Effects of Ionizing Radiation on Deoxyribonucleic Acid and Related Systems. Part 1. The Rôle of Oxygen

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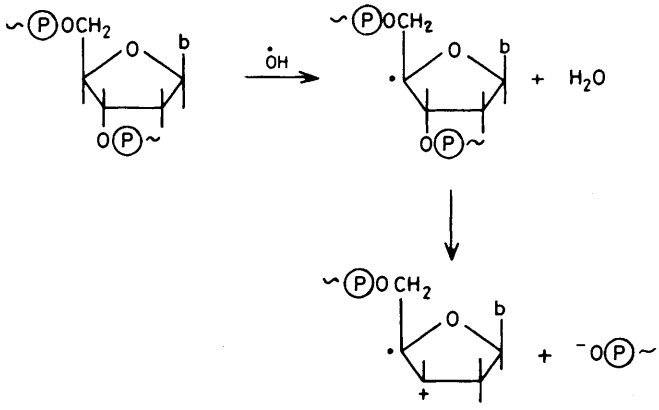
Exposure of aqueous solutions of calf-thymus DNA to ^{60}Co γ-rays at 77 K results in the formation of equal yields of G^+ and T^- centres, detected by e.s.r. spectroscopy. In the presence of oxygen, O_2^- ions are also formed at the expense of T^-, the primary yield of G^+ centres being unaffected. On annealing above 77 K the e.s.r. features for O_2^- ions were initially modified as a result of changes in solvation, and then, at *ca.* 193 K, they were lost irreversibly. A large growth in signals due to $RO_2\cdot$ radicals was observed simultaneously. These radicals are formed from T^- anions or the protonated form, TH, and probably also indirectly from G^+ radicals. In parallel experiments, under comparable conditions, we have analysed for single and double strand breaks using plasmid DNA (pBR 322). Both single and double strand breaks persist under conditions in which G^+ and T^- are the only detectable primary radical products. The presence of oxygen causes only a slight increase in damage to DNA.

Studies of the effects of ionizing radiation on DNA are of more than academic significance in view of the real possibility that the resulting damage plays an important rôle in cell death and the development of cell abnormalities. Furthermore, modification of DNA damage by radiosensitisers may possibly be important in cancer therapy.

There has been an extensive onslaught on the mechanism of direct radiation damage to DNA constituents,[1] and these studies have led, for example, to a set of characteristic e.s.r. spectra for various base and sugar radicals which can aid in the study of radicals formed in DNA.[2–4]

In studies on DNA itself, two different approaches have been used. In one, damage is primarily indirect since dilute aqueous solutions are studied at ambient temperatures. Under these conditions damage is largely confined to water molecules, and the major attack on DNA comprises addition of solvated electrons to base units and attack by ·OH radicals at various sites, the latter being thought to be of major importance. Probably the most significant reaction of ·OH radicals is hydrogen atom abstraction from the deoxyribose unit with consequent β-elimination leading to chain breaking[5,6] (Scheme).



Scheme. ~Ⓟ = the phosphate groups in DNA

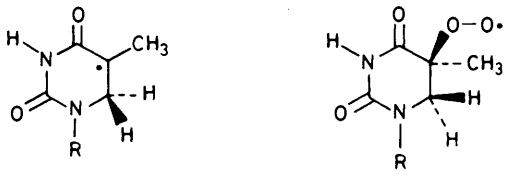
In the other extreme method, damage is almost entirely primary. The systems used comprise either 'dry' DNA or frozen aqueous solutions.[7–9] In the latter, phase separation occurs on freezing. Damage to the ice crystals is thereby effectively confined, and can be ignored. Attention is then focused on direct damage to the saturated DNA phase. The relative importance of indirect *versus* direct damage has not been resolved. We subscribe to the view that the actual concentrations of DNA in the nuclei of mammalian cells do not approximate to a *dilute* solution and therefore that direct ionization of DNA may constitute a major source of damage.

We have been principally interested in studying the primary radical products by e.s.r. spectroscopy. It is only by resorting to low temperatures that these primary radicals can be trapped in sufficient concentrations for detection by e.s.r. spectroscopy. By using a range of irradiating temperatures below the softening point, we have been able to establish that there is no major change in mechanism over a wide temperature range. We are therefore confident that these same radicals will be the primary, albeit transient, damage centres when DNA is irradiated under ambient conditions.

γ-Irradiation of native DNA *in vitro* and *in vivo* ultimately gives rise to a number of well characterised lesions which include single and double strand breaks, release of bases from the intact chain, and various base modifications.[1] Most, if not all, of these lesions appear to be repaired with varying degrees of efficiency and it is not clear what characterises the events that are cytotoxic or mutagenic. Despite this uncertainty it is still probable that the interruption of the nucleotide strand is one of the most serious kinds of radiation damage to the macromolecular structure of DNA. We have therefore chosen to study single (ssb) and double strand breaks (dsb) as indices of biological damage. Hitherto strand break studies have been carried out under conditions that are grossly different from those for e.s.r. experiments. For example, differences in temperature, concentration, and phase are normal. Also, the presence of other biological components severely complicates any attempt at comparison of e.s.r. and strand-break results.

A major aim of the present study was to discover to what extent the radicals formed in DNA at 77 K that are readily detected by e.s.r. lead ultimately to strand breakage (single and double), and to probe the effects of irradiation temperature and phase on the yields of such breaks.

At the cellular level oxygen is known to be a powerful radiosensitising agent. Although there are reports that oxygen may modify the direct effects of radiation on DNA these reports range from radioprotection through to radiosensitisation.[10] The mechanism by which the oxygen effect is expressed *in vivo* remains unclear. Another aim of this study therefore was to probe the effects of oxygen on the extent of chain breaks and to relate this to the yields of primary radicals detected by e.s.r.



(a) (b)

spectroscopy. It has been suggested that a major rôle of highly electron-affinic radiosensitisers, such as metronidazole, is to capture electrons.[11] Since oxygen has a high electron affinity we wished to study the extent to which O_2^- was formed and to see how this might affect DNA damage.

There have been two major e.s.r. studies of radicals formed in frozen aqueous DNA solutions irradiated at 77 K.[9,12] Both agree that the resulting damage is remarkably simple, the products being only the guanine cation (G^+) and the thymine anion (T^-). No sugar or phosphate radicals were detected despite the fact that sugar radicals are the normal primary electron-loss products from mononucleotides.[13,14] Also, it is surprising that T^- should be formed exclusively, since it is probable that cytosine has a higher electron affinity than thymine. The e.s.r. evidence for G^+ and T^- is based on careful computer techniques involving simulations using authentic spectra for these radicals,[9] and also using spun DNA ribbons which are strongly oriented along the major axis.[8] For our present purposes, we accept these assignments.

In the absence of oxygen, protonation of T^- gradually proceeds on annealing to give the well established eight-line spectrum for •TH radicals (**a**). Their subsequent reactions on further annealing are unknown. The G^+ signals decay with no clear formation of other radicals detectable by e.s.r. spectroscopy. In the presence of oxygen, •TH radicals are converted rapidly into TH–O_2• radicals, presumably (**b**),[9] whilst G^+ radicals probably also give RO_2• radicals by some unknown process. Again, subsequent reactions of these radicals are unknown.

A variety of questions remain to be answered in connection with direct radiation effects on DNA, perhaps the most important being (i) do subsequent reactions involving the radicals so far detected by e.s.r. spectroscopy lead ultimately to single and/or double strand breaks? Related questions include (ii) what other radicals are involved in reactions leading to strand breaks? (iii) Do oxygen molecules act as electron traps thereby modifying the initial yields of T^- and G^+? (iv) If so, what rôle may O_2^- anions play in subsequent reactions of DNA? (v) Do G^+ radicals react with oxygen? (vi) What is the effect of oxygen on the ultimate yields of single and double strand breaks? The aim of the present study was to attempt to provide answers to some of these questions. A longer term aim of our work on DNA is to probe the effects of a wide range of additives on direct radiation processes, bearing in mind the possibilities, however remote, of radioprotection or radiosensitisation.

Results and Discussion

Detection of O_2^- *and* HO_2•.—In our previous studies of O_2^- ions,[15,16] we have established that solvation in glassy protic solvents at 77 K is extensive, leading to well defined e.s.r. features. Our postulate that well defined, in-plane, hydrogen bonding is responsible for the strong lifting of π-orbital degeneracy, as indicated in Figure 1, has been nicely confirmed by recent electron spin-echo studies of Kevan and his co-workers.[17] We conclude that different g_z features (z is the molecular axis) will be characteristic of different types of

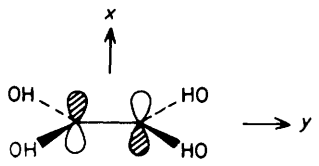


Figure 1. Solvation of O_2^- ions in water. The structure is ---- $(\pi_y^*)^2(\pi_x^*)^1$, the π-orbital degeneracy being lifted by hydrogen bonding. Nevertheless, for field along z orbital angular momentum is induced and the g_z feature is shifted to low field. The extent of this shift is a function of the x–y splitting and hence of the extent of solvation.

solvated O_2^- ions, and the narrower the features the more precise the solvation.

After irradiation of oxygenated DNA solutions at 77 K a relatively narrow feature at g 2.103 was detected. This moved slightly towards the free-spin value on annealing. A broader more intense peak at 2.090 (Figure 2) was also present whose intensity was proportional to [DNA]. It is only possible to measure the g_z features for O_2^- since the g_x and g_y features are both expected to be close to the free-spin value and hence are obscured beneath other more intense features. (These include strong features from •OH radicals, as indicated in Figure 2. We stress that these •OH radicals are almost all formed in ice crystallites formed during phase separation on freezing. On annealing, these are lost at 110—130 K which is the annealing temperature for •OH radicals in ice.[18,19] They are not able to diffuse across phase boundaries and attack DNA molecules.)

The sharp g 2.103 band is assigned to O_2^- ions formed from oxygen located close to specific regions of the DNA molecules or the sodium ions. When solutions of NaO_2 in water are frozen normal solvated O_2^- ions are not observed, only an extremely broad feature thought to be associated with phase-separated hydrated NaO_2 being detected. The width must be due, at least in part, to spin–spin broadening, which would not be expected in our systems. The second feature, at g 2.090, is more significant since it represents the bulk of the O_2^- anions formed. It is probable that these O_2^- ions are formed in glassy disorganized regions of water molecules close to the DNA molecules. These would all have slightly different g_z values which accounts for the large width of this feature.

We believe that this is the first time that O_2^- ions have been observed in frozen aqueous solutions of DNA. From our above reasoning we conclude that all the O_2^- ions that we detect are formed close to DNA.

We also detected doublet features in the g_z 2.032 region (A_H 16 G) which we assign to HO_2• radicals.[20] These flank the g_z feature for RO_2• radicals which grows in on annealing (Figure 2*b*). These features were clear on annealing to *ca.* 135 K, when the obscuring features due to •OH radicals were lost. We conclude that some O_2^- ions must be formed so close to proton donors that protonation is facile. These are not normal water molecules, but could be unusually acidic water, or possibly acidic N–H protons.

On further annealing the O_2^- features were lost irreversibly at *ca.* 193 K, and the HO_2• features were also lost, or hidden beneath the more intense RO_2• features. This is the temperature at which RO_2• features grow in strongly, and it is tempting to infer that there is an interconversion. However, as discussed below, we are more inclined to the view that this is coincidental. In the absence of oxygen approximately equal yields of G^+ and T^- are formed as expected since the number of electron-gain centres must equal the number of electron-loss centres.

When oxygenated systems were used, there was a clear decrease in the yield of T^- radicals, corresponding to *ca.* 10% loss (Figure 3), a fact not noted in previous studies.[9] Careful computer subtraction established that it is indeed only T^- that

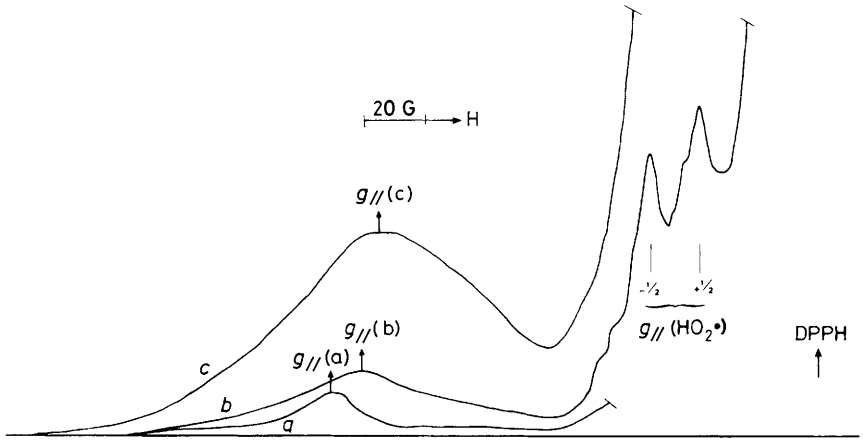


Figure 2. First-derivative x-band e.s.r. spectra for oxygenated solutions of DNA in water after exposure to ^{60}Co γ-rays at 77 K and annealing to *ca.* 130 K, showing the $g_{||}$ component of the spectrum for O_2^- as a function of DNA concentration: *a* 10 mg ml^{-1}, *b* 50 mg ml^{-1}, *c* 100 mg ml^{-1}. Also shown are the $M_I(^1H) = \pm\frac{1}{2}$ parallel features assigned to $HO_2\cdot$ radicals

is lost (Figure 3*c*). It is significant that the yield of G^+ radicals was not greatly altered under these conditions. The results suggest that the loss of T^- is approximately equal to the gain of O_2^- radicals. We conclude that oxygen competes directly with DNA for electrons thereby reducing the total yield of DNA radicals. We had anticipated that such electron scavenging might lead to an *increase* in G^+ yields as well as a decrease in $[T^-]$, by preventing electron return. This could occur on annealing, as in reaction (1), or even during the radiation

$$G^+ + T^- \longrightarrow G + T \qquad (1)$$

process at 77 K. Our results suggest that reaction (1) is not important, otherwise loss of T^- would mean a reduction in the extent of electron return and hence a detectable increase in $[G^+]$. Such an increase has, however, been reported in the presence of other electron scavengers.[11]

Reactions of O_2^- *and* $HO_2\cdot$.—The most probable reactions are electron donation by O_2^- and hydrogen extraction by $HO_2\cdot$. The former would result in formation of T^- (or ·TH) radicals. This was not observed. The latter would be expected to give some sugar-centred radicals, but these are also not clearly detected. A third possibility is addition (2) of O_2^- to carbonyl groups. We have suggested that a species formed from O_2^- in dimethylformamide, having 'normal' $RO_2\cdot$ e.s.r. parameters, is such an addition product. As mentioned above, in the present study, the $RO_2\cdot$ signal grows in as the O_2^- signal is lost, which would accord well with reaction (2). However, for the

$$>C{=}O + O_2^- \longrightarrow >C\begin{matrix}\diagup O\text{–}O\cdot \\ \diagdown O^-\end{matrix} \qquad (2)$$

dimethylformamide reaction, it is necessary to have the solvent very dry,[21,22] or normal solvated O_2^- ions are detected. We therefore suspect that, in aqueous systems, reaction (2) is improbable, but nevertheless cannot be dismissed. We also cannot exclude an alternative formation of $RO_2\cdot$ from O_2^- as shown in reaction (3). Even if the first equilibrium is

$$O_2^- + T \rightleftharpoons O_2 + \dot{T}^- \xrightarrow{H^+} \dot{T}H \xrightarrow{O_2} RO_2\cdot \qquad (3)$$

unfavourable with respect to formation of T^-, the equilibrium may be drawn over by conversion into $RO_2\cdot$. Although we cannot detect TH· formation, conversion into $RO_2\cdot$ is likely to be fast because the O_2 would be close to the newly formed radical and would not have to diffuse through the matrix.

Strand Breaks.—A number of methods have been used to analyse single and double strand breaks. *in vivo* Irradiations and *in vitro* studies using chromosomal DNA have usually relied on average molecular weight determinations by hydrodynamic methods under native (dsb) and denaturing (ssb) conditions.[1] Alternative methods that analyse for the production of specific end groups, detectable either enzymically or chemically, have also been developed. The improved methods for extracting and purifying plasmid DNA offers a third assay method based on gel electrophoresis. Of the methods currently available the last would appear to be the simplest and most accurate.

Plasmid DNA can exist in three forms, the covalently closed superhelically twisted form (**I**), the 'nicked,' relaxed, open circular form (**II**), and the linear form (**III**) (Figure 4). These three forms can be readily separated by gel electrophoresis and can be quantified by staining[23] or radiolabelling. Cleavage at a single site on one chain of the plasmid allows the superhelical twists of form (**I**) to relax generating form (**II**). Breakage of both stands at a coincident site generates form (**III**).

(We are presuming that two breaks in opposite strands within about five base pairs of one another will behave as a double strand break while two breaks further than about ten base pairs apart will behave as independent single strand breaks.) Clearly, in a γ-irradiation experiment starting with form (**I**), form (**II**) reports numbers of single strand breaks while form (**III**) reports double strand breaks. The simplicity of this method arises from the fact that there is no molecular weight change on going from form (**I**) to forms (**II**) and (**III**) and that migration of forms (**II**) and (**III**) on gel electrophoresis is independent of the actual site of chain scission such that all variants comigrate.

The most important result is that irradiation at 77 K under conditions that are close to those in the e.s.r. studies does result, ultimately, in both single and double strand breaks. Since G^+ and T^- are the only detectable primary radiation products this result seems to establish that at least one of these centres can initiate a sequence of reactions which ultimately result in strand breaks, both in the presence or absence of oxygen (Figure 5).

This conclusion is only valid if the yield of strand breaks is of the same order of magnitude as that of G^+ and T^- ions. This is, in fact, the case in our system. The estimated *G* value (number of strand breaks, or radicals per 100 eV dose) for single strand breaks is 0.4—0.7, whilst our *G* value for G^+ and T^- formation

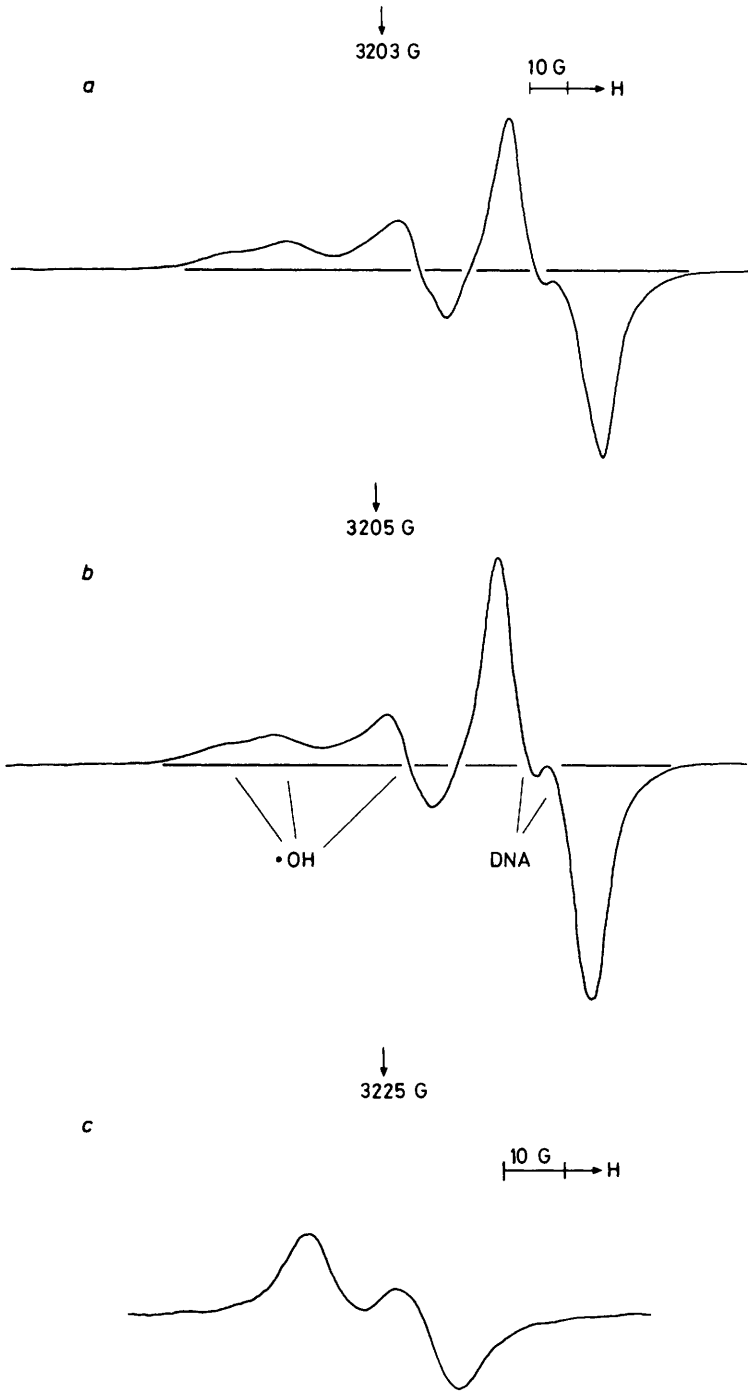


Figure 3. First-derivative *X*-band e.s.r. spectra for oxygenated (*a*) and deoxygenated (*b*) aqueous DNA after exposure to ^{60}Co γ-rays at 77 K, showing features assigned to •OH radicals and DNA radicals (G^+ and T^-), together with the difference spectrum (*c*) assigned to T^-

is *ca.* 1.5 which compares well with the literature values of 1—3.[24,25] The increase in damage in the presence of oxygen is most simply explained in terms of RO_2• radicals being converted into strand breaks with higher efficiency.

The second important result is that the fraction of double strand breaks is at least an order of magnitude greater than that predicted if two random independent single-break processes were involved. As shown in Figure 4 it is expected that at higher doses a significant fraction of plasmid molecules will have two or more strand breaks. If these strand breaks are sufficiently far apart (>10 bases) the electrophoretic properties of the molecule will not change and these multiply damaged plasmid molecules will all comigrate as form (**II**). Assuming damage is completely random, it is possible to calculate the fractions of molecules that are expected to contain two, three, four, *etc.*, strand breaks for a given dose. From a statistical analysis when 50% of form (**I**) has been converted into form (**II**) *ca.* 10% of the latter would be expected to contain two breaks. If, in order to get a double strand break, it is required that the second break must occur in the opposite strand, ±5 residues from the initial strand break, then the fraction of linear molecules would be *ca.* 10 × 10/8 000 = 0.01% (pBR 322 has *ca.* 8 000 bases). At doses

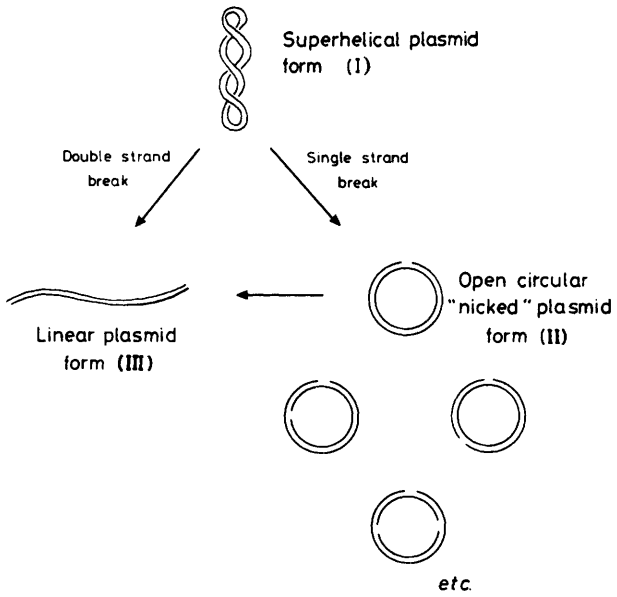


Figure 4. Protocol for the analysis of γ-radiation-induced single and double strand breaks using plasmid DNA

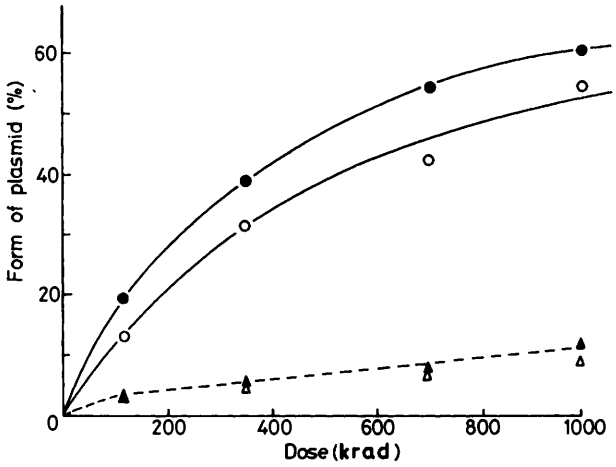


Figure 5. The oxygen effect on strand breaks induced by γ-irradiation of plasmid DNA (pBR 322) at 77 K. The percentage form (II) indicates single strand breaks produced in the presence (●) and absence (○) of oxygen. Double strand breaks formed in the presence (▲) and absence (△) of oxygen are indicated by form (III)

sufficient to convert 50% of the form (I), we observe the linear form (III) at levels between 5 and 10% (Figure 5).

We can most readily explain the high incidence of double strand breaks by postulating that both $\dot{T}^-$ and $\dot{G}^+$ may ultimately give rise to strand breaks. Electron ejection and electron capture must initially occur close together; however, since G^+ and T^- formation is specific, transfer between bases must occur. If G^+ and T^- were trapped close together and if both centres can ultimately give rise to strand breaks then this would account for the high incidence of double strand breaks. Had T^- and G^+ been trapped within *ca.* 10 Å we would have expected to be able to detect e.s.r. evidence for pair-trapping.[26] No features for such triplet-state pairs either in the *g* 2 or 4 regions were seen. We conclude from these observations that an appreciable number of T^- and G^+ centres are trapped in different chains within the range 10—30 Å and that there is a

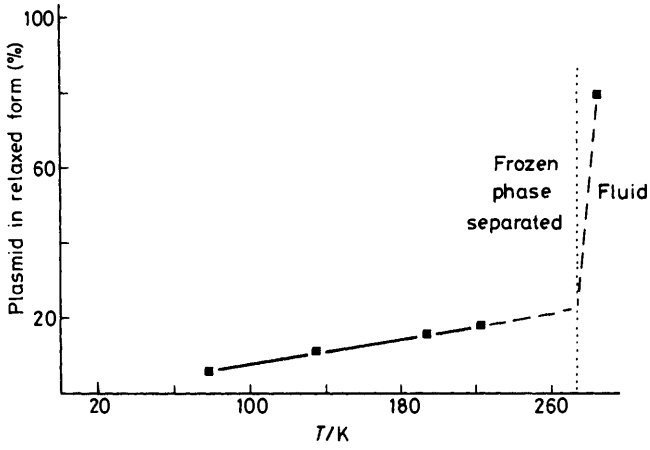


Figure 6. The effect of temperature and phase on γ-radiation-induced damage to DNA. Identical samples of pBR 322 (*ca.* 80 μg mol^{-1}) were subjected to the same dose (*ca.* 10 krad) at the temperatures indicated. Subsequent analysis for strand breaks was always conducted at room temperature

significant probability that both react ultimately to give strand breaks.

The foregoing discussion depends entirely on the conclusion that T^- and G^+ are the only radicals formed in significant concentrations. It has been argued that there must be radicals present in these irradiated samples that are not detected by e.s.r. spectroscopy.[27] If this is the case, then our conclusion that both $\dot{G}^+$ and $\dot{T}^-$ centres must react at least in part to give strand breaks is no longer compelling. Whilst it is not possible to disprove this suggestion, we are nevertheless unable to formulate any chemically reasonable radical centres which would not be expected to give well defined e.s.r. spectra. The requirement is, essentially, that spectral features are so broad that they are lost in noise. This is only likely to arise if there is large hyperfine or *g*-anisotropy. The only nuclei likely to provide large anisotropic coupling are ^{14}N and ^{31}P. Base-centred radicals with considerable ^{14}N anisotropy, such as $\dot{G}^+$, are in fact formed, and are readily detected by e.s.r. spectroscopy. Thus this cause of broadening can be dismissed. Of possible radicals exhibiting ^{31}P coupling, electron-loss radicals, such as $(RO)_2PO_2^{\bullet}$, exhibit only small ^{31}P splittings which are almost isotropic, since the SOMO is non-bonding on oxygen.[28] Such centres give well defined spectra and are certainly not formed in irradiated DNA. Electron capture at phosphorus can give rise to dissociative electron capture, giving alkyl radicals [reaction (4)] which are not detected, phosphoryl radicals [reaction (5)], or phosphoranyl radicals [reaction (6)].[29,30] Phosphoryl and phosphoranyl radicals exhibit very

$$(RO)_2PO_2^- + e^- \longrightarrow R\cdot + (RO)PO_3^{2-} \quad (4)$$

$$(RO)_2PO_2^- + e^- \longrightarrow RO^- + \cdot P(O_2)OR \quad (5)$$

$$(RO)_2PO_2^- + e^- \longrightarrow \begin{matrix} & & OR & \\ & & | & \\ & & O^{2-} & \\ & \diagup & & \\ \cdot P & & & \\ & \diagdown & & \\ & & OR & \\ & & | & \\ & & OR & \end{matrix} \quad (6)$$

large, fairly isotropic splittings, and are readily detectable at 77 K. There is no sign of such features in irradiated nucleotides or DNA.

Radicals exhibiting marked *g*-anisotropy are the most likely to be e.s.r. 'silent,' since Δg is frequently governed by solvent

interactions, as outlined above for O_2^-, and as is the case for ·OH radicals.[31] The most likely candidate is the RO· radical. These are important electron-loss centres in many irradiated nucleosides and nucleotides. They are formed by electron loss from ROH giving $\dot{ROH}^+$ which promptly lose their protons. Although they are generally detectable in single crystals, they might well not be in non-crystalline samples. However, we cannot envisage any route to such radicals from DNA, since there are no OH groups present. Electron loss from ether oxygen gives $(\dot{ROR})^+$ radical cations, which have well defined e.s.r. spectra,[32,33] and there is no evidence that such cations break down to give RO· and carbocations, and indeed, this would seem to be a highly improbable step.

We conclude that there are no reasonable candidates for radicals in concentrations comparable with those for $\dot{G}^+$ and $\dot{T}^-$ centres which are expected to give very broad e.s.r. features. In the absence of any specific suggestions for alternative radical products we feel justified in concluding that $\dot{G}^+$ and $\dot{T}^-$ are the major DNA radical centres formed, and that these must therefore be ultimately responsible for strand breaks.

Finally, we have observed that there is a gradual increase in the number of strand breaks on increasing the irradiation temperature, and a dramatic rise for irradiated fluid systems (Figure 6). The latter effect was expected, since the indirect mechanism of ·OH radical attack, known to lead efficiently to strand breaks (Scheme), is now operative. In the frozen systems, water damage is largely confined to the ice crystallites, and anneals out prior to melting. In the fluid solutions these water radicals will attack DNA, as observed.

Conclusions.—In agreement with previous studies we have observed that exposure of frozen, phase-separated aqueous DNA solutions results in direct electron loss from DNA. Although the ionization must initially be indiscriminate, the 'holes' rapidly end up on G while electrons become localised on T. Since both single and double strand breaks persist at high levels under conditions where only G^+ and T^- are detectable by e.s.r. and since we have no evidence for any other radical product that would not be detectable by e.s.r., we conclude that these primary radical products must be capable of giving rise to strand breaks. From the relatively high proportion of double strand breaks, we conclude that the original T^- and G^+ centres are trapped close together, and can both ultimately lead to strand breaks, although the chemical pathways remain obscure. We have noted for the first time that oxygen competes with DNA for electrons, giving rise to a reduction in the yield of T^- and formation of O_2^-. We do not observe an increase in G^+ which suggests that substantial electron return does not occur in our system. T^- Centres readily protonate at C_6 to give ·TH radicals which react efficiently with oxygen to give RO_2· radicals. G^+ Centres are also being lost at temperatures where RO_2· begins to grow in. This suggests that G^+ can form RO_2·; however, G^+ is a highly delocalised radical and we doubt that this would add oxygen directly. Probably some neutral intermediate radical is involved.

The presence of oxygen results in a modest increase in damage to DNA; we interpret this in terms of RO_2· being converted into strand breaks with a higher efficiency than that for the non-oxygenated radicals.

Experimental

Chemicals.—pBR322 DNA was isolated according to the procedure of Birnboim and Doly.[34] Typically, pBR322 DNA preparations contained *ca.* 95% of the superhelical form (I) DNA. Tris, EDTA, and ethidium bromide were obtained from the Sigma Chemical Company and agarose-ME was obtained from the Miles Laboratories Ltd. Calf thymus DNA for the e.s.r. studies was obtained from the Sigma Chemical Company.

γ-Irradiation and Assays for DNA Breaks.—Form (I) DNA (80 μg ml^{-1}) in 10mM-Tris HCl buffer, pH 7.6, containing 1mM-EDTA was gas purged for 60 min with oxygen or oxygen-free nitrogen. To ensure complete deoxygenation the nitrogen was further 'scrubbed' with an alkaline pyrogallol solution. Samples of *ca.* 20 μl were sealed and γ-irradiated in a ^{60}Co source under the relevant conditions. Following irradiation 6 μl of a dye–EDTA mixture containing 56% glycerol (v/v), 50mM-EDTA, and 0.05% Bromophenol Blue (w/v) was added. Portions were then taken and assayed for the production of ssb and dsb by agarose gel electrophoresis as described below.

Agarose Gel Electrophoresis.—Portions of the reaction mixtures which contained 0.7—1 μg of DNA were layered onto 1% agarose slab gels and electrophoresed in a horizontal slab gel apparatus for *ca.* 16 h at room temperature with a 40mM-Tris HCl buffer containing 20mM-sodium acetate and 1mM-EDTA at pH 8.2. After electrophoresis, gels were stained with 2.5 μg ml^{-1} ethidium bromide in the electrophoresis buffer for *ca.* 15 min. The stained gels were then excited with a transilluminator and photographed with a Polaroid MP-4 Land Camera using a red filter (Kodak Wratten filter No. 9) and Polaroid type 55 film. The negative films of gel were used for densitometric scanning.

Quantitation of Single Strand and Double Strand DNA Breaks by Densitometric Scanning of Negative Films of Gels.[23]—The negative films of the ethidium bromide stained patterns of the γ-irradiated pBR 322 DNA were scanned with a scanning microdensitometer (Mk III CS, Joyce, Loebl and Co. Ltd., Gateshead on Tyne). The production of the relaxed form (I) DNA arises from single strand breaks while the linear form (III) DNA results from a double strand break. We have assumed the form (I) pBR 322 DNA shows a staining efficiency of 70% that for forms (II) and (III) as has been demonstrated for PM2 DNA[35] and have used this factor to normalise our data.

γ-Irradiation and E.s.r. Measurements. [Type (I) Sodium Salt DNA].—Frozen samples were prepared by cooling a Pyrex tube containing a solution of 50 mg ml^{-1} DNA in liquid nitrogen. Extrusion of this frozen solution from the tube produced uniform solid cylinders 2.5 cm long. The spectra were obtained from an *X*-band Varian E-109 spectrometer of 100 kHz field modulation. Measurements at 77 K were made with the sample placed in a quartz Dewar flask containing liquid nitrogen. The Dewar was then inserted directly into the spectrometer cavity. Annealing was achieved by decanting the liquid nitrogen and continuously monitoring the spectrum as the sample warmed up until a significant change was observed, when the sample was immediately recooled with liquid nitrogen. Control at temperatures from *ca.* 90 K and upwards was possible by the use of a variable-temperature accessory designed and constructed in these laboratories. Nitrogen gas, cooled by passing through a coil immersed in liquid nitrogen, was pre-heated to a set temperature, then blown over the sample held in the spectrometer cavity. Samples were γ-irradiated by exposure to a ^{60}Co source in a Vickrad whose dose was *ca.* 0.7 MRad h^{-1}. Double integrations, subtractions, and storage of spectra were performed on a Hewlett-Packard 9835B computer.

Acknowledgements

Our thanks go to the Commission of the European Communities, the Cancer Research Campaign for financial

support, and to Professor G. E. Adams and Dr. S. Gregoli for helpful discussions.

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Received 10*th October* 1983; *Paper* 3/1787