Radiation Effects on 5-Bromo-5,6-dihydrothymine and Related Compounds: an Electron Spin Resonance Study

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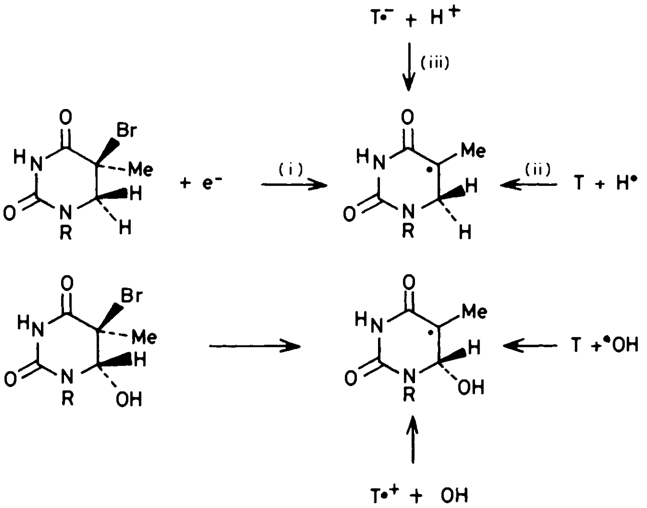
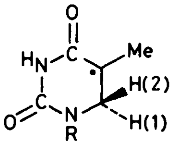
Exposure of 5-bromo-5,6-dihydrothymine, 5-bromo-6-hydroxy-5,6-dihydrothymine, and the two corresponding nucleoside derivatives to ^{60}Co γ-rays at 77 K gave paramagnetic centres identified as the 5-yl radicals formed by dissociative electron capture with concurrent formation of bromide ions. In some cases, electron loss centres formed by loss of a C-6 proton from the parent cations were also detected. The 5-yl radicals are of interest since they are related to radicals which may be formed in the radiolysis of DNA. For the hydroxy derivatives, the evidence suggests that they are initially formed in a conformation such that the C-6 proton is constrained to lie close to the radical plane, resulting in unusually small proton hyperfine coupling constants. This result has significant implications for the use of standard e.s.r. parameters in fingerprinting' the presence of such radicals.

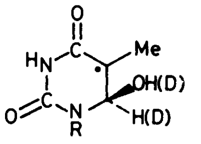
Besides its specific chemical interest, a study of the radiolysis of brominated thymine derivatives is of some biochemical significance, because it provides alternative routes to intermediates and products relevant to the interaction of radiation with DNA. In the present work, we are concerned with primary ionic and radical processes. This will eventually be linked with quantitative studies of overall reaction products. For 5-bromo derivatives, bromine is selectively lost on electron addition, the resulting radicals being just those that are frequently invoked in interpretations of the radiolysis of DNA. Indeed, for 5-bromo-5,6-dihydrothymine, the resulting radical is the well-known species formed by the addition of H$^{\bullet}$ (or e$^-$ + H$^+$) to the thymine base in DNA (Scheme).



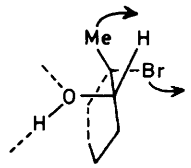
Scheme.

The e.s.r. results are also of interest in giving information about various different conformers of the radicals studied and about their decay profile as a function of temperature. Thus, in our view, the major significance of this study is that the number of primary radical centres is limited to two and hence the task of unravelling the mechanism of product formation is less


(I)


(II)

R = H, or deoxyribose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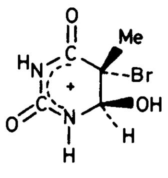
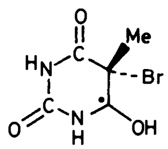
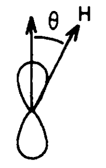
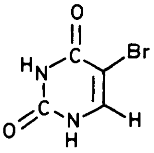
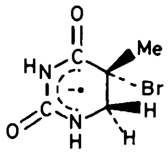

(VI)


(Va)


(IV)


(VII)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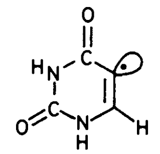
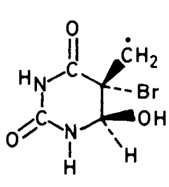

(IX)


(Vb)

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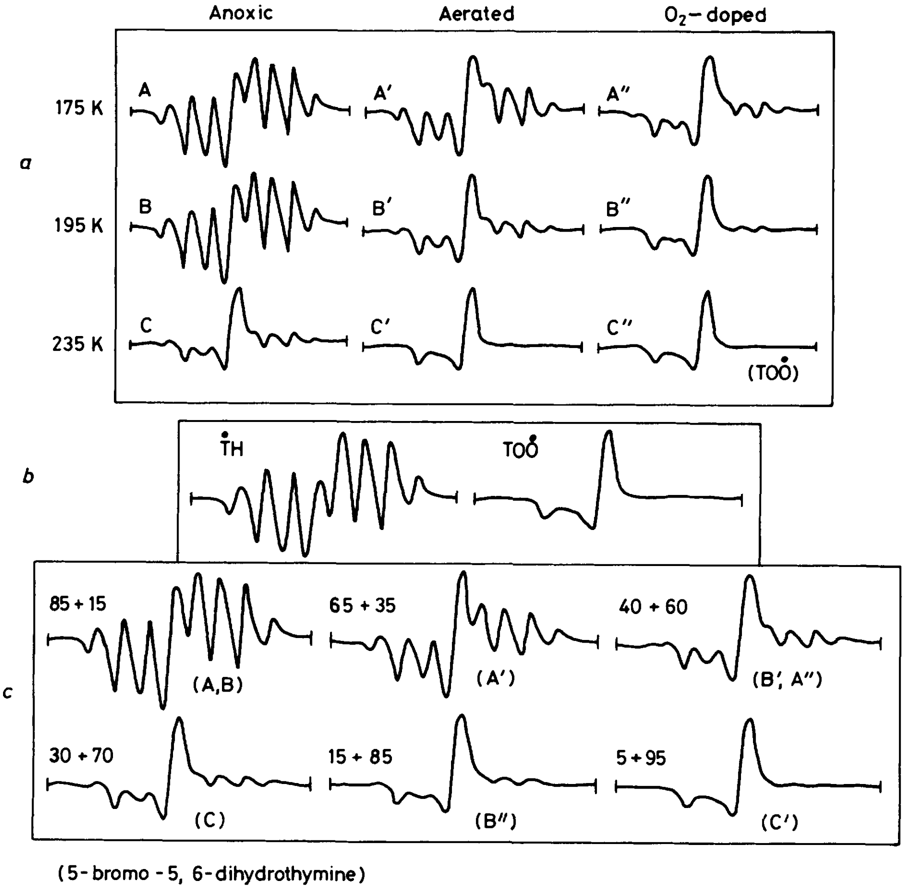


Figure 1. Various first-derivative *X*-band e.s.r. spectra for aqueous solutions of 5-bromo-5,6-dihydrothymine after exposure to ^{60}Co γ-rays at 77 K, annealing for 60 s at the temperatures indicated and recooling to 77 K for measurement. *a* Experimental spectra in the absence of oxygen, in normal aerated solutions and in oxygenated solutions ((I) (TH$^{\cdot}$) and $TO_2^{\cdot}$). *b* Pure spectra for the two major radicals. *c* Reconstruction of some of these spectra, using the percentages of (I) and $TO_2^{\cdot}$ indicated

formidable than is frequently the case. This largely removes the usual ambiguities encountered in systems for which there is a wider range of radicals and of products.

Previous studies of 5-brominated 5,6-dihydrothymine derivatives include the important work of Sevilla and Engelhardt[1] who studied electron attachment to 5-bromo-6-hydroxy-5,6-dihydrothymidine dissolved in 8 mol dm^{-3} NaOD (D_2O) and in 12 mol dm^{-3} LiCl (D_2O) glasses. They observed loss of bromide ion with the formation of radical (II). Gregoli and co-workers[2] and also Sevilla and Engelhardt[1] have postulated the formation of (II) by OH^- addition to the radical-cation of thymine in aqueous systems and Riederer and Hüttermann have detected the same species in aqueous sulphuric acid solutions.[3]

Results

These are summarised in the Figures, giving representative real and computer-constructed spectra selected from many hundreds recorded during the progress of this work.

(i) *Central Spectral Region.—5-Bromo-5,6-dihydrothymine.* All spectra obtained from this derivative were satisfactorily analysed in terms of two species, the thymine radical (I) giving the well-known eight-line spectrum and a peroxyl radical, having three characteristic *g*-values (Figure 1). The spectra at 77 K are grossly overlapped by $^{\cdot}OH$ radical features but these centres, formed in ice crystallites, are irreversibly lost at *ca.* 130 K, so our analyses all refer to temperatures above this.

The decay of the individual radicals and the radical yields as a function of temperature are shown in Figure 2. We stress that these are the results of 'pulse' heating, as described in the Experimental section, rather than being a set of results obtained at the indicated temperatures. Note that the total concentration of radicals in the central region in the presence of oxygen is actually slightly greater than that in its absence. This suggests that some of the electron-loss centres, which do not make a significant contribution to this spectral region, are also converted into $RO_2^{\cdot}$ radicals.

The temperature profiles in Figure 2 (and in Figure 4) show that there is a definite conversion of radicals (I) and (II) into $RO_2^{\cdot}$ type radicals on adding oxygen. This is almost certainly *via* addition to C-5. Their formation is largely quenched when oxygen is removed, but our inability to remove all traces (Figure 2*a*) illustrates the difficulties involved in deoxygenation procedures. When the concentration of dioxygen is high, conversion into $RO_2^{\cdot}$ is more efficient. Probably the reaction rate is diffusion controlled under these conditions.

All radicals are lost as the medium in the vicinity of the radicals becomes mobile (*ca.* 235 K). We were unable to detect the formation of any other radicals, so they must be lost at rates greater than their rates of formation, so that only low stationary concentrations are obtained. Some mechanistic aspects of the reactions of these radicals will be presented in a later paper.

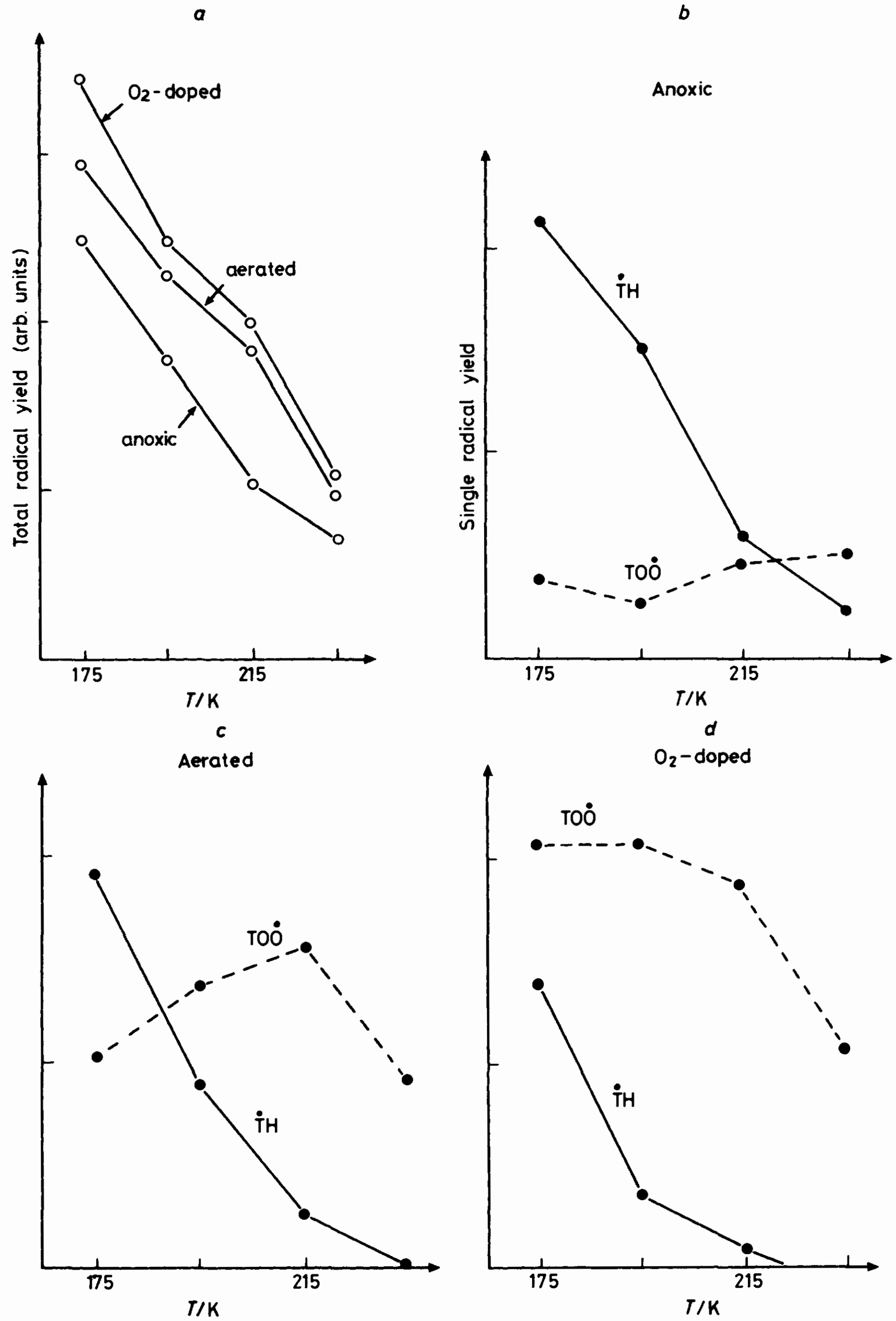


Figure 2. Trends in radical yields as a function of temperature for irradiated aqueous solutions of 5-bromo-6-hydroxy-5,6-dihydrothymine. *a* Total radical concentrations. *b* Yields of TH' and TO_2' in deoxygenated systems. *c* Yields in normal aerated systems. *d* Yields in solutions saturated with oxygen

5-*Bromo*-6-*hydroxy*-5,6-*dihydro-thymine and -thymidine*.—The results (Figures 3 and 4) were similar. However, on annealing, there were marked irreversible changes in the e.s.r. spectra. As discussed below, we nevertheless postulate contributions from only two radicals in this spectral region, type (**II**) and RO_2. We have computed a series of spectra having a fixed coupling to the methyl protons (22.5 G) and a coupling for the C-6–H proton varying from 3—20 G. All the observed spectra for (**II**) were completely accommodated within this range of values, there being a steady increase in the latter coupling as the anneal temperature increased. We stress that, had we not recognised this possibility and subtracted features assigned to radical (**II**) using literature data,[1-3] a new set of lines would have been obtained which would be completely spurious. This is a real danger in the task of computer analysis of spectra, especially when subtraction procedures are used. Indeed, the same problem arises even with the spectra for radical (**I**), since the parameters are temperature- and medium-dependent and, in particular, the two methylene protons are not always equivalent. We have found that subtraction of spectra based on literature data can reveal apparently quite significant residual features which, in reality, are pure artefacts. In our view, the proper procedure is to discover parameters for a single species which leave no such residues on subtraction. If this proves to be possible, then only one species is present.

Results for the thymidine derivatives, showing only an apparent quartet splitting rather than the expected quintet[1-3] (Figure 3), were so surprising to us that we studied the C-6 deuterio analogues both as the OH and as the OD derivative. [Both *trans* diastereoisomers of 5-bromo-6-hydroxy-5,6-dihydrothymidine (5*R*,6*R*) and (5*S*,6*S*) were used: the results were identical.] The latter substitution simply reduced the line width but the former gave a quartet (Figure 3*b*) which no longer required an extra 6 G splitting for good simulation. We conclude firmly that this 6 G splitting stems from the C-6 proton and that the radical is indeed (**II**), with a remarkably constrained conformation (see below).

We also studied the thymine derivative for further confirmation. This gave spectra at 175 K with clearly resolved coupling to the C-6 proton (10 G), and this increased to *ca.* 11.5 G at 215 K. However, at 235 K the residual radical spectrum included a remarkably narrow line quartet, the coupling to the C-6 proton being only *ca.* 3 G, as estimated by computer synthesis. The decay profile for these radicals is indicated in Figure 4.

(ii) *Outer Spectra Regions.—Bromine-containing radicals.*

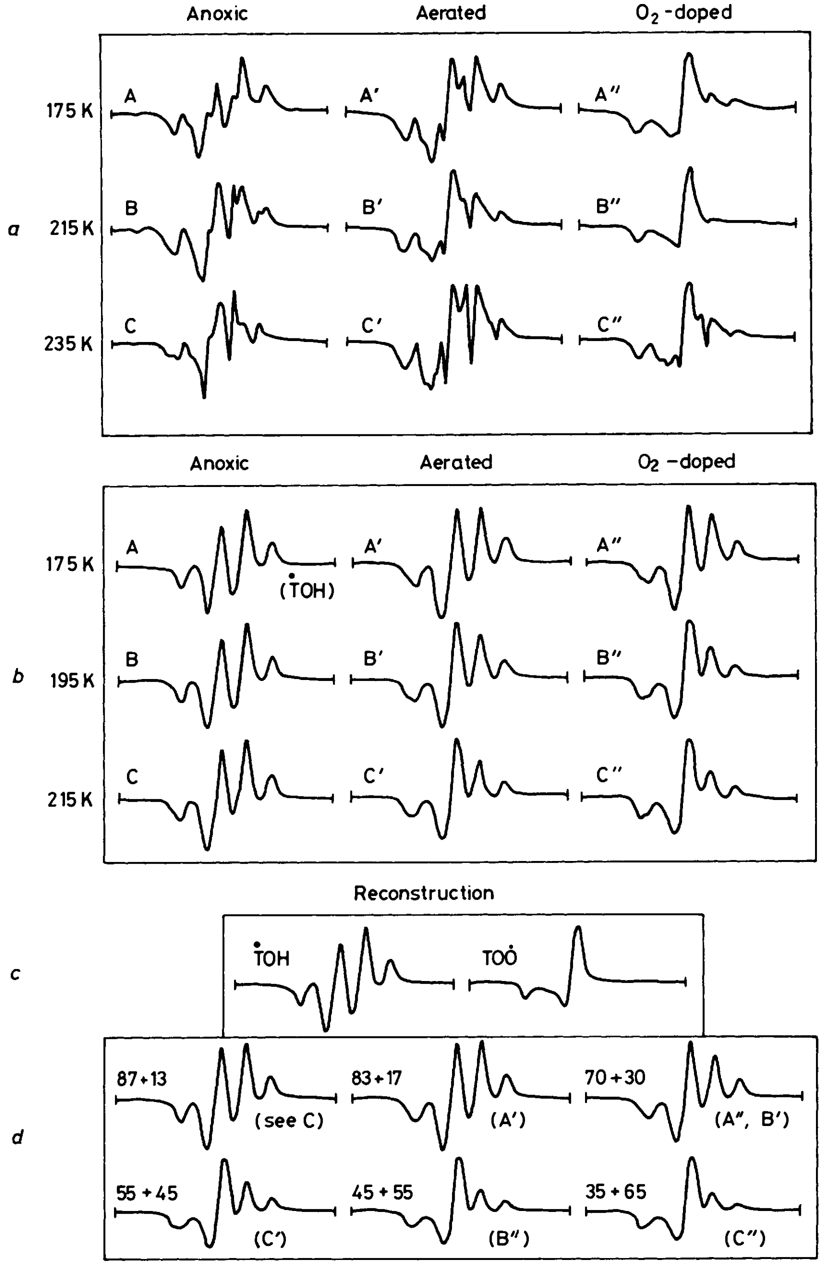
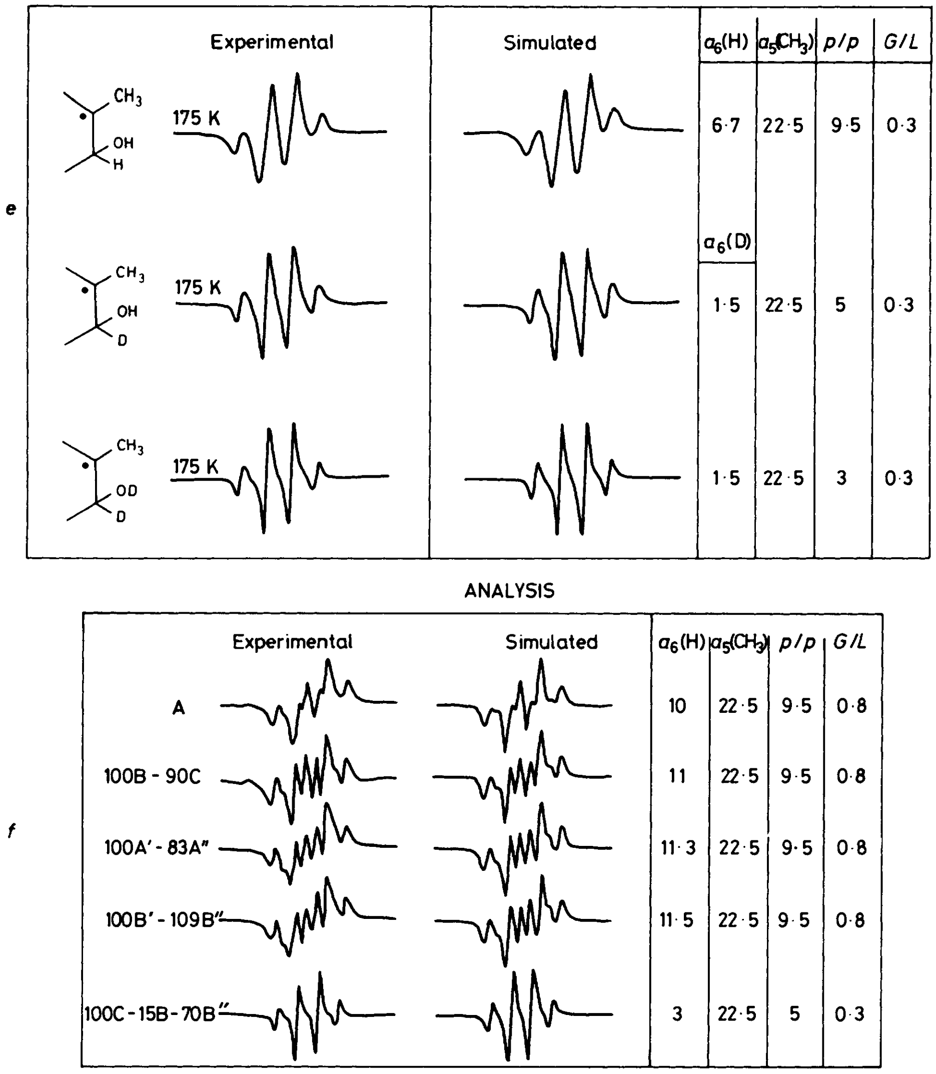


Figure 3. First-derivative *x*-band e.s.r. spectra for aqueous solutions of 5-bromo-6-hydroxy-5,6-dihydro-thymine and -thymidine after exposure to ^{60}Co γ-rays at 77 K, annealing, for 60 sec, to the temperatures indicated, and recooling to 77 K for measurement. *a* Experimental spectra for the thymine derivative. *b* For the thymidine derivative. Some reconstructed spectra using those for radical (**II**) ($^{\bullet}$TOH) and $TO_2^{\bullet}$ shown in *c* for the thymidine derivative are shown in *d*. In *e*, the effect of selective deuteriation is indicated for radical (**II**), together with simulated spectra using the data indicated. Variations in the form of the spectra for (**II**) derived from the thymine derivative are indicated in *f*. Simulated spectra are also given together with the hyperfine coupling constants required



(5-bromo-6-hydroxy-5,6-dihydrothymine)

Figure 3 (*continued*)

Because of mechanistic expectations (see below), we also looked for radicals having large hyperfine coupling to bromine nuclei. These are not detected in the normal spectra at low gain, low power, and low modulation and hence can be readily missed. In all cases, there was some evidence for extra features in the outer regions, but the degree of resolution was only good for the 6-hydroxy derivatives (Figure 5). Even in this case, the lines are very broad compared with the central features. This is, unfortunately, almost always the case for powder spectra of radicals having large *g*- and *A*-anisotropy.

Discussion

Nature of Frozen Solutions.—In contrast with the glasses used, for example, by Sevilla and Engelhardt,[1] the aqueous solutions used herein undergo phase-separation on freezing. This yields essentially pure ice crystallites together with an organic-hydrate phase which is either glassy or polycrystalline.[2,4] On irradiation at 77 K there is a high yield of $^{\bullet}OH$ radicals, most, if not all, of which are confined to the ice phase, as can be assessed from the form of the e.s.r. spectral features.[5] These have been formed from the initial H_2O^+ and e^- products[6] and decay rapidly at the characteristic temperature range of 120—130 K. We consider that all radiation-produced radicals formed in the ice crystallites are confined therein and can be ignored when considering the radical chemistry of the organic solutes.[4]

Solutions saturated with oxygen will lose some oxygen on freezing since oxygen molecules are excluded from the ice crystallites. However, the concentration of oxygen in the organic-rich phase must be high and the results suggest that oxygen molecules are often near-neighbours to organic radicals.

Identification of Radicals.—There can be no doubt that the 8-line spectra obtained from irradiation of 5-bromo-5,6-dihydrothymine are all due to radical (I), formed by dissociative electron capture (equation 1). On annealing, definite changes in

$$R\text{–}Br + e^- \longrightarrow R^{\bullet} + Br^- \qquad (1)$$

the e.s.r. spectra were observed (Figure 1*b*). In our view, these are caused by changes in the conformation of these radicals (I), rather than being due to the formation of new radicals. The same applies, to a far greater degree, to the spectra of radicals (II) formed from the hydroxy derivatives.

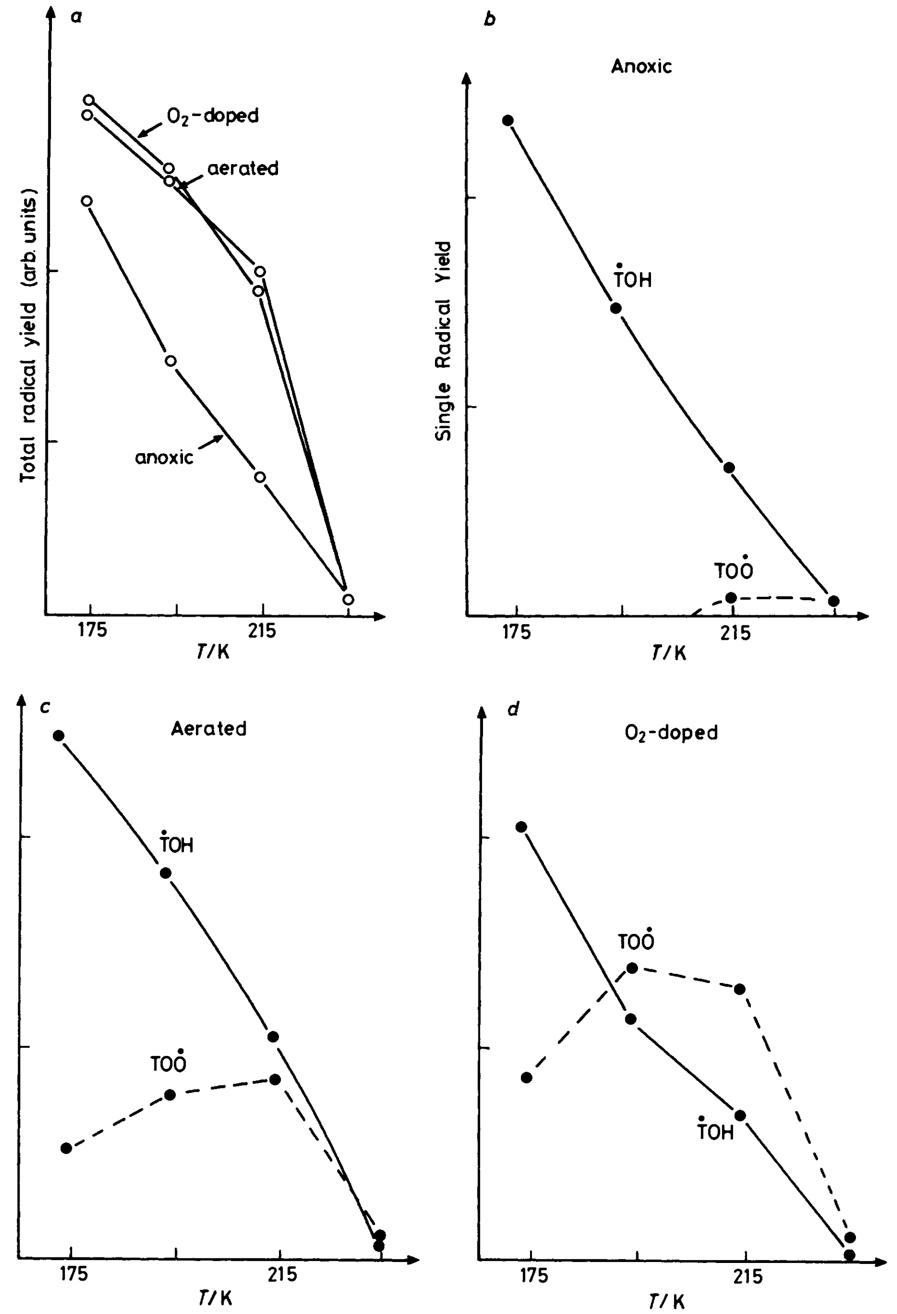


Figure 4. As for Figure 2, for the radicals [(II) and $TO_2^•$] formed in irradiated solutions of 5-bromo-5,6-dihydrothymine

In view of the complex changes observed in the e.s.r. spectra for these systems (Figure 3), this statement requires justification. This is attempted in the discussion of conformation changes given below.

The asymmetric singlet obtained from oxygenated solutions is undoubtedly due to $RO_2^•$ radicals, which have characteristic g-values almost independent of the nature of R. Unfortunately, nothing can be deduced from the e.s.r. spectra about the nature of the group R. From these spectra, however, the fact that loss of signals due to radicals (I) or (II) is coupled by growth of the $RO_2^•$ signal suggests strongly that R is either (I) or (II). Nevertheless, it remains possible that other radicals are also scavenged by oxygen. The fact that both $R^•$ and $RO_2^•$ centres decay during the conversion makes it difficult to draw quantitative conclusions.

Conformational Changes.—Since the conformation at C-5 for radical (I) is expected to be planar, the two hydrogens of the $>CH_2$ group should become equivalent in the absence of external constraints. However, in the parent bromo derivative, they occupy sites which can be described as 'axial' and 'equatorial', and when the bromide ion is lost we expect that there will be an initial 'memory' of this conformation, possibly in the form of an environmental constraint from the bromide ion. These are not able to move far from their original sites, thus conferring an asymmetric environment on the radical. Since such asymmetry is not expected to be precise in the low-temperature systems, the lines are broadened.

This situation is far more marked for the hydroxy derivatives [radicals (II)]. In these cases, the parent compounds always have the OH and Br groups *trans* to each other, as indicated in

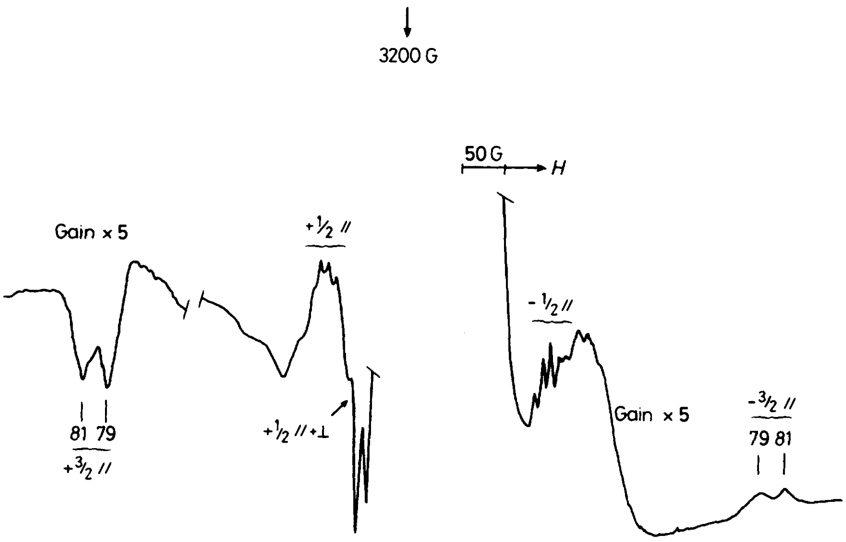


Figure 5. First-derivative x-band e.s.r. spectrum for an aqueous solution of 5-bromo-6-hydroxy-5,6-dihydrothymine after exposure to ^{60}Co γ-rays at 77 K showing the $M_I = \pm\frac{3}{2}$ and $\pm\frac{1}{2}$ features assigned to radical (V)

(III). Furthermore, the OH group is expected to be involved in two hydrogen bonds,[7] and hence to be strongly locked to the matrix. In the case of the thymidine derivative, this is also true of the R-group. On loss of bromide the configuration about C-5 can become trigonal-planar by a minor movement of the Me group [see arrows in (III)], but the C-6 proton remains initially close to its original 'equatorial' site relative to the ring. This is because neither the OH-group nor the ring can move far from their initial locations. This concept satisfactorily explains the remarkably small initial value for the C-6–proton hyperfine coupling, since this depends strongly on the twist angle θ, defined in (IV), according to equation (2).

$$A(^1\mathrm{H}) = A_0 \cos^2\theta \qquad (2)$$

On annealing, the radical should relax towards its equilibrium conformation. However, these ice-media are so rigid that this was not attained prior to radical decay. For the more weakly hydrogen-bonded glasses used by Sevilla and Engelhardt,[1] a marked increase in this coupling constant was indeed detected on annealing. (It is noteworthy that we actually recorded a slight initial fall in this coupling constant on annealing. We suggest that this was due to a preferential loss of radicals with a larger coupling constant rather than a change in conformation because it is difficult to see why there should be a change of θ in the wrong direction.)

Comparison with Thymine Addition Radicals.—As stressed above, radicals (I) and (II) have also been prepared by reaction involving thymine, the overall effect being either H-atom or OH-radical addition to C-6 (Scheme).

The e.s.r. spectra for radical (I) formed by these alternative procedures differ slightly, the best-fit parameters suggesting a minor increase in spin-density on the methyl protons and loss on the methylene protons for the radical formed from the bromo derivative relative to the H-atom addition species. This suggests a shift in spin-density from N-1 to C-5: thus the CH_2 group loses spin-density on one side and gains it on the other, there being a net loss because the extra 'conjugation' is lost. For the methyl group there is only a gain. This perturbation may be due to a slight residual buckling of the ring. It is probably not due to weak interaction with the ejected bromide ion since this normally gives rise to $^{81-79}$Br hyperfine coupling and a small reduction in spin-density on the radical,[8,9] neither of which are observed.

The differences are more marked for the hydroxy derivatives (II). In this case, radicals formed from thymine have C-6–H proton couplings of *ca.* 21 G,[10] which is very much greater than that observed for the radical formed by loss of bromide ion (3—6 G). This result agrees nicely with the theory outlined above. For OH addition to thymine, the ring is initially planar but as $^{\bullet}$OH or OH$^-$ adds, the C–H proton is relatively free to move to its preferred site. Since the OH group was free to attack the thymine ring, it must also be sufficiently free to approach its most favoured site.

We conclude that the radicals formed by these alternative routes are chemically the same and they are expected to undergo the same reactions. They differ only in their conformations, these differences arising because of strong matrix effects. We stress that similar effects may be detected for other processes of this type when constrained matrices are used.

Bromine-containing Radicals.—Our experience with e.s.r. studies of bromo-organic radicals, both α-bromo ($R_2\dot{C}$–Br)[11,12] and β-bromo ($R_2\dot{C}$–CH_2Br),[13,14] is that hyperfine coupling to bromine (^{79}Br and ^{81}Br have $I = \frac{3}{2}$ and similar magnetic moments and abundancies) is large and very anisotropic. Since there was no evidence for the spectra of electron-loss species in the central region, we searched the outer regions of the spectra at high gains and high microwave powers and, indeed, managed to detect such features.

A relatively well-resolved quartet signal was obtained from the hydroxy derivatives, as shown in Figure 5. We assign this to a β-bromo radical such as (**Va**) or possibly (**Vb**), for the following reasons. Similar spectra have been obtained from irradiated alkyl bromides, which were assigned to $R_2\dot{C}$–CH_2Br radicals.[13,14] Although a completely different, narrow line spectrum has also been assigned to such radicals,[15] we think there are good reasons for ignoring this assignment.[16,17] The maximum hyperfine splitting to ^{79}Br and ^{81}Br (*ca.* 280 G) is reduced from that for $R_2\dot{C}CH_2Br$ radicals (*ca.* 360 G). This result seems to us to be reasonable for radical (**Va**), which can be formed from the parent π-cation (VI) by proton-loss, because orbital overlap is less favourable and the spin-density on adjacent carbon atoms is less than unity. However, they are not reasonable for the parent cation, which can only have low spindensities adjacent to the C–Br group; the major density being expected to be centred on the two nitrogen atoms.[18] The results are less reasonable for (**Vb**), since we would expect this to resemble the $R_2\dot{C}CH_2Br$ species and have a larger maximum bromine coupling.

In addition to the doublet splitting arising from ^{81}Br and ^{79}Br features, which is normal for these β-bromo radicals (in contrast with the situation for α-bromo radicals[12]), there is a small extra splitting of *ca.* 9 G, which appears as a triplet. We tentatively assign this to the parallel coupling for one of the ^{14}N nuclei. This result eliminates radical (**Vb**) since this should exhibit a 22 G triplet from the CH_2 protons. The $M_I = \pm\frac{1}{2}$ feature is intense because the *x*, *y*, and *z* components are close together. For the 5,6-dihydro derivatives, we have only been able to detect a broad line in this region, so our identification in this case must remain tentative.

Mechanism of Radical Formation.—According to recent analyses,[4,10] we expect that for the pure compounds and the phase-separated aqueous systems, the major reactions leading to the observed radicals should be equations (3) and (4). The

$$RBr \xrightarrow{\gamma} (RBr)^+ + e^- \qquad (3)$$

$$e^- + RBr \longrightarrow R^{\bullet} + Br^- \qquad (4)$$

facility of the dissociative electron-capture step (4) is in marked contrast with the relative inefficiency of this reaction for 5-bromouracil and related compounds (**VII**). This arises, in our view, because electron addition to the –CO–NH–CO–NR– unit of the present bromo derivatives gives an anion (**VIII**), the bromine atom being in a β-position to the electron, which will result in considerable σ–π delocalisation.[13,14,19] In contrast, addition into the π-ring for (**VII**) gives a radical which will acquire π spin-density on bromine but no σ spin-density. Furthermore, loss of Br^- for the 5,6-dihydro derivatives leads to a resonance-stabilised π-radical, whereas loss of Br^- for the anion of (**VII**) leads to the far less stable 'σ' or 'nb' radical (**IX**).[20]

The radical cation (**IV**), $(RBr)^+$, has not been studied by e.s.r. spectroscopy. In an aqueous glass, it is expected to lose a proton to give (**Va**) which, having a β-bromine atom, should give a large bromine hyperfine coupling. In our view, this is most probably the species detected herein.

Conclusions

We conclude that radiation damage to these thymine derivatives proceeds *via* electron-loss and electron-gain. The former cationic centres deprotonate to give trapped β-bromo radicals whilst the latter undergo dissociative electron capture with loss of bromide ions.

The ways in which these radicals lead to the final stable products will be discussed in a subsequent publication.[21]

Experimental

Sample Preparation.—Thymine, 5,6-dihydrothymine, and thymidine, obtained from Sigma, were used without further purification. Synthesis of 6-deuteriothymidine (isotropic labelling up to 90%) was accomplished by a selective base-catalysed exchange procedure.[22] The 5-bromo-5,6-dihydrothymine was prepared by mild bromination of 5,6-dihydrothymine in dioxane solution.[23] The *trans*-5-bromo-6-hydroxy-5,6-dihydrothymine was synthesised according to Baudish and Davidson.[24] The (+)-*trans*-(5*R*,6*R*) and (−)-*trans*-(5*S*,6*S*) diastereoisomers of 5-bromo-6-hydroxy-5,6-dihydrothymidine were prepared by the addition of bromine to aqueous solution of thymidine containing sodium acetate and further purified by reversed-phase h.p.l.c.[25]

Experimental Apparatus and Computer Techniques.—Details of the experimental apparatus as well as the computer techniques used for the analysis and reconstruction of composite e.s.r. spectra have been extensively described.[26]

Experimental Procedure.—Frozen samples were prepared by cooling, in liquid nitrogen, Pyrex tubes containing 0.3 ml of solution. Extrusion of the frozen solution from the tube produced uniform solid cylinders 3 cm long. Samples were systematically submitted to the following irradiation, annealing, and e.s.r. recooling routines:

Irradiation: 5 Mrad at 77 K in a Gamma cell 200 (^{60}Co) at a dose rate of 4×10^5 mrad h^{-1}.

Annealing: Steps of 5 min at 105, 115, 135, 175, 195, 205, 215, 225, and 235 K.

E.s.r. Recooling: When different conditions are not mentioned, it is intended that the e.s.r. spectra have been recooled at 77 K and at a microwave power of 0.02 mW.

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