear to add to DNA or contribute to strand breaks.[9,10]

Clearly there are some major differences between the results of solid-state γ-radiolysis of DNA and liquid phase photoionisation studies. To bridge the gap between the two methods we have undertaken an EPR study of photoionised DNA and its constituents at low temperature (77 K). Our work on DNA is not yet complete, but has forced us to study the individual bases because the results are difficult to interpret. Here we report our work on thymine and its derivatives, and compare the laser-damage results with those for ^{60}Co γ-ray damage.

Experimental

Materials.—Thymine, 1-methylthymine, thymidine, thymidine 5′-monophosphate (TMP), 5-methyluridine, 5-ethyl-2′-deoxyuridine, were all obtained from Sigma. Magnesium perchlorate, sodium perchlorate, sodium hydroxide, lithium chloride and D_2O (99.8%D) were obtained from Aldrich. These materials were used without further purification. Water was purified using a millipore Multi Q system.

Matrices.—Three low-temperature matrices were used in this investigation. Frozen aqueous solutions, and solutions of 3 mol dm^{-3} $Mg(ClO_4)_2$ (pH 5.5) and 8.5 mol dm^{-3} $NaClO_4$ (pH 8) which form transparent glasses when cooled to 77 K. The glass solutions (H_2O or D_2O) were made using solute concentrations of 0.05 mol dm^{-3} whereas solute concentrations of 0.2 mol dm^{-3} were employed for the frozen aqueous solutions. Generally for the isolation and identification of radicals under neutral conditions $Mg(ClO_4)_2$ glasses were employed, since they made better glasses than 8.5 mol dm^{-3} $NaClO_4$. However, in a number of cases alkaline glasses were required. In such cases 8.5 mol dm^{-3} $NaClO_4$ glasses with added NaOD (pD 11.5) were employed. For irradiation purposes cylindrical pellets (approximately 3 mm × 2 cm) were made by cooling the solutions to 77 K in cylindrical tinfoil moulds. Frozen aqueous solutions were prepared by freezing *ca.* 0.3 cm^3 of solution in a Pyrex tube (id = 3 mm). Unless otherwise stated, all solutions were deoxygenated by being degassed with N_2 (BOC) for 10–15 min prior to freezing.

The photoionisation is biphotonic, generating a radical cation and a photoejected electron. In the perchlorate glasses, the perchlorate scavenges the electron [reaction (2)] producing ClO_3^- and $O^{\bullet -}$. Thus, radical anion formation is suppressed in these glasses.

$$X + ClO_4^- \xrightarrow{2h\nu} X^{\bullet +} + O^{\bullet -} + ClO_3^- \qquad (2)$$

The signal due to $O^{\bullet -}$ is a singlet which shows characteristic parallel and perpendicular *g*-values. The *g*-values measured for $O^{\bullet -}$ in 8 mol dm^{-3} $NaClO_4$ by Kevan[33] are $g_\perp = 2.054$ and $g_\parallel = 2.003$. The value for $g_\perp$ is significantly less than that in sodium hydroxide glasses ($g_\perp = 2.09$ and $g_\parallel = 2.002$)[34,35] but is similar to that found for 3 mol dm^{-3} $Mg(ClO_4)_2$ in this study. Annealing the perchlorate glasses resulted in a large loss of radicals near their glass-transition temperatures (*ca.* 155 K for $Mg(ClO_4)_2$ and *ca.* 180 K for $NaClO_4$).

Irradiation Technique.—A Questek KrF excimer laser at the Laser Support Facility, Rutherford Appleton Laboratory, was used for photolysis. The laser energy at 248 nm was between 200 and 350 mJ per pulse, the pulse duration was 20 ns, the area of beam cross section was between 2–3 cm^2, and the pulse repetition rate was 5 Hz. Laser intensities varied between approximately 3.3×10^{10} W m^{-2} and 8.8×10^{10} W m^{-2}. As a result of the above parameters of pulse length and laser intensity in conjunction with the increased excited state lifetimes at 77 K, photoionisation is expected to occur *via* both the triplet and singlet excited-state manifolds.

Frozen 'pellets' were photolysed in a quartz Dewar flask at 77 K. The total irradiation time was varied between 30 s and 2 min and the irradiated samples were then stored in liquid nitrogen and transported to Leicester for EPR analysis.

EPR Analysis and Sample Annealing.—EPR spectra were recorded at 77 K on a Jeol JES-REIZ X-band spectrometer interfaced with an Archimedes computer. The EPR spectra were obtained directly at 77 K and after annealing using a copper block cryostat ('annealing' refers to the warming of the sample to a prescribed temperature for 8 min followed by recooling to 77 K). At 77 K the irradiated perchlorate glasses have composite EPR spectra comprising features from the thymine radicals, $O^{\bullet -}$ and $H^{\bullet}$ or $D^{\bullet}$. At 77 K the features of $O^{\bullet -}$ and $D^{\bullet}$ overlay those of the radical cation but $D^{\bullet}$ atoms were lost on annealing to 115 K for the $Mg(ClO_4)_2$ glasses and 130 K for the $NaClO_4$ glass. Further annealing to the melting points of the glasses [$\geqslant$ 155 K for $Mg(ClO_4)_2$ and $\geqslant$ 180 K for $NaClO_4$] led to the irreversible loss of $O^{\bullet -}$ thereby revealing the pure solute radical spectra. However, in a number of cases secondary reactions took place at the glass transition temperatures and spectral analysis of the parent radicals was therefore more difficult.

To overcome these difficulties, the solute radical spectra were isolated from the $O^{\bullet -}$ spectrum by computer subtraction of the spectra obtained at high and low microwave powers (Fig. 1).

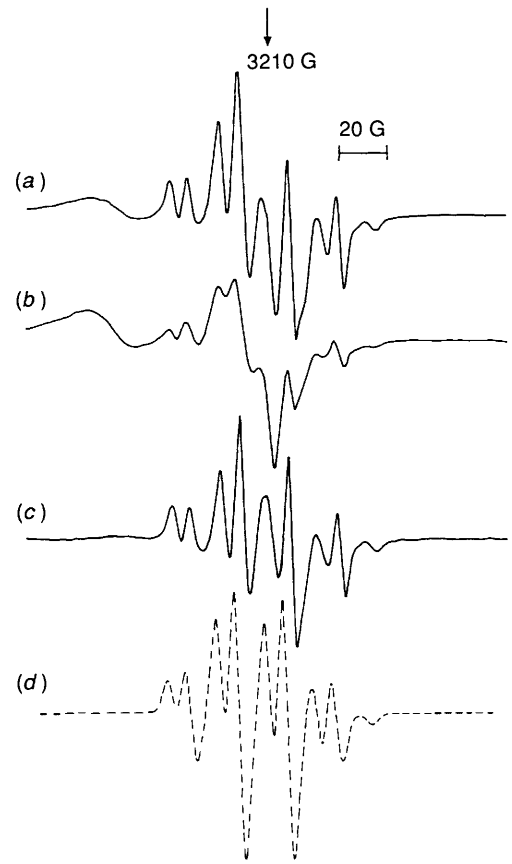


Fig. 1 X-Band EPR spectra of photoionised thymine in $Mg(ClO_4)_2$–D_2O glasses after annealing to 115 K to remove the $D^•$ radicals: (*a*) at 0.01 mW showing the $O^{•-}$ and $T(-H^+)^•$ radicals; (*b*) at 10 mW showing the partial saturation of the $T(-H^+)^•$ radical; (*c*) subtraction of the high microwave power spectrum from the low power spectrum using the outer perpendicular features of the $O^{•-}$ spectrum; in this manner the $O^{•-}$ spectrum can be subtracted so as to reveal the organic radical; (*d*) simulation of $T(-H^+)^•$ based on the parameters in the text

Table 1 EPR parameters for the thymine π-radicals associated with electron loss

	Hyperfine coupling/G			
Compound/radical	N $A_{\parallel}$	N $A_{\perp}$	H a(Me)	H a(N–CH_n)
Thymine[36]	14	2.6	19.1	—
Thymine[42]	12	0	20.2	—
Thymine[a]	12	< 2.5	20	—
TMP[41] alkaline pH[b]	13.1	< 3.1	21.3	8.3
1-Methylthymine[41] alkaline pH	13	< 2.5	21.1	8.4
1,3-Dimethyl thymine[48]	16	0	22	10

[a] This study. [b] Similar parameters were found in this study.

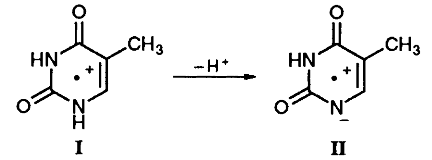
This method depends on the fact that the spectra for the radical cations and their products saturate more readily than that for $O^{•-}$ so that at high powers the $O^{•-}$ signal dominates. Using the outer perpendicular feature of the $O^{•-}$ spectrum as a measure, it can be accurately subtracted to reveal the organic radical spectra (Fig. 1). Typically a low microwave power of 0.01 mW, an intermediate power of 0.2 mW, and a high power of 10 mW were employed for each study.

Results

The most curious aspect of our results is the prevalence, in many systems, of the $TCH_2^•$ radical (**IV**). This is not detected in DNA under any circumstances following irradiation and is not expected to be a product of direct ionisation. The results are described for each radical in turn and are discussed and compared with the EPR results obtained after γ-radiolysis.

Thymine.—Both $NaClO_4$ and $Mg(ClO_4)_2$ glasses gave a well resolved quartet features ($A \cong 20$ G), each component flanked by parallel-type features assigned to a ^{14}N coupling of *ca.* 12 G (Fig. 1). The EPR spectra were identical for H_2O and D_2O glasses, which indicates that none of the couplings are due to exchangeable protons. Also, for alkaline $NaClO_4$ glasses (pH $\cong$ 11.5) the spectra were unaltered. These 'quartet' spectra closely resemble those assigned to the π electron-loss radical reported in other studies, using γ-irradiated single crystals,[36,37] photoionisation,[38–41] and chemical oxidation.[42] The 20 G quartet is assigned to hyperfine coupling to the three methyl protons and on the basis of theoretical spin density calculations N^1 is implicated as the source of the nitrogen coupling.[38,43]

Sevilla *et al.* propose that the π-radical cation loses a proton from N^1 to form radical (**II**).[42] Although we agree with this hypothesis, our experimental evidence, based on the EPR spectra, is insufficient to confirm proton loss. In fact the primary evidence for N^1 deprotonation is that in neutral glasses N^1 substituted derivatives do not appear to form stable π-radical cations, but instead lose a proton from the C^5 methyl group to form the $TCH_2^•$ radical (**IV**).

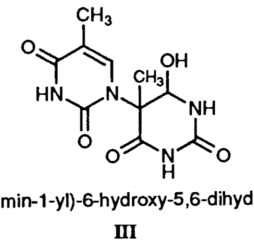


I Thymine π radical cation

II Thymin-1-yl radical

The simulated spectrum [Fig. 1(*d*)] is based on parameters taken from single crystal[36,37] and powder spectra.[38,39,42] The best fit was obtained using the parameters of Sevilla *et al.*[42] who reported a methyl coupling of 20.2 G, a nitrogen coupling of $A_{\parallel} \cong 12$ G and $A_{\perp} \leqslant 0$ G, a g-value difference of $g_{\perp} - g_{\parallel} = 0.003$, and a linewidth of 3 G (Table 1).

Annealing the thymine–perchlorate glasses led to a decrease in the concentration of the π-radical with no gain of other features. This observation is consistent with the product analysis results of Wagner *et al.*[44] who identified 6-hydroxy-5-(thymin-1-yl)-5,6-dihydrothymine (**III**) as an important product in the menadione-mediated photooxidation of thymine. They suggested that this was formed by addition of radical **II** to another thymine molecule in aerated aqueous solution.



5-(Thymin-1-yl)-6-hydroxy-5,6-dihydrothymine

III

Thymidine and TMP in Perchlorate Glasses.—In marked contrast with thymine, these N^1 derivatives gave a multi-line spectrum (Fig. 2) which is thought to be characteristic of the $TCH_2^•$ radical (**IV**) formed by net hydrogen-atom loss from the C^5-methyl group. Confirmation that the quartet spectrum was due to the $TCH_2^•$ radical was achieved by photoionisation of thymidine deuteriated at the C^6 position, which gave a triplet spectrum.

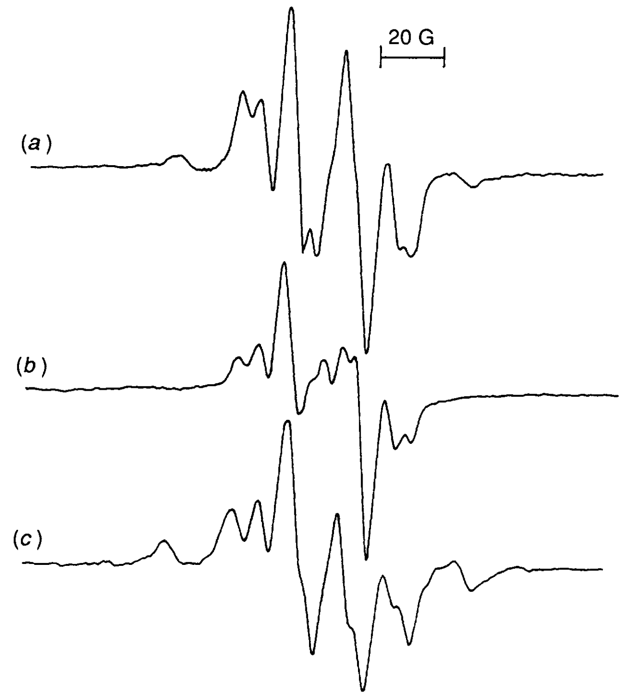


Fig. 2 X-Band EPR spectra assigned to the $TCH_2^\bullet$ radical arising through proton loss from the C^5-methyl group on photoionisation of (*a*) TMP and (*b*) 5-methyluridine in $Mg(ClO_4)_2$–D_2O glasses. The spectra were recorded at 0.01 mW after annealing to 115 K; (*c*) after annealing TMP in $Mg(ClO_4)_2$–H_2O to > 155 K; the spectrum is a composite and consists of features assigned to the $TCH_2^\bullet$ and $TH^\bullet$ radicals

Table 2 EPR parameters for the $TCH_2^\bullet$ radical, the $U\dot{C}HCH_3$ radical, and the quintet successor radical to $TCH_2^\bullet$

	Hyperfine coupling/G						
Compound/radical	CH_n $A(x)$	CH_n $A(y)$	CH_n $A(z)$	H(6) $A(x)$	H(6) $A(y)$	H(6) $A(z)$	H a(Me)
Thymine[45] $TCH_2^\bullet$	8.1	22.4	15.6	7.3	14.8	11.7	—
Thymidine[46] $TCH_2^\bullet$	7.9	25.1	15.3	5	14.9	10.6	—
	7.3	23.5	15.3				
Thymidine[37] $TCH_2^\bullet$	7	23	15	5	14	10	—
5-Ethyl-2′-deoxy-uridine[a]	7.5	24.3	15.3	5	14.9	10.6	14
TMP Quintet species[b]	—	—	—	20 (iso)			20

[a] $U\dot{C}HCH_3$. [b] The parameters assigned to the dimeric **VIII** and/or cyclic **IX** radicals.

$TCH_2^\bullet$ radicals have been detected in irradiated crystals of thymine and its derivatives,[36,45,48] and in irradiated frozen aqueous TMP at 77 K[47,48] (Table 2). The results show that there are two equivalent protons with A_{iso}15–17 G and a third proton with A_{iso}10–12G. All these have anisotropic couplings which are characteristic of α-protons. These are clearly the $-CH_2^\bullet$ protons and the C^6–H proton in an allylic-type centre (**IV**), where most of the spin density is confined to this unit. The powder spectrum for $TCH_2^\bullet$ radicals should have six components but the inner four lines will overlap to give a doublet if the lines are too broad. In some of our studies on TMP only such a quartet has been resolved (Fig. 2) but for the N^1–CH_3 derivatives all six lines were detected (Fig 4). It is possible that the poor resolution for the TMP derivative arises because of an extra doublet species, with $A(^1H)$ *ca.* 16 G. The spectrum obtained for 5-methyluridine (which is the ribose analogue of thymidine) gave a similar spectrum to TMP, which supports the assignment of $TCH_2^\bullet$, as do the results of the N^1–CH_3 derivatives discussed below.

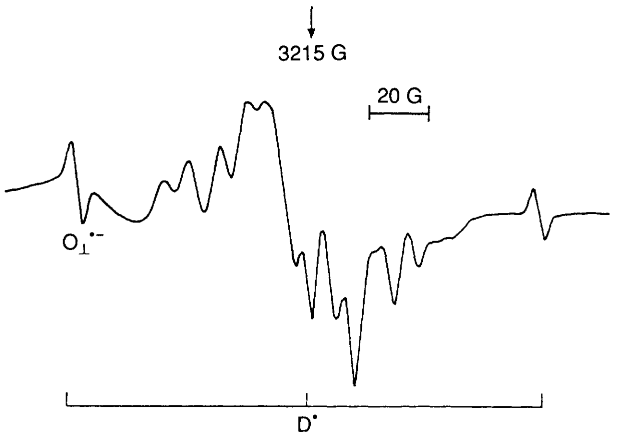
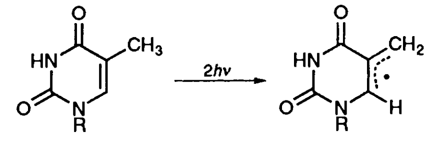


Fig. 3 X-Band EPR spectrum of photoionised TMP in alkaline $NaClO_4$–D_2O (pD ≅ 11.5). The spectrum was recorded at 77 K, a microwave power of 0.01 mW. It was not possible to isolate the organic radical spectrum from the solvent radical features, nevertheless, they were assigned to the neutral π-radical **V** associated with electron loss.

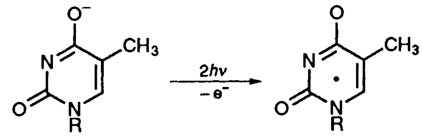


N'-substituted thymine derivative

$TCH_2^\bullet$ **IV**

On annealing, these features were not replaced by any others prior to complete radical loss. This does not mean that secondary radicals are not formed. The lack of any detectable successor radicals may be a kinetic phenomenon in which the secondary species are more labile than the $TCH_2^\bullet$ radical. However, in the H_2O–perchlorate glasses, the octet spectrum of the $TH^\bullet$ radical in which a hydrogen is added to C^6, was detected on annealing to 155 K (Fig. 2). This was a minor component and was probably formed by $H^\bullet$ addition to the thymine residues and/or C^6 protonation of the low concentration of $T^{\bullet-}$ centres in these glasses.

When the $NaClO_4$ glass was made alkaline (pH ≅ 11.5), $TCH_2^\bullet$ radicals were no longer formed and the spectrum of the π-radical was once more observed (Fig. 3). At this pH the N^3–H proton has been lost (pK_a = 9.7), so the species is in fact the neutral π-radical (**V**). This system gave some $TCH_2^\bullet$ radicals on annealing, but this was concomitant with the loss of $O^{\bullet-}$ radicals, and we suggest that these $TCH_2^\bullet$ radicals were formed *via* attack by $O^{\bullet-}$ (or $^\bullet OH$) radicals.



Neutral π radical **V**

A similar study using a low-pressure mercury arc to generate radicals has been reported by Sevilla.[41] Alkaline $NaClO_4$ glasses were employed since the resulting anions have long triplet lifetimes, and photoionisation from the triplet state can occur in good yield. As in the present study, Sevilla[41] observed the neutral π-radical (**V**) for all the N^1-thymine derivatives. The magnitude of the β-proton couplings from the R-groups correlated well with the magnitude of the parallel hyperfine splitting to ^{14}N, showing that this splitting is indeed from N^1 not N^3 (Table 1). Clearly the formation of the neutral π-radical

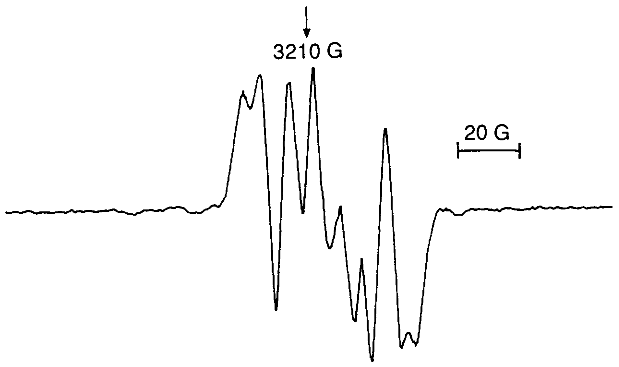


Fig. 4 X-Band EPR spectrum assigned to the TCH$_2^\bullet$ radical, arising from the photoionisation of 1-methylthymine in Mg(ClO$_4$)$_2$–D$_2$O at 77 K, and annealing to 115 K so as to remove the D$^\bullet$ features

as opposed to TCH$_2^\bullet$ is due to pH. At pH $\cong$ 11.5 the parent molecule will deprotonate at N^3 to give an anion which yields a neutral π-radical on electron loss and makes proton loss unnecessary.

TMP in Frozen Aqueous Matrices.—Frozen aqueous (H$_2$O and D$_2$O) solutions of TMP were photoionised at 77 K and the EPR spectra were recorded at this temperature before and after annealing (Figs. 5 and 6). On annealing, the radicals present at 77 K were converted into secondary radicals and finally into diamagnetic products. Since no electron scavengers were employed in the frozen aqueous irradiations, a 50:50 ratio of radical anions and radical cations was expected. However, in practice a number of TMP radicals probably associated with electron loss and gain centres were detected, but it was not possible to ascertain their relative concentrations accurately.

The spectrum for TCH$_2^\bullet$ radicals is clear at 77 K, and is stable on warming to approximately 220 K with little if any decay. In addition there are strong features for TH$^\bullet$ in the H$_2$O matrix but only weak TD$^\bullet$ features in the D$_2$O matrix. The doublet spectrum of the thymine electron adduct T$^{\bullet -}$ may also be present but, if so, it is obscured by the features of the TCH$_2^\bullet$, TH$^\bullet$, or TD$^\bullet$ radicals, so T$^{\bullet -}$ does not seem to be a major product.

Annealing the samples above 220 K led to the conversion of TCH$_2^\bullet$ into a radical with a well defined quintet spectrum. The spectrum has a line intensity ratio of approximately 1,4,6,4,1 with an equidistant spacing of *ca.* 20 G (Figs. 5 and 6). The simulated spectrum (Fig. 5) using four proton couplings of 20 G and a line-width of 4.5 G is in good agreement with the experimental spectra (Table 2). From this result it would appear that the unpaired electron is coupled to three β-methyl protons and a fourth β-proton probably at the C^6 position.

1-*Methylthymine and* 1,3-*Dimethylthymine.*—Photoionisation of both 1-methylthymine and 1,3-dimethylthymine in Mg(ClO$_4$)$_2$ glasses gave rise to electron-loss radicals with spectra which we have assigned to the TCH$_2^\bullet$ radical (Fig. 4). There are some clear differences between the TCH$_2^\bullet$ spectrum derived from TMP and those observed for these derivatives. We suggest that the spectral differences are probably due to small differences in the spin distribution of the different TCH$_2^\bullet$ radicals. It is noteworthy that γ-radiolysis of dilute solutions of the dimethyl derivative in freon gave only the π-radical cation,[49] with very similar EPR parameters to the neutral π-radical described herein. Also, well defined coupling was observed for the N^1–CH$_3$ protons but not for the N^3–CH$_3$ protons. This confirms that the spin density at N^3 is low in the π radicals.

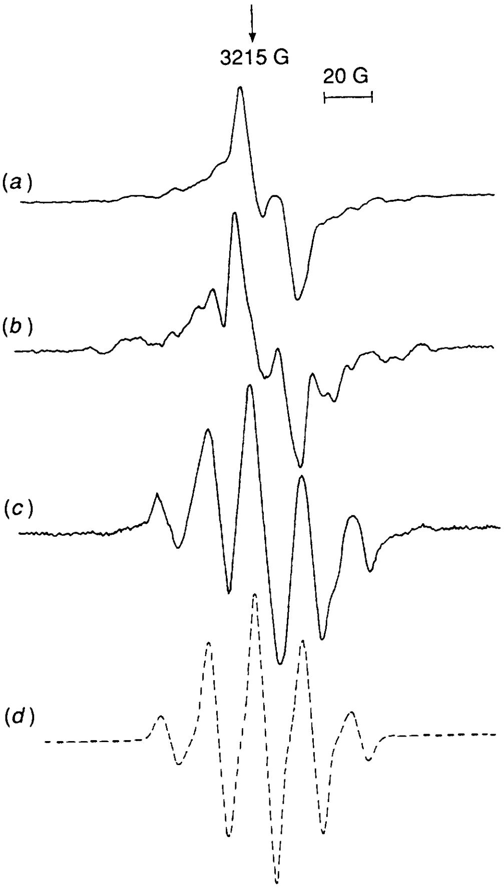
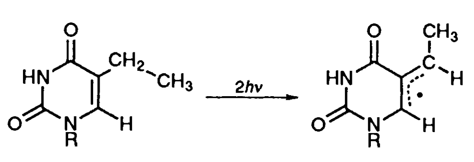


Fig. 5 X-Band EPR spectra of photoionised TMP in frozen aqueous D$_2$O; (*a*) at 77 K showing a composite spectrum due to TCH$_2^\bullet$ and probably TH$^\bullet$, T$^{\bullet -}$ and TD; (*b*) after annealing to 220 K showing a spectrum dominated by the features assigned to the TCH$_2^\bullet$ radical; (*c*) after annealing to 240 K showing the quintet spectrum due to the dimeric and/or cyclic radicals; (*d*) simulation of the quintet radical based on parameters in the text

5-*Ethyl*-2′-*deoxyuridine.*—This derivative is of interest because it is the ethyl analogue of thymidine. Photoionisation of 5-ethyl-2′-deoxyuridine in 3 mol dm^{-3} Mg(ClO$_4$)$_2$ and frozen aqueous D$_2$O matrices gave a radical with a sextet EPR spectrum. The spectrum is present at 77 K but is best resolved in frozen aqueous D$_2$O after annealing to between 200–240 K (Fig. 7). This species was readily identified from its EPR spectrum as the U$\dot{\mathrm{C}}$HCH$_3$ radical (**VI**), formed by proton loss from the methylene position of the ethyl moiety [reaction (3)].



5-Ethyl-2′-deoxyuridine U$\dot{\mathrm{C}}$HCH$_3$ **VI** (3)

The spectrum was well simulated using two anisotropic α-proton couplings and three β-methyl proton couplings (Fig. 7). The anisotropic couplings were H^6 (5 G, 10.6 G, 14.9 G) A_{iso} = 10.2 G and H^7 (7.5 G, 15.3 G, 24.3 G) A_{iso} = 15.7 G. The methyl proton couplings were 14 G (Table 2). Computer simulation using the above parameters with a line-width of 4 G was in good agreement with the experimental spectrum, which suggests that the radical assignment is correct (Fig. 7). Furthermore, these hyperfine parameters compare closely to those for TCH$_2^\bullet$ radicals which also supports our assignment.

In irradiated frozen aqueous solution, doublet features for the radical anion were detected, together with a sextet assigned to the ethyl analogue of TH$^\bullet$. On annealing the systems containing

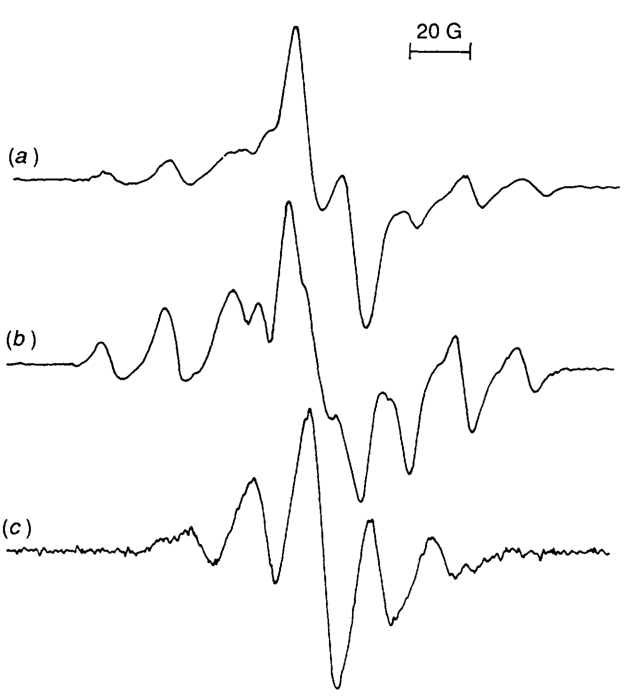


Fig. 6 X-Band EPR spectrum of photoionised TMP in frozen aqueous H_2O: (*a*) at 77 K showing a composite spectrum assigned to the $TCH_2^{\bullet}$, $T^{\bullet -}$ and $TH^{\bullet}$ radicals; the spectrum is dominated by the octet spectrum of $TH^{\bullet}$; (*b*) after annealing to 220 K showing the decay of the central doublet features; (*c*) after annealing to 240 K showing the quintet spectrum assigned to the successor radical of $TCH_2^{\bullet}$

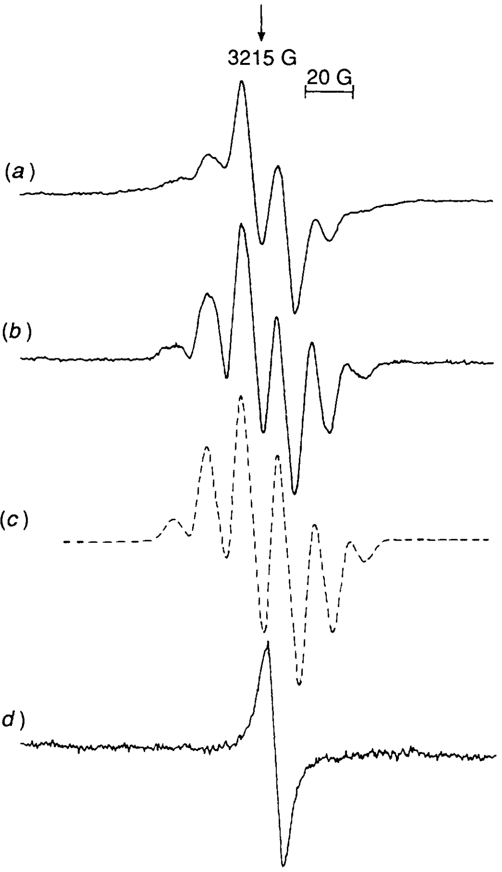


Fig. 7 X-Band EPR spectra of photoionised 2′-deoxy-5-ethyluridine in D_2O: (*a*) at 77 K showing the sextet spectrum assigned to the $UCHCH_3^{\bullet}$ radical; the central region of the spectrum probably contains an additional doublet due to the electron adduct radical; (*b*) after annealing to 240 K showing the well resolved sextet spectrum; (*c*) simulation of the sextet spectrum based on parameters in the text; (*d*) the singlet spectrum that appears on annealing to 260 K

the $U\dot{C}HCH_3$ radical, no features analogous to the quintet formed from $TCH_2^{\bullet}$ were detected. In the D_2O matrix annealing above 240 K led to the rapid decay of the sextet which was succeeded by a sharp singlet spectrum (Fig. 7). The singlet spectrum constituted only *ca.* 4% of the radical concentration at 240 K and it was not possible to assign it to a particular radical.

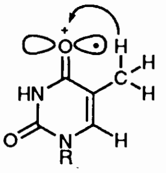
Discussion

There are three main problems that arise from the laser studies. (*i*) Why are $TCH_2^{\bullet}$ and related radicals detected rather than the π-radicals when $>N^1\text{–}H$ is replaced by $>N^1\text{–}R$? (*ii*) On annealing the frozen aqueous TMP samples what is the quintet radical formed from $TCH_2^{\bullet}$? (*iii*) Why, when electron capture to give $T^{\bullet -}$ radicals is expected in the frozen aqueous matrices, are $TH^{\bullet}$ and related radicals detected at 77 K? We address these problems in turn.

The $TCH_2^{\bullet}$ *Radical.*—For reasons given above, we accept that the species formed from $>N^1\text{–}CH_3$ and TMP derivatives are $TCH_2^{\bullet}$ radicals (**IV**), formed effectively, by hydrogen-atom loss from the methyl group. The normal explanation is that it is formed by proton-loss from the methyl group of the parent radical cation.

There are several problems associated with this explanation. One is that the N^3–H proton is far more acidic than the methyl protons, so why is this proton not lost preferentially? Another is that there must be an available proton acceptor for the methyl proton. This means an acceptor with a 'free' lone-pair of electrons. This is the case for the N^3–H proton, which is surely H-bonded to a neighbouring water molecule in these matrices. However, the water in the vicinity of the $-CH_3$ group is expected to be fully hydrogen bonded, as, for example, in chlathrate compounds. It is possible that if the positive charge generated by π-electron loss is sufficiently delocalised on the methyl protons that water–water bonds are induced to break, in order to solvate these protons. This would facilitate transfer of H^+ to a liberated lone pair of electrons. However, this requires that the methyl group should cease to rotate, which is not found experimentally. Also, from the large ^1H hyperfine coupling of *ca.* 20 G for the radical cation it seems that the spin-density on C^5 is very high ($\geqslant 50\%$) which means that charge density on the methyl group is relatively low. In summary, the N^1,N^3-dimethylthymine radical cation (in freon),[49] and the neutral π-radicals deprotonated at either N^1 or N^3 are clearly stable, with no tendency to lose a proton from the methyl group. The spin-density at the methyl protons, and on C^5, is almost constant for these radicals. The N^1–R, N^3–H π-radical cation is expected to deprotonate far faster from N^3 than from the methyl group, to form the neutral π-radical which is quite stable. Since this does not occur at neutral pH, we conclude that the favoured mechanism involving deprotonation of the radical cation to form $TCH_2^{\bullet}$ is rather unlikely.

It has been suggested that for γ-irradiated frozen aqueous TMP, the $TCH_2^{\bullet}$ radical is formed by $^{\bullet}OH$ radical attack.[48] This cannot explain our results since electrons are ejected from TMP and not from the water so $^{\bullet}OH$ radicals are not formed in the bulk water. Picosecond high intensity UV photolysis of dilute aqueous solutions at room temperature have been shown to give rise to ionisation and dissociation of the water.[5,50] However, at 77 K the $^{\bullet}OH$ radicals are readily identifiable by their characteristic EPR spectrum. We have found no evidence for this radical, probably because the laser intensities were 1–2 orders of magnitude below the threshold at which photoionisation of the water becomes effective (*i.e.*, 10^{12} W m^{-2}). Also, the optical spectrum of water narrows and shifts to lower wavelength on cooling to 77 K.[31] Hence, we can rule out attack by •OH radicals in the present work.

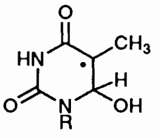


A

We tentatively suggest that our method of double quantum photolysis involves, at least in part, electron-loss from the non-bonding in-plane 2p orbital on oxygen. It is well established that such structures often undergo hydrogen atom transfer, as indicated in A, to give the distonic cation, protonated on oxygen.[51] This can explain why $TCH_2^•$ radicals can be formed on photolysis at 77 K. Clearly the balance between loss from the π-system and the (C=O)n system must be subtle. It seems that if the N^1–H protons are unsubstituted or the N^3–H protons are removed, loss from the π-system is favoured. However, when N^1–H is converted into N^1–R, and the N^3–H proton is present, loss from the (C=O)n level is favoured. Other systems that have been shown by EPR spectroscopy to react in this way are the duroquinone radical cation,[52] a range of esters,[53] and certain ketones.[54]

We stress that this scheme is only intended to explain the direct formation of $TCH_2^•$ species at 77 K. In other studies such as γ-irradiated frozen aqueous TMP,[47,48] it may well be formed by •OH radical attack directly on the methyl group on annealing, or by $O^{•-}$ attack for the perchlorate system, where loss of $O^{•-}$ coincides with the appearance of the $TCH_2^•$ radical.

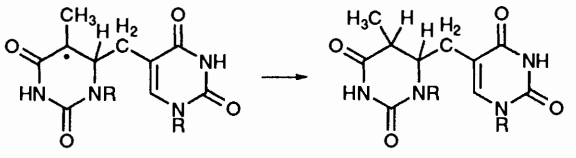
The Quintet Radical Centre.—This centre, which appears to be formed from $TCH_2^•$ centres on warming, has been previously identified as the TOH• species (**VII**) formed by the addition of water molecules to $TCH_2^•$ radicals [reaction (4)]. Recently this centre (**VII**), together with the C^5–OH adduct has been clearly identified as the major product of attack by •OH radicals in aqueous solution at room temperature.[55] The data for thymine are $A_{CH_3} = 22.3$ G and $A_{H^6} = 15.3$ G, and for thymidine $A_{CH_3} = 23.3$ G and $A_{H^6} = 10.9$ G. These values cannot account for the five-line spectrum observed herein, with $A_{4H} \cong 20$ G. Also, the hydration process indicated in reaction (4) seems to us to be most improbable. This is supported by the fact that there is no sign of the deuteriated analogue of TOH• in the D_2O matrix. (In this case a deuterium would be incorporated in the methyl group and a quartet spectrum would be expected.)



TOH• **VII**

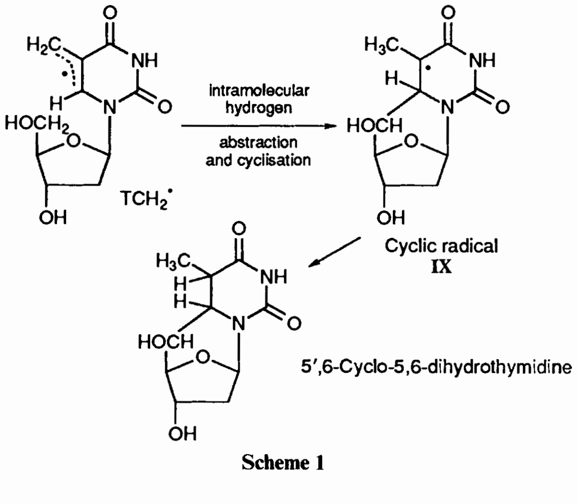
$$TCH_2^• \xrightarrow{H_2O} TOH^• \qquad (4)$$

An alternative explanation which can accommodate the observed results is that the $TCH_2^•$ adds to the C^6 position of a neighbouring TMP molecule to form a dimeric radical **VIII**. This has a CH_3 group with high adjacent spin-density at C^5 so that a 20 G coupling from the methyl protons is reasonable, and 20 G is also quite feasible for the C^6–H hydrogen. The identification of 6-thymidyl-5,6-dihydrothymidine in γ-irradiated frozen aqueous thymidine is compatible with the assignment of the dimeric radical.[56] Furthermore, if the quintet species apparent in the frozen aqueous media is indeed the dimer adduct **VIII**, then the absence of these features on annealing the glassy systems is understandable, since base stacking is not significant in the glasses.



Dimeric radical **VIII** 6-Thymidyl-5,6-dihydrothymidine

Another likely explanation for the quintet centre is the reaction shown in Scheme 1. This comprises hydrogen-atom transfer from the sugar followed by cyclisation to give the cyclic radical **IX**. As with the dimeric radical, the cyclic radical has a structure similar to that of TH• (**XI**), so that a methyl proton coupling of *ca.* 20 G is reasonable. A coupling of 20 G to the C^6 proton is then rather small by comparison, but this may be a steric effect for the cyclic structure forcing the C^6 hydrogen towards the plane of the ring, and thereby reducing the splitting. This explanation seems feasible because the cyclic product, 5′,6-cyclo-5,6-dihydrothymidine, has been isolated as a significant product in frozen aqueous thymidine after γ-irradiation at 77 K.[57] Mechanistically it is quite feasible and requires intramolecular hydrogen abstraction by $TCH_2^•$ from the $C^{5'}$ hydrogen of the deoxyribose moiety followed by cyclisation. In view of the product analysis results it seems likely that both the dimeric radical **VIII** and the cyclic radical **IX** are good candidates for the quintet spectrum.

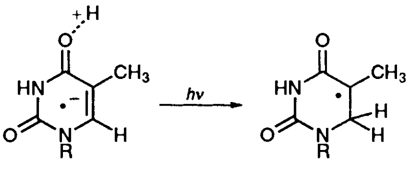


Scheme 1

Formation of TH• at 77 K.—The last problem that arises from this study is the clear formation of TH• in high yield for the frozen aqueous TMP (H_2O) (Fig. 6). In contrast, the exposure of frozen aqueous TMP to γ-radiation at 77 K gives $T^{•-}$ centres (doublet spectra)[47,48,58] just as is found for $DNA^{•-}$ itself,[17] and TH• centres (octet spectra) grow in only on annealing to *ca.* 150 K and above. Hydrogen atoms will certainly add to TMP to give TH• but this should not happen under our conditions because there are certainly no hydrogen atoms generated in these systems. We suggest that the presence of TH• at 77 K in this study, is due to the photoinduced protonation of $T^{•-}$ [reaction (5)].

$$T^{•-} \xrightarrow[H_2O]{h\nu} TH^• \qquad (5)$$

Once again we are faced with the same problem as for $TCH_2^{\bullet}$ formation since there are no readily available proton donors near C^6 that are not strongly bonded to by hydrogen bonds. If protonation is induced it is far more likely to occur at one of the C=O groups, that are already H-bonded and are available to undergo proton-transfer. One possibility is that $T^{\bullet -}$ is formed, and is protonated at >C=O to give >C=OH$^+$. The problem is explicable if the latter species absorbs a photon from a successive laser pulse to create an excited state capable of internal proton transfer. However, there may well be equally valid alternatives to this explanation.



$T^{\bullet -}$ X TH• XI

Comparison of Photoionisation with the Effects of Ionising Radiation.—One of the main unresolved problems associated with radiation damage is concerned with tracks and spurs, where, especially for spurs, there may be several local sites damaged in one event. Also, electrons are ejected from a range of orbitals depending on the energy of the high-energy electron at impact. For the former, EPR spectroscopy has, as yet, been of little help. In rare cases, pair trapping of radicals has been observed[59] but never for DNA systems. Thus it is difficult to see how these phenomena can be responsible for the major differences observed between the experimental EPR results and the track and spur models of radiation damage. However, the latter differences may well be significant. Thus, population of the specific n(C=O) level postulated herein will be a rare event so that the initial formation of the $TCH_2^{\bullet}$ centre is also improbable.

Perhaps the most striking contrast between the two modes of damage is the fact that in frozen aqueous solutions, in particular D_2O, the radical anion appears to dominate the γ-irradiated samples at 130 K, whereas for laser photolysis, the $TCH_2^{\bullet}$ radical is present in far greater abundance. The question that arises is what has happened to the hole-centres in the radiolysis and what has happened to the electrons in the photolysis? At present, the EPR spectra alone do not seem to provide clear answers. For the thymine derivatives, base stacking may be important, but it is still difficult to understand the contrasting behaviour between radiolysis and photoionisation.

Acknowledgements

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