The Effects of Ionising Radiation on Frozen Aqueous RNA: an Electron Paramagnetic Resonance Study

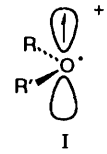
Philip J. Boon, Aidan O'Connell, Ian D. Podmore and Martyn C. R. Symons*
Department of Chemistry, The University, Leicester, UK LE1 7RH

Exposure of frozen aqueous solutions of various RNA samples to ^{60}Co γ-rays at 77 K gives well defined parallel components ($g_z = 2.077$) which are assigned to alkoxyl radicals (RO•) formed from the ribose units. The formation of these radicals, presumably by electron loss from ROH units followed by very rapid proton transfer to water molecules hydrogen bonded to the –OH group, establishes that electron loss from the sugar moieties is significant. No primary sugar radicals have been detected for DNA irradiated under the same conditions. This has been explained in terms of rapid electron-donation from proximal bases (*i.e.*, positive 'hole' transfer to a neighbouring base) which must also occur for RNA. For the latter, however, proton-transfer is able to compete with electron-transfer.

The major electron-gain and -loss centres have combined EPR spectra that closely resemble that for DNA. Loss is confined to the purine bases and gain to the pyrimidine bases. Unfortunately, addition of an electron to cytosine, thymine and uracil in these polymeric systems generates remarkably similar EPR doublet spectra, and it is, in our view, extremely difficult to make an accurate estimate of the relative yields of these centres by computer synthesis. In the case of DNA, the one-electron adduct of thymine is converted into the 5,6-dihydrothymine radical (•TH) on warming to *ca.* 200 K, i.e., protonation at the C6 site of the pyrimidine ring. Spectra for the analogous 5,6-dihydrouracil radical (•UH) have been recorded, but these features are completely absent on annealing irradiated frozen aqueous RNA systems. Possible reasons for this contrast are discussed.

One of the compounds most widely studied by radio-chemists and biologists is DNA.[1–3] EPR spectroscopy has helped to shed light on aspects of primary damage and on some of the initial reactions, but it seems that many of the expected initial processes are too fast to be detected by this method even at 4 K. At the molecular level, for small molecules, the initial steps can be viewed as electron-ejection from molecular orbitals of the whole molecule, and a range of levels are involved, depending on the energies on the high energy incident electrons. However, for large polymers such as DNA polyanions, it is reasonable to consider localised loss from all parts of the ion. Thus, we envisage loss from the phosphate ions to give $(RO)_2PO_2^•$ radicals, from the deoxyribose units, giving in particular $ROR^{+•}$ type radicals, as well as loss from any of the four bases.[4] However, the only centres detected by EPR spectroscopy are base ions, electron-loss being mainly from guanine and possibly adenine. The same considerations are thought to apply to irradiation of DNA with low energy electrons. These might be expected to react with phosphate centres to give a range of products such as phosphoranyl radical-anions, and phosphoryl radicals or alkyl radicals by dissociative electron capture. Indeed, we have recently shown that α-glucose 6-phosphate reacts at low temperatures to give all these products.[5] However, no such centres are detected for irradiated DNA, even at 4 K. The only detectable electron-gain centres are found on the pyrimidine bases.[6–9] Again, therefore, it is necessary to postulate rapid electron transfer to the bases. This is so rapid that the shape change stabilising phosphoranyl radicals and the bond-breaking that gives the other products are apparently slower than intramolecular electron transfer. It was originally supposed that electrons and 'holes' (electron-loss centres) could move freely along the DNA strands, and hence that recombination would be efficient.[10] However, it is now realised that the hole-centres are trapped by proton-loss and the electron-gain centres by proton-gain.[6–9,11,12] Once these reactions have occurred the centres are fixed and the resultant species detected by EPR spectroscopy.

Another area of extensive study by EPR spectroscopists has been radiation damage to DNA-base nucleosides and nucleotides. A major difference, compared with DNA, is that primary sugar radicals are detected in these systems. Following the extensive work of Box and co-workers,[13] many workers have established that a major electron-loss centre in these compounds is the alkoxyl radical (RO•).[14–16] Studies of simple alcohols confirmed these assignments.[17,18] These radicals must be formed from primary $ROH^{+•}$ centres by proton-loss. Thus, in these systems, proton-transfer can compete with electron-transfer from the bases. We stress that electron-loss to form $ROH^{+•}$ cations is no more favourable than loss to form $ROR^{+•}$ cations (**I**). However, these cannot be rapidly stabilised in any



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way, and hence highly favourable electron-transfer from any proximal base occurs efficiently. However, the –OH protons of sugar –OH groups will always be strongly hydrogen bonded to adjacent water molecules,[19] and hence proton-transfer from the very strongly acidic $ROH^{+•}$ radical can be extremely fast and can compete efficiently with electron-transfer.

RNA polyanions have not been studied in this way by EPR spectroscopy, so far as we know. A liquid phase study, using aminoxyl-forming spin-traps has been reported,[20] but this does not give direct information about primary processes. RNA differs from DNA in three major respects. One is that it is single- rather than double-stranded, with a tertiary, folded conformation. Since our studies are of highly localised radicals, this

Table 1 EPR parameters for various electron-gain centres formed on cytosine, thymine and uracil (for cytosine *N*-protonation can take place at two different sites. For RNA and DNA this is believed to occur at N^3, however, no EPR data are available)

Compound	Matrix	*T*/K	Radical centre	A_{iso} (C6)	A_{iso} (Other)	g_{iso}
Thymidine[a]	Single Crystal–D_2O	4.2	$T^{-\bullet}(D^+)$	11.7	—	—
Thymidine[b]	Single Crystal–H_2O	10	$T^{-\bullet}(H^+)$	11.8	11.8	—
Thymidine[c]	12 mol dm^{-3} LiCl–D_2O	4	$T^{-\bullet}$ or $T^{-\bullet}(H^+)$	12.4	—	2.0030
Thymidine 5′-monophosphate[d]	10 mol dm^{-3} LiCl–H_2O	77	$T^{-\bullet}$ or $T^{-\bullet}(H^+)$	11.8	—	—
DNA[e]	Fibres	77	$T^{-\bullet}$ or $T^{-\bullet}(H^+)$	13.6	3.9	2.0033
Cytidine[f]	10 mol dm^{-3} LiCi–H_2O	77	$C^{-\bullet}(H^+)$	12.9	11.3	—
Cytidine 5′-monophosphate[f]	10 mol dm^{-3} LiCl–H_2O	77	$C^{-\bullet}(H^+)$	13.1	11.5	—
Uridine[d]	10 mol dm^{-3} LiCl–H_2O	77	$U^{-\bullet}(H^+)$	12.1	—	—
Uridine 5′-monophosphate[d]	10 mol dm^{-3} LiCl–H_2O	77	$U^{-\bullet}(H^+)$	12.3	—	—

[a] Ref. 14. [b] Ref. 30. [c] Ref. 31. [d] Ref. 32. [e] Ref. 33. [f] Ref. 34.

Table 2 EPR parameters for various alkoxyl radicals, RO•

Alkoxyl radical (RO•)	*T*/K	g_z	*A*
RNA[a]	10	2.077	—
$RCH_2O^{\bullet}$ (Thymidine)[b]	4.2	2.083	71, 80
$RCH_2O^{\bullet}$ (Thymidine)[b]	77	2.102	42, 85
$RCH_2O^{\bullet}$ (Thymidine)[c]	4.2	2.083	71, 80
$(CD_3O^{\bullet})$[c]	4.2	2.064	—
$(CH_3O^{\bullet})$[d]	4.2	2.088	52
$(Me_3CO^{\bullet})$[c]	4.2	2.110	—
$(C_6H_{11}O^{\bullet})$[c]	4.2	2.087	70

[a] Present study. [b] Ref. 18. [c] Ref. 14. [d] Ref. 17.

probably has little significance in the present work. Another is that it uses ribose rather than 2-deoxyribose used in DNA. The third is that the thymine bases are replaced by uracil, the other three bases remaining the same. The major aim of the present study was to see if we could detect specific electron-loss from the sugar units. The other aim was to see if there were any major differences in the reactions of the bases when uracil replaces thymine.

Experimental

Ribonucleic acid (calf liver), uridine and cytidine were obtained from Sigma. NaCl was purchased from Aldrich, and D_2O from Goss Scientific Instruments. These materials were used without further purification. H_2O was purified using a Millipore 'Multi-Q' system.

Procedure.—Two media were employed for this investigation; frozen aqueous 5.5 mol dm^{-3} sodium chloride and frozen aqueous solutions. The sodium chloride solutions were made up using a uridine concentration of 100 mmol dm^{-3} and an RNA concentration of 25 mg cm^{-3}. Beads were formed by allowing droplets of a given solution to freeze in liquid nitrogen. Frozen aqueous samples of uridine (0.25 mol dm^{-3}), RNA (100 mg cm^{-3}) and DNA (50 mg cm^{-3}) were prepared as cylindrical pellets by freezing *ca.* 0.3 cm^3 of the solution in a Pyrex tube.

Samples were irradiated either at 77 K using a Vickrad ^{60}Co γ-ray source or at 4 K with an X-ray source; irradiation being carried out to a dose of 1 Mrad for the sodium chloride solutions and 3 Mrads for the frozen aqueous systems. EPR spectra were recorded at 77 K on a JEOL JES-RE1Z X-band spectrometer, interfaced with an Archimedes computer.

Samples were annealed either by decanting the coolant from the insert Dewar and recooling when significant spectral changes were detected, or by the use of a copper-block cryostat. In the latter the samples were allowed to warm to a predetermined temperature which was maintained for 7 min before being recooled to 77 K.

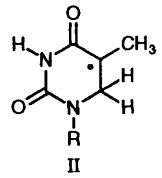
One problem continually experienced in these studies of RNA has been the appearance of an asymmetric singlet ($g_{\parallel}$ = 2.033, $g_{\perp}$ *ca.* 2.002) which grew in at *ca.* 200 K and thus obscured the spectra. This is identified as the $RO_2^{\bullet}$ radical, formed from reactive DNA centres. All systems were deoxygenated, using oxygen-free nitrogen gas which was further purged of O_2. These precautions have always removed all trace of $RO_2^{\bullet}$ signals in our other studies using frozen aqueous or glassy systems, including studies of irradiated DNA. It seems that RNA anions are somehow able to occlude molecular oxygen, and we hope to study this effect in the future.

Results and Discussion

These are shown in Figs. 1–5 and the EPR parameters are summarised in Tables 1 and 2, together with results from other systems.

Base-centred Radicals.—At low microwave powers the g = 2 region of these spectra closely resembled those for DNA in the same media, as shown in Fig. 1. This means that the hole-centres remain primarily $G^{+\bullet}/A^{+\bullet}$. A rough estimate shows that the contribution from RO• centres is *ca.* 5% of the total, which would have no detectable affect on the $G^{+\bullet}$ yield. We mentioned that the hole centres are usually referred to as $G^{+\bullet}$. Unfortunately, the spectra for $A^{+\bullet}$ centres are quite similar, so it is difficult to prove the extent to which $A^{+\bullet}$ may contribute. Sevilla and co-workers have attempted to differentiate between the two, using 'bench-mark' spectra and careful computer simulation. They suggest that, of the total radical yield, there is >45% $G^{+\bullet}$ and <5% $A^{+\bullet}$ in irradiated frozen aqueous double-stranded DNA, and 26–28% $G^{+\bullet}$ and 8–17% $A^{+\bullet}$ in irradiated frozen aqueous single-stranded DNA.[8,21] We have shown that, at least for DNA strand breaks, there is no detectable preference for one base site over another,[22] which may also point to the formation of $A^{+\bullet}$ as well as $G^{+\bullet}$.

The same problem arises for electron-capture centres. That $T^{-\bullet}$ is formed in irradiated DNA is clearly established because it is converted into •TH radicals (**II**) by protonation at C6 on warming to *ca.* 200 K. This gives a very characteristic octet EPR



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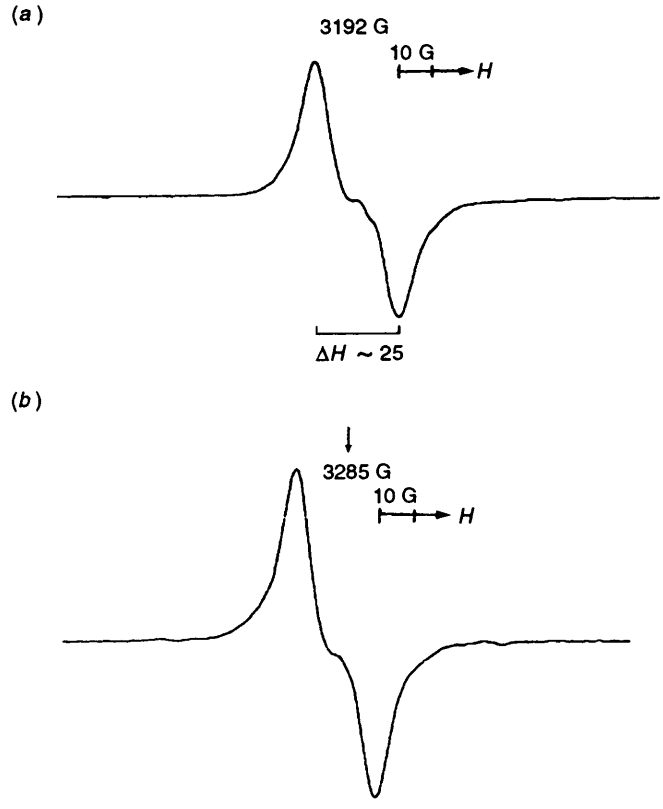


Fig. 1 First derivative X-band EPR spectra for frozen aqueous solutions of (*a*) RNA and (*b*) DNA, after exposure to ^{60}Co γ-rays at 77 K, and annealing to 130 K to remove features from 'OH radicals trapped in the ice. These broad features are the superimposition of a singlet from $G^{+\bullet} + A^{+\bullet}$, and a doublet (*ca.* 16 G) from $C^{-\bullet}$ and $T^{-\bullet}$ for DNA (or $C^{-\bullet}$ and $U^{-\bullet}$ for RNA).

spectrum. We have analysed this system on the assumption that most of the $T^{-\bullet}$ reacts to form 'TH, the results showing that at least 36% of the anion radicals must be $T^{-\bullet}$.[9] Hüttermann and co-workers have also used 'TH as a measure of $T^{-\bullet}$.[6] However, both Sevilla and co-workers[8,21] and Bernhard and co-workers[7] claim to be able to analyse the doublet obtained from DNA in terms of two doublets; one for $C^{-\bullet}$ and one for $T^{-\bullet}$. They conclude that $C^{-\bullet}$ dominates very strongly. The former assigned the $C^{-\bullet}$ contribution in irradiated frozen aqueous double-stranded DNA as *ca.* 77%, and the remainder to $T^{-\bullet}$. However, it is difficult to understand these results in terms of the relative high yields of 'TH formed on annealing.[9]

In the present case the same problem arises. The spectrum for $U^{-\bullet}$, shown in Fig. 2, is very similar to that for $C^{-\bullet}$ when formed in DNA or oligomers. For reasons given elsewhere,[9] we are of the opinion that environmental effects can lead to small modifications of both spectra that are as large as the differences between them, and hence that computer analysis is unreliable. As can be seen from Fig. 2, the doublet assigned to pyrimidine anions found in RNA is quite well defined when frozen aqueous sodium chloride solvents are used. This is thought to be because, on freezing, there is an extensive glassy region surrounding the RNA ions that is rich in NaCl. [We were unable to use the 10 mol dm^{-3} LiCl matrix, favoured for isolating DNA radical anion spectra,[9] because RNA was not sufficiently soluble.] On radiolysis, electrons formed in this glassy region have a high probability of reaching the polymer to give $C^{-\bullet}/U^{-\bullet}$, whereas the holes are captured as Cl$^{\bullet}$ bound to water or to Cl^- ions ($ClOH^{-\bullet}$ or $Cl_2^{-\bullet}$, respectively) and remain inactive at low temperatures. The doublet obtained in this way from RNA is less well resolved than that obtained by adding the individual $C^{-\bullet}$ and $U^{-\bullet}$ doublets together in any proportion. Thus it seems

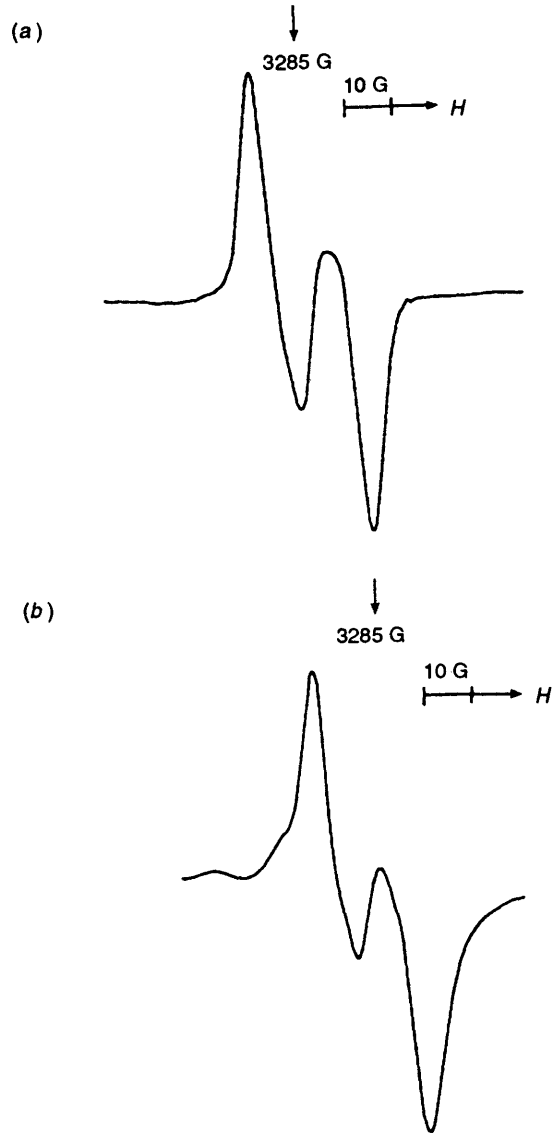
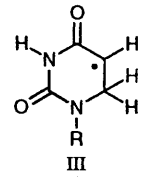


Fig. 2 First derivative X-band EPR spectra for solutions of (*a*) uridine and (*b*) RNA in 5.5 mol dm^{-3} NaCl–D_2O solvent after exposure to ^{60}Co γ-rays at 77 K, showing characteristic doublets assigned to (*a*) $U^{-\bullet}$ and (*b*) RNA containing $C^{-\bullet}$ and/or $U^{-\bullet}$

that there is a third, minor component which is probably the broad singlet due to $G^{+\bullet}$ centres. In this respect RNA differs from DNA, which is almost completely free of radical cations under these circumstances and gives a better resolved doublet.

Since we are unable to estimate the amount of $U^{-\bullet}$ formed from these spectra, we have made a thorough search for any conversion into 'UH on careful annealing. The spectra for 'UH (III), obtained from uridine in 5.5 mol dm^{-3} NaCl/H_2O is shown in Fig. 3. However, these well defined features were never



III

detected in either the frozen aqueous or frozen sodium chloride systems containing RNA. This may mean that for RNA, $C^{-\bullet}$ completely dominates. Unfortunately, the secondary radicals formed from $C^{-\bullet}$ are so unstable that they never accumulate to detectable concentrations in our systems, so there is no method

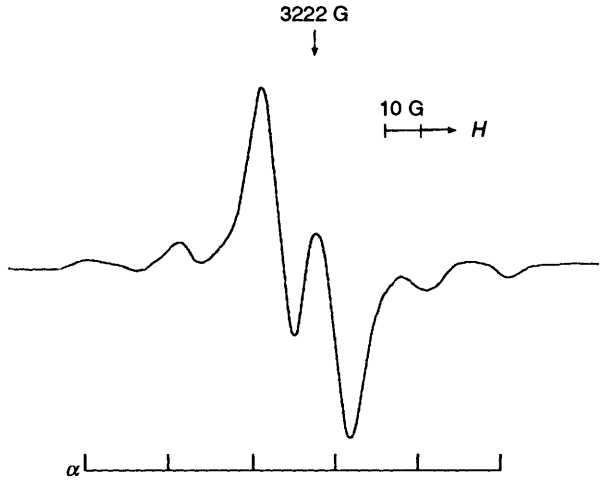


Fig. 3 First derivative X-band EPR spectrum for uridine in 5.5 mol dm^{-3} NaCl–H_2O after exposure to ^{60}Co γ-rays at 77 K and warmed to 183 K, showing features α assigned to •UH radicals

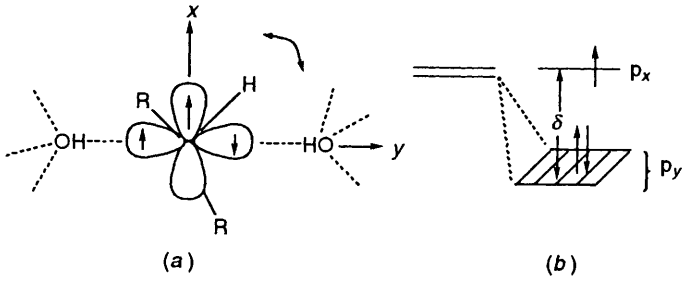


Fig. 4 This shows the degenerate p_x and p_y orbitals on the oxygen of RO• radicals, and the way in which solvation can fix these axes (*a*) and induce a splitting, δ, of the p_x and p_y orbitals (*b*). As δ increases, so g_z shifts towards the free-spin *g*-value (2.0023). The double-headed arrow in *a* indicates variability in the directions of the orbital axes relative to the molecular frame.

for estimating $C^{-\bullet}$ yields other than from the primary doublet. It seems surprising that $U^{-\bullet}$ should not form since $T^{-\bullet}$ is formed extensively in DNA. The two key parameters involved are the relative electron affinities, which should be quite comparable, and the proton affinities, which should also be comparable. In our view, protonation of $U^{-\bullet}$ or $T^{-\bullet}$ must initially occur at one of the carbonyl groups.[9] This depends on the presence of hydrogen-bonded water which should be at least as prevalent for uracil as it is for thymine. Thus, it is difficult to see why $U^{-\bullet}$ should fail to form in RNA given that $T^{-\bullet}$ forms in DNA.

Another possibility is that •UH is less readily formed than •TH. It is well established that •TH decays rapidly in frozen aqueous systems in the 200–240 K temperature range. We have suggested that this reaction involves transfer of hydrogen atoms from nearby C–H units of deoxyribose, and that this is an important route to strand-breaks.[8,23] It may be that this reaction is more facile for •UH, and hence that these radicals fail to accumulate sufficiently for EPR detection. Unfortunately, our results using the present techniques are unable to distinguish between these various possibilities.

Alkoxyl Radicals (RO•).—The difficulty associated with alkoxyl radicals (RO•) is that the p(π) orbitals on oxygen are almost degenerate and it is not possible to select specific *x* and *y* axes (the C–O direction is taken as *z*). As with •OH radicals,[24] or $O_2^{-\bullet}$ ions,[25] the axes are thought to be controlled by solvation, and well defined spectra are obtained only if solvation is well defined. Otherwise extremely broad features are obtained especially in the parallel region.[26,27]

For RO• radicals the situation is depicted pictorially in Fig. 4. Before electron-loss, we expect that both oxygen and hydrogen will be involved in hydrogen bonding to water. On electron loss, the O–H proton moves a short distance on to the H-bonded water oxygen, but is expected to remain strongly H-bonded to RO•. The other H-bond is expected to remain intact, as shown in Fig. 4. In our solvation model, these hydrogen bonds are responsible for lifting the degeneracy of the oxygen 2p(π) orbitals, and hence define the *x* and *y* axes. The filled orbital (p_y) is involved in hydrogen bonding, whilst the half-filled orbital is perpendicular to this, as shown in Fig. 4. We suggest that the orbital splitting, δ, is caused largely by hydrogen-bonding, and controls the shifts in g_z. Given that in these glassy systems, δ will vary considerably from centre to centre, g_z will also vary, giving a broad spread of g_z values.

There is only one β proton for this radical but the magnitude of its hyperfine interaction is again controlled by the hydrogen bonding. This is because it varies with $\cos^2\theta$, where θ is the angle between the plane containing the H–C–O unit and the *x*-axis which defines the $2p_x^1$ orbital. To a first approximation $A(^1H) = A^\circ \cos^2\theta$, A° being the maximum possible coupling.[28] Using the hyperfine splitting for the methyl protons of $H_3CO^\bullet$ radicals as a gauge,[17] A° is approximately 100 G. The minimum value is close to zero. The values obtained for RNA in a glassy medium could be expected to vary considerably, as the tilt of the *x*-axis changes.

At high microwave powers and low temperatures (4–20 K), frozen aqueous RNA gave a broad 'parallel'-like feature at $g = 2.077$ (Fig. 5). This feature was lost when the temperature was raised to 77 K. There is no sign of ^1H hyperfine coupling, and from the line-width we can say that $A(^1H) \leqslant 60$ G. Probably there is a distribution of *A* values, as discussed above. Nevertheless, the g_z-value is close to those for well authenticated RO• spectra, such as that for thymidine (see Table 2). Unfortunately, the maximum splitting for the β-proton is too small for us to detect perpendicular features, even if these were quite narrow, since they would be completely concealed by the central features. Thus, the results accord well with expectation, and we conclude that the sugar –OH group in RNA undergoes electron- followed by proton-loss to give RO• radicals, which are trapped at very low temperatures.

The well defined parallel feature was lost irreversibly at 40–50 K, but at 70 K, at high gain, modulation and power, a very broad, flat-topped feature covering *ca.* 120 G and centred approximately at $g_z = 2.1$ was obtained. Also, a relatively narrow pair of lines grew in on either side of the main features separated by *ca.* 120 G and centred on $g = 1.999$ [Fig. 5(*b*)]. These features are so weak that they may simply stem from impurities, but they do appear to be related. The doublet is not the spectrum for HCO• radicals, which could be formed from methanol impurities, for example, since the characteristic parallel and perpendicular splitting of the high field $(-\frac{1}{2})$[29] line is not seen, this feature being narrow and symmetrical, as expected for an RO• centre. In fact, these features could be a strongly broadened version of the four-line spectra found for RO• in thymidine.[18] Since RNA strands are relatively short, these could be formed from 5′ and 3′ end groups. Another possibility is that the *x* and *y* axes for the 2′-RO• species formed at low temperatures have changed, so that maximum C–H overlap is achieved with the px^1 orbital. [$A^\circ \approx 100$ G is implicated from results for $H_3CO^\bullet$ radicals, but 120 G is certainly not impossible even though it is surprisingly large.] So we consider reasons why this might possibly occur.

A factor that might influence the direction of *x* is the σ–π overlap that is largely responsible for the unusually large value for A°. This arises because of the high electron affinity of the oxygen atom, which draws electrons from the C–H bond, leaving high spin-density on hydrogen. Most EPR results suggest that the hyperconjugation energy for C–H bonds is not

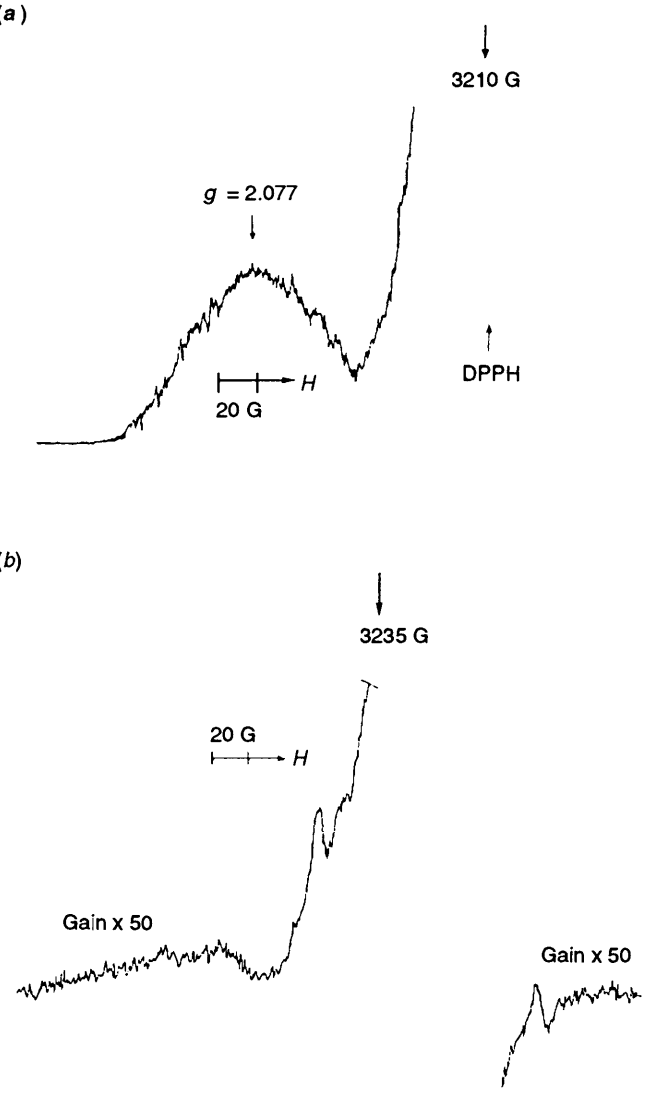


Fig. 5 First derivative X-band EPR spectra for a solution of RNA in water: (*a*) after X-irradiation at 4 K, and annealing to 10 K. This shows the g_z = 2.077 feature assigned to 2'-RO' radicals. The expected doublet splitting has been lost in the line-width, or there is a distribution of $A(^1H)$ coupling constants. (*b*) After irradiation at 77 K, showing very broad low-field features plus the 120 G doublet discussed in the text.

much greater than those for C–C bonds. If they were equal there would be no preference, and results for other RO' radicals, given in Table 2, seem to show that this is not the controlling factor, since the ^1H splittings can be large or small. Nevertheless, if, on annealing, the hydrogen bonds tend to weaken or be lost, the hyperconjugation difference might come into play. This would result in an increase in $A(^1H)$ and also in g_z, as observed.

We conclude that these results prove that electron-loss from the ribose units does occur, but that the hole-centre is only trapped if proton-loss is extremely rapid. Otherwise, as postulated for DNA, electron transfer from the nearest base is very efficient and no primary sugar radicals are detectable.

Acknowledgements

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