The Effects of Ionizing Radiation on Deoxyribonucleic acid. Part 5.† The Rôle of Thiols in Chemical Repair

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Exposure of frozen, deoxygenated, aqueous solutions of DNA to ^{60}Co γ-rays at 77 K results in the formation of guanine-centred radical-cations ($G^{\bullet+}$) and thymine-centred radical-anions ($T^{\bullet-}$). Both these primary centres are thought to be capable of inducing DNA strand-breaks, both single (SSB) and double (DSB). When low concentrations of a range of water-soluble thiols were added, there was no change in the initial yield of $G^{\bullet+}$ and $T^{\bullet-}$ as judged from the e.s.r. spectra. However, on annealing, the normal pattern of radical reactions was abruptly modified at *ca.* 200 ± 5 K, with the DNA-centred radicals being dramatically reduced in concentration with the concomitant growth of e.s.r. signals characteristic of $RS^{\bullet}$–SR^{-} radical-anions. For example, for solutions containing one thiol molecule per 25 base-pairs, there was a loss of *ca.* 50% in the concentration of DNA radicals at this temperature.

Using plasmid DNA, the change in the numbers of SSBs and DSBs was monitored when various thiols were present. There was a marked fall in the yields of both these events, in accord with the e.s.r. results.

It is concluded that these thiols react by hydrogen-atom donation to various DNA radicals thereby forming $RS^{\bullet}$ radicals which rapidly form $RS^{\bullet}SR^{-}$ radical-anions. It seems that, under our conditions, neither of these sulphur radicals is able to react with DNA.

In the presence of oxygen, the results are less definitive, the degree of repair being a function of the relative concentrations of oxygen and thiol. E.s.r. evidence for the formation of DNA-centred peroxy radicals and their reaction with thiols is presented, and also there is evidence for the addition of oxygen to $RS^{\bullet}$ radicals to give $RSO_2^{\bullet}$ radicals. The latter are probably able to react with DNA.

The lethal and mutagenic effects of ionizing radiation on cellular systems are assumed to be associated with the radical-induced chemical changes to key biological macromolecules, particularly deoxyribonucleic acid (DNA), that accompanies exposure to such radiation.[1] Many different radiation-dependent modifications to DNA have been characterized ranging from various base modifications through to single and double strand-breaks.[2] The latter must be considered to be a very serious form of damage since the informational content of the macromolecule cannot easily be restored. In discussing the fundamental mechanisms of these processes it is useful to distinguish between the direct component, in which the radical centres are formed directly within or very near to the macromolecule, and the indirect component, in which the radical centres are formed as a result of interaction with the radiolysis products of bulk water ($OH^{\bullet}$, e^{-}, $H^{\bullet}$). We have been studying the direct damage mechanism and ourselves and others have previously argued that the contribution that this may make to the *in vivo* damage mechanism may be of proportionally greater significance.[3–8]

Not surprisingly, living systems have evolved a variety of ways of dealing with the hazards associated with ionizing radiation. It is generally accepted that various small molecules play important rôles as radical scavengers, particularly with respect to the reactive hydroxyl radical, thereby preventing their interaction with DNA and other macromolecues.[9–11] This is clearly of importance with respect to the indirect damage mechanism. At the next level of protection it has been suggested that radicals formed within the DNA may be 'repaired'.[12–15] Such action may serve to intercept such radicals before they react further to give strand-breaks, *etc.*, and perhaps even preventing radical chain reactions from occurring. Finally, in addition to these chemical 'repair' processes, it is apparent that there are a number of efficient enzymatic repair pathways that can specifically recognize and excise a range of modifications to DNA.[16]

† Part 4, ref. 6.

It has been known for many years that thiols are efficient mediators of radical reactions, their action being hydrogen-atom donation,[17–20] as in (1), in competition with oxygen fixation.[21–25]

$$R^{\bullet\prime} + RSH \longrightarrow R'H + RS^{\bullet} \qquad (1)$$

$RS^{\bullet}$ radicals are far less reactive, and generally react selectively to give $RS–S^{\bullet}R^{-}$ molecules. There has recently been an upsurge of interest in these repair reactions, particularly with respect to the rôles of the tripeptide glutathione in modifying the response of cells to ionizing radiation[25–27] and efficient repair of damaged DNA is strongly implicated in most cases.

In the present study, we return to the problem of the chemistry involved when thiols interact with DNA radicals. Previous studies have concentrated on the rôle of thiols in chemical 'repair', under conditions of indirect damage, where the initial events involve hydroxyl radical and hydrogen atom ($H^{\bullet}$) attack on DNA or related model materials.[20,28–30] We and others have argued that in the cells of eukaryotes the nuclear DNA is not well modelled by a dilute aqueous solution and that because there is not a substantial amount of bulk water in the nucleus, the direct-damage pathway will make a substantial contribution.[3,31] In view of this we considered it to be of importance to determine the effects of thiols on the primary and secondary radicals produced under conditions of direct damage. Frozen aqueous solutions have allowed us in our previous studies to focus on the direct-damage component. The bulk water is phase-separated from the DNA on freezing and does not contribute to the DNA damage, while the DNA phase comprises the DNA plus tightly bound solvating water.[3,31] The frozen aqueous solutions also provide the advantage that the primary radicals can be trapped and studied directly by electron spin resonance (e.s.r.) spectroscopy.[3,31] The aims of this present study were to establish the effects of thiols on the direct-damage pathway by our combined approach of e.s.r. spectroscopy and strand-break analysis.

Experimental

Materials.—Plasmid DNA (pBR 322) was isolated according to a modified procedure of Birnboim and Doly.[32] Typically pBR 322 DNA preparations contained *ca.* 85% of the superhelical Form I DNA. Tris(hydroxymethyl)methylamine (Tris) buffer, (ethylenedinitrilo)tetra-acetic acid (EDTA), 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide), and calf thymus DNA (for e.s.r. studies) were obtained from the Sigma Chemical Company. Agarose-Me was obtained from the Miles Laboratory.

The thiol and thiol derivatives employed, γ-L-glutamyl-L-cysteinyl-glycine (glutathione), 2-aminoethanethiol hydrochloride (cysteamine, AET), L-cysteine, DL-2,3-dihydroxybutane-1,4-dithiol (dithiothreitol), 2,2′-dithiobis(ethylamine) dihydrochloride (cystamine), DL-cystine dihydrochloride, and 5-methyl-L-cysteine, were purchased from Sigma Chemical Company and were of the highest quality.

γ-Irradiation and Assays for DNA Breaks.—The assay used has been described in our previous publications.[3–6] Variations were introduced in order to optimize the separation of the linear Form III DNA from the relaxed Form II DNA. A solution of thiol or thiol derivative (10 μl) was added to the plasmid DNA solution (90 μl; 80 μg ml^{-1}) in Tris hydrochloride buffer (10 mM; pH 7.6) containing EDTA (1 mM) to give the appropriate final additive concentration. Samples with and without additive were γ-irradiated in a Vickrad ^{60}Co γ-ray source under the appropriate conditions. Oxygenated and deoxygenated samples were prepared by gas purging with oxygen or thoroughly deoxygenated nitrogen for 60 min, then sealed and frozen prior to irradiation. Following irradiation and annealing to room temperature, a dye–EDTA mixture (6 μl; 56% glycerol v/v; 50 mM EDTA; 0.05% Bromophenol Blue w/v) was added and portions removed for analysis by agarose gel electrophoresis.

Gel Electrophoresis.—Samples of irradiated plasmid DNA (0.7—1 μg) were layered onto agarose slab gels (1.3%) and subjected to electrophoresis in a horizontal gel apparatus at 40 V for *ca.* 16 h at room temperature using Tris buffer (90 mM; pH 8.3) containing boric acid (90 mM) and EDTA (2.5 mM). After electrophoresis the gels were stained with ethidium bromide (2.5 μg ml^{-1}) in electrophoresis buffer for at least 15 min and the excess removed by washing. 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 the gels were used for densitometric scanning.

Quantitation of Single and Double Strand-breaks.—The negative films of the ethidium bromide-stained gels were scanned using a u.v.–visible spectrophotometer with gel scanning attachment (Pye Unicam SP8-100). The superhelical Form I of the plasmid takes up less ethidium bromide than the other two Forms. We have used a staining efficiency of 80% (as demonstrated for pBR 322[33]) to normalize our data. No further uptake of ethidium bromide was observed on prolonged incubations. All samples were analysed in duplicate and average values reported in the Results section.

γ-Irradiation and E.s.r. Measurements.—Frozen samples were prepared by cooling in liquid nitrogen a Pyrex tube containing an oxic or anoxic solution* of DNA (50 mg ml^{-1}; Type I sodium salt DNA, Sigma) with and without additive (0.1—140 mM). On extrusion from the tube, samples were irradiated as described above, and the e.s.r. spectra recorded (*X*-band Varian E-109 spectrometer, 100 KHz field modulation) at 77 K. The samples were annealed to a particular temperature for 2 min through the use of a second spectrometer (a Varian E-3 *X*-band) equipped with a variable-temperature accessory. The samples were then recooled (77 K) prior to the e.s.r. spectra being recorded using the E-109 spectrometer.

To judge the decay of the spectral features of the DNA ± additive samples with temperature, the spectra (at $g = 4.3$) of a bead containing tris(acetylacetonato)di-iron(III) was recorded in tandem with the DNA samples under identical e.s.r. parameters and this was employed as an internal intensity standard. In conjunction with the Fe^{III} bead samples of DNA not containing additive, were co-irradiated with the DNA–additive samples. These were employed to normalize further the DNA-additive spectra.

Storage, manipulations, and double integrations of spectra were performed with a Hewlett-Packard 9835B computer which was interfaced with the E-109 spectrometer and a Hewlett-Packard 9845A external flexible disc memory. All spectra were digitized at 1 024 equidistant points and stored on disc. *G* Values (numbers of radicals or strand-breaks per 100 eV) were estimated by comparison with the double integral value taken from a spectrum of dibutylaminoxyl of known spin concentration.

The spectra of 5,6-dihydro-5-thymyl radicals, TH•, for computer subtraction were obtained from a wet DNA sample (100% relative humidity), γ-irradiated at 77 K, and successively annealed up to 285 K, as described by Gregoli *et al.*[31]

E.s.r. data at *Q*-band frequencies were recorded using a Bruker e.s.r. E.R. 200D-SRC spectrometer with a Bruker ERO5 *Q*-band microwave bridge. Accurate *in situ* temperature control was achieved by an attached Bruker E.R. 4111 VT Variable Temperature unit.

Results and Discussion

It is now generally accepted that γ-irradiation of frozen aqueous solutions of DNA at 77 K, or dry films of DNA, give rise to a remarkably simple pattern of primary damage, the initial ionic radicals being exclusively the guanine cation ($G^{\bullet+}$) and the thymine anion ($T^{\bullet-}$) in approximately equal yields (as judged from their e.s.r. spectra).[3,31,34] On annealing, in the absence of oxygen, the thymine radical-anion protonates on carbon to give the 5-thymyl radical, TH•, that is unambiguously characterized by its well-established eight-line spectrum [Figure 1(a)]. $G^{\bullet+}$ decays without accumulation of any secondary radical that can be easily distinguished from $G^{\bullet+}$ by e.s.r. spectroscopy under our conditions.

A recent study suggests that loss of a proton from nitrogen to give a neutral radical (symbolized as GN•) with high spin density localized on nitrogen (N3) occurs at *ca.* 220 K for oriented fibres.[35] Under oxic conditions TH• radicals are rapidly converted into peroxyl radicals, $THO_2^{\bullet}$, whilst $G^{\bullet+}$ *via* (GN•) radicals probably also give $RO_2^{\bullet}$ radicals [Figure 1(c)]. We have previously shown that under both oxic and anoxic conditions single and double strand-breaks occur at significant levels.[3] In order to account for this we have proposed that a significant number of these radicals react *via* an intramolecular hydrogen-atom abstraction from an appropriately positioned deoxyribose moiety,[36] as shown in Figure 2. Subsequent immediate decay of the resulting sugar radicals must occur since under normal conditions no further radicals are detected. The further reaction of sugar radicals to give strand-breaks presumably occurs *via* a β-elimination mechanism analogous to that described for the hydroxyl radical-initiated reaction.[37] In support of this we have shown that when sugar-centred radicals are generated at lower temperatures they are indeed detect-

* Oxic and anoxic solution of DNA are defined as samples that have been gas-purged with oxygen and nitrogen respectively.

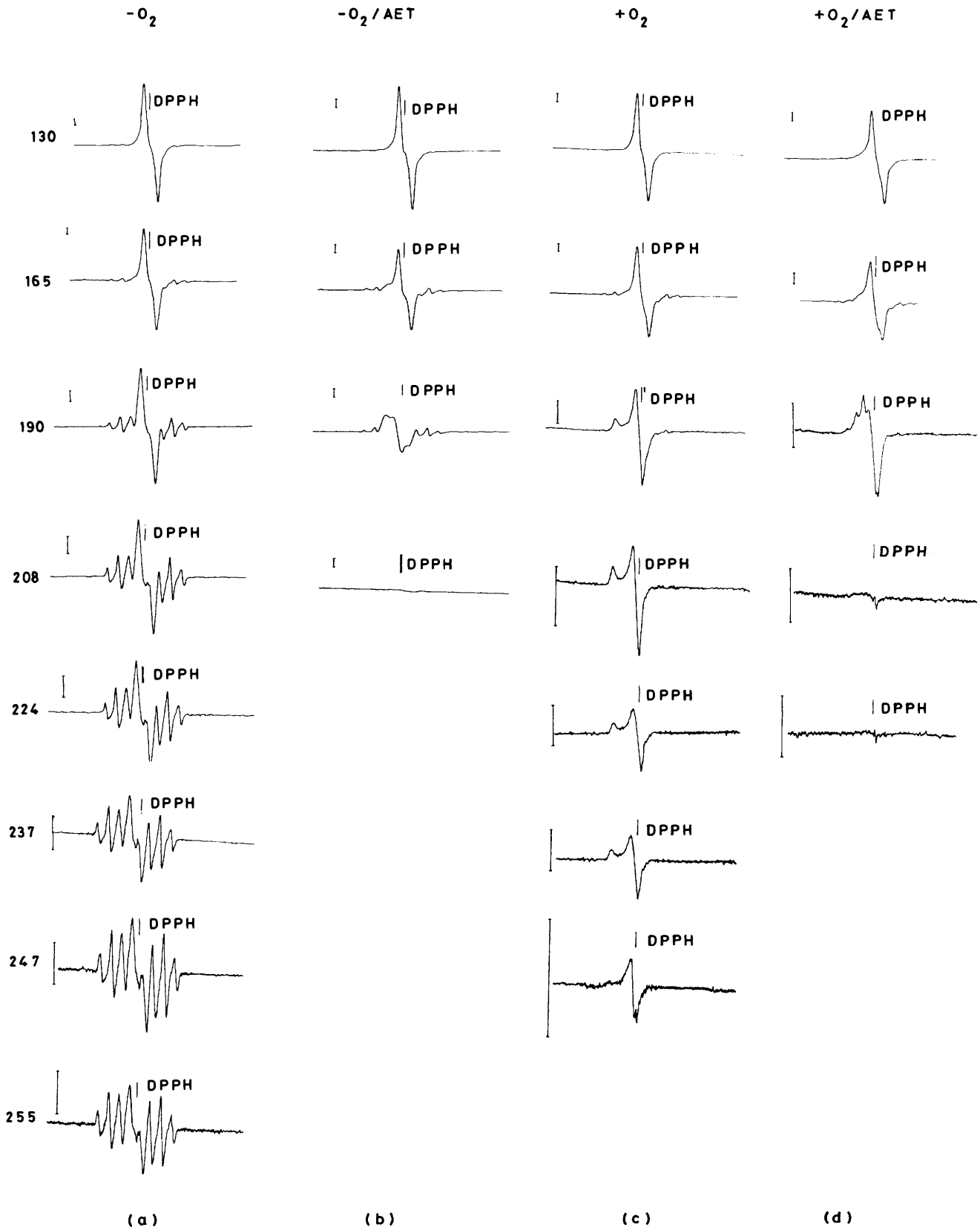
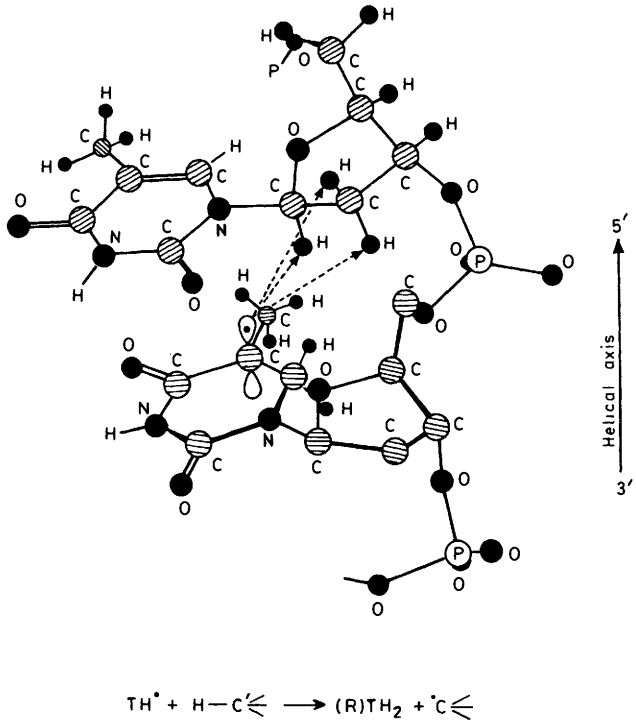


Figure 1. Representative steps in the evolution of e.s.r. spectra arising from thermal annealing of frozen aqueous solutions of DNA (~50 mg ml^{-1}) either in the (a) absence of oxygen. (b) absence of oxygen plus thiol (AET ~40 mM), (c) presence of oxygen, and (d) presence of oxygen plus thiol (AET, ~40 mM). Irradiation and measurement at 77 K. Dose, ~0.8 Mrad; microwave power 0.02 mW; modulation 4 Gauss



$TH^{\bullet} + H-C' \leqslant \longrightarrow (R)TH_2 + {}^{\bullet}C \leqslant$

Figure 2. Intramolecular hydrogen-atom abstraction from the deoxyribose unit

able[5,6] and we find that they decay to give non-radical products at lower temperatures than those for the normal DNA radicals. In our view, the key to understanding these reactions is the favourable proximity of certain sugar C–H units. Thermal motions will bring these well within tunnelling distances, so that these reactions can occur with high frequency. This contrasts with the situation normally encountered in which collisions between reactants are infrequent and radical + radical reactions can compete favourably. It is encouraging that similar base-mediated intramolecular hydrogen-atom abstraction reactions have recently also been proposed as steps in the reactions of $H^{\bullet}$ and $HO^{\bullet}$ base adducts leading to strand-breaks and base release in dilute aqueous solutions of poly(U).[38,39]

Assuming that hydrogen-atom abstraction would be most favourable from the nearest available site on the adjacent sugar we have inspected the B-DNA structure[40] using molecular graphics (Chem-X, Chemical Designs Limited). The hydrogens attached to C-1′ and C-2′ of the ribose unit 5′ to the 5-thymyl bearing nucleoside residue are significantly closer than other alternative centres. We predict, therefore, that the TH_2 formed by the intramolecular hydrogen-atom abstraction under strictly anoxic conditions would have the *R*-configuration. Subsequent to our prediction Furlong *et al.*[41] have recently shown that this is indeed the case.

This would also predict that the strand-breaks occurring under conditions of direct damage arise through a mechanism that is distinct from the indirect pathway in which hydroxyl radicals apparently predominantly abstract the C-4′ hydrogen atom.[37]

Effects of Thiols (E.s.r. Studies of Anoxic Systems).—The addition of moderate concentrations of a range of thiols (glutathione, cysteine, 2-aminoethanethiol, and dithiothreitol) (0.1—10 mM) to frozen aqueous solutions of DNA did not alter the initial absolute or relative yields of either $G^{\bullet+}$ or $T^{\bullet-}$ following irradiation at 77 K. However, at high concentrations of 2-aminoethanethiol (40—140 mM) a slight decrease in the yield of $T^{\bullet-}$ was observed initially. We suggest that this is due to traces of oxidized 2-aminoethanethiol (RSSR) present in the commercial material or formed during incubation with DNA. The disulphide is an effective electron scavenger (see below).

On annealing irradiated frozen aqueous DNA solutions in the presence of a variety of thiols all e.s.r. signals were abruptly lost at 200 ± 5 K [Figure 1(b)]. Concurrent with this loss of the DNA radical signals, we observed the growth and subsequent decay of signals from sulphur-centred radicals ($RS^{\bullet}SR^{-}$) derived from transient thiyl radicals, $RS^{\bullet}$ (see below). This abrupt loss of *all* DNA radicals was demonstrated under anoxic conditions at concentrations of thiols down to *ca.* 0.5 mM (1:150 base-pairs). (Below this concentration some DNA radicals survived.)

Effect of Thiols (E.s.r. Studies of Oxic Systems).—In the absence of thiols, the DNA radicals ($TH^{\bullet}$ and probably $GN^{\bullet}$) are efficiently converted into $RO_2^{\bullet}$ at *ca.* 190 ± 10 K, the $RO_2^{\bullet}$ radicals being characterized by a broad singlet with $g_{\parallel} = 2.034$ and $g_{\perp} \sim 2.000$ [Figure 1(c)].

When thiols were included, the $RO_2^{\bullet}$ radical yields were reduced and they again decayed abruptly at 200 ± 5 K [Figure 1(d)]. However, greater [RSH] (10 mM) was required to remove the $RO_2^{\bullet}$ signals completely at this temperature.

Sulphur Radicals.—At the temperature at which the DNA radicals decayed as a result of thiol action (under anoxic conditions) new e.s.r. features developed, which are characteristic of $RSS^{\bullet}R^{-}$ radical-anions. The features for these anions were well defined at *Q*- and *X*-band frequencies [Figure 3(a) and 3(b) respectively]. An intense yellow colour appeared together with these features, this colour being also characteristic of $RSS^{\bullet}R^{-}$ radicals ($\lambda_{max.} \approx 400$ nm). From a comparison with literature values (Table) the species formed under anoxic conditions is conclusively identified as $RSS^{\bullet}R^{-}$. The e.s.r. signals for $RSS^{\bullet}R^{-}$ radicals were lost by *ca.* 210 K, the temperature at which *all* spins were lost (Figure 4).

Table. Comparison of e.s.r. parameters of the sulphur-centred radicals formed in oxic and anoxic DNA/AET binary systems after annealing to 190—208 K, with experimental and reported data for $RSOO^{\bullet}$[a,b] and $RSS^{\bullet}R^{-}$[d,f]

	g-Tensor components		
	g_x	g_y[e]	g_z
$DNA/AET/O_2$	2.025	2.017	2.002
D_2O/Glutathione/O_2[a]	2.026	2.016	2.000
Aminopentanethiol/O_2[b]	2.026	2.107	2.000
E. coli B/r/0.1M-MPA/O_2[b]	2.026	2.107	2.002
$DNA/AET/-O_2$[c]	2.024	2.011	2.002
Cystine HCl/Crystal[d]	2.0178	2.0174	2.002
Glutathione oxidized/D_2O–CD_3OD (*ca.* 4:1 v/v)[f]	2.017	2.107	2.002
Dithiothreitol/D_2O–$CD_3OD/-O_2$/pH 10[a] (*ca.* 4:1 v/v)	2.022	2.011	2.003
Glutathione reductase[f]	2.024	2.015	2.002
Lysozyme[f]	2.0024	2.017	2.002

[a] G. D. D. Jones, J. S. Lea, and M. C. R. Symons, unpublished results. [b] Ref. 48. [c] Parameters measured after computer subtraction of $TH^{\bullet}$ + residual $G^{\bullet+}$ and $T^{\bullet-}$. [d] H. C. Box, H. G. Freund, K. T. Lilga, and E. E. Budzinski, *J. Phys. Chem.*, 1970, **74**, 40. [e] In most cases, g_z and g_y were not sufficiently defined to draw a clear distinction. [f] D. N. R. Rao, M. C. R. Symons, and J. M. Stephenson, *J. Chem. Soc., Perkin Trans. 2*, 1983, 727.

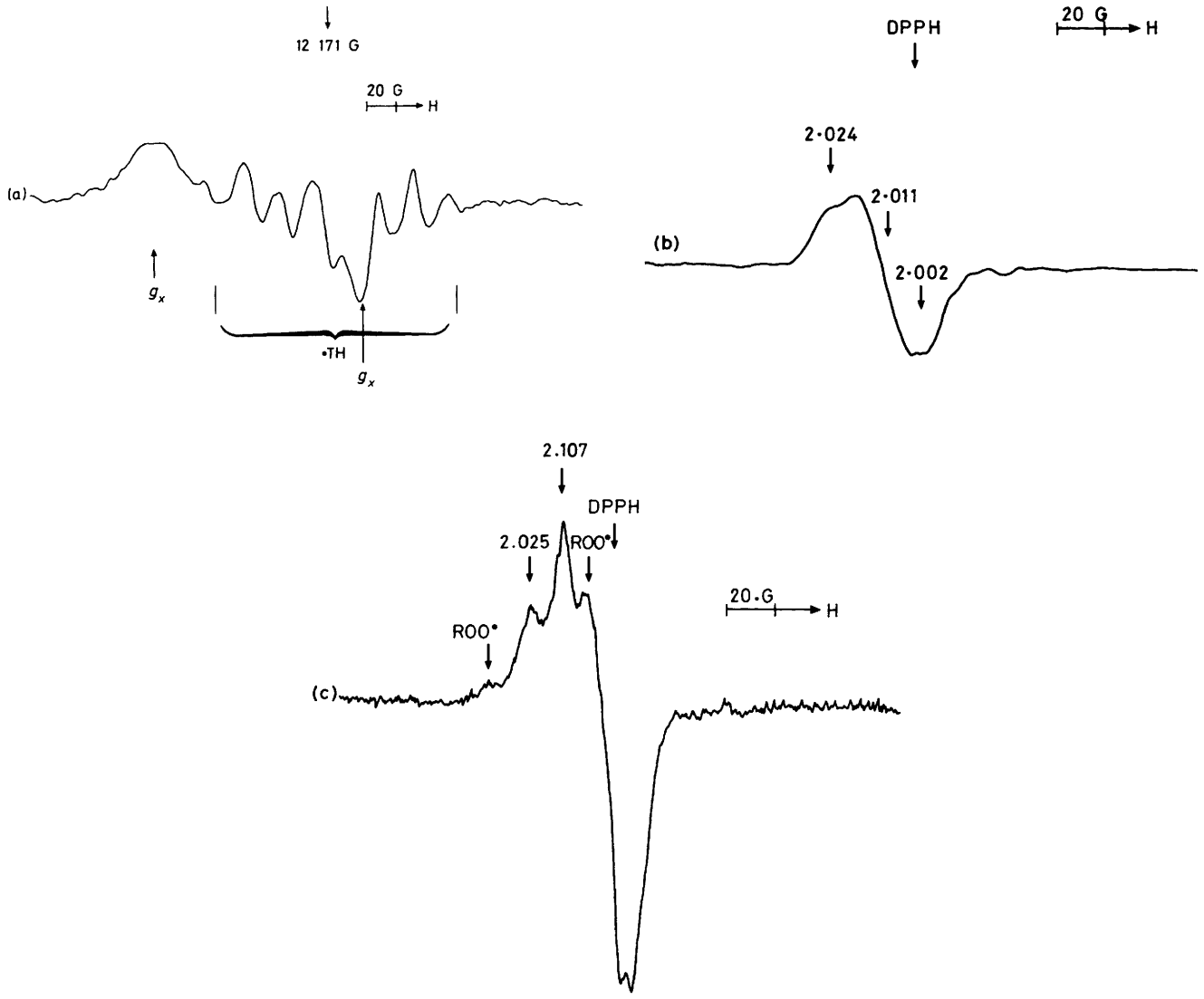


Figure 3. (a) First-derivative *Q*-band e.s.r. spectrum at *ca.* 100 K for an anoxic solution of DNA (~50 mg ml^{-1}) containing 2-aminoethanethiol (40 mM) after exposure to ^{60}Co γ-rays at 77 K and annealing to 195 K showing g_x and g_z features for $RSS^{\bullet}R^-$ radicals, together with features for residual $TH^{\bullet}$ radicals. Dose 1—4 Mrad; microwave power 1.1 μW; modulation 4 Gauss. (b) The resultant *X*-band first-derivative e.s.r. spectrum at 77 K for an anoxic DNA solution (~50 mg ml^{-1}) containing 2-aminoethanethiol (40 mM), after exposure to ^{60}Co γ-rays at 77 K and annealing to 190 K [*i.e.* Figure 1(b), 190 K], on computer subtraction of overlapping signals from $TH^{\bullet}$ (as judged by cancellation of wing features); *g*-tensor components for $RSS^{\bullet}R^-$ radicals can be seen and are compared with those from other experimental and literature values (Table). Dose. ~0.8 Mrad; microwave power 0.02 mW; modulation 4 Gauss. (c) First-derivative *X*-band spectrum at 77 K for an oxic solution of DNA (~50 mg ml^{-1}) containing 2-aminoethanethiol (40 mM) after exposure to ^{60}Co γ-rays at 77 K and annealing to 190 K showing *g*-tensor components of $RSOO^{\bullet}$ radicals and residual peroxyl radicals. [See Table for comparisons with other experimental and literature values.] Dose, microwave power, modulation as in (b)

The $RS^{\bullet}$ radicals thought to be formed directly as a result of reaction with DNA radicals [equation (1)] have not been detected by e.s.r. spectroscopy. This is not surprising since their spectra are controlled by the environment [as a result of hydrogen-bonding which lifts the degeneracy of the $3p(\pi)$ orbitals] which is itself expected to be variable in our systems.[41,42] However, reaction (2) is facile and we have, therefore, looked carefully for the radical $RS^{\bullet}$–S(H)R.[42,43]

$$RS^{\bullet} + RSH \rightleftharpoons RS^{\bullet}\text{–}S(H)R \qquad (2)$$

Surprisingly, no sign of this species was observed, only the conjugate base $RS^{\bullet}$–S^-R being detected. It seems, therefore, that reaction (2) is facile but that, under our conditions, the effective pH is such that deprotonation is complete. Our own studies[44] together with those of others[45–47] indicate p*K* of disulphide radical-anions to be ~5.5. Such a value would allow the deprotonation (3) to be favourable in our system.

$$RS^{\bullet}\text{–}S(H)R + (B) \rightleftharpoons RS\text{–}S^{\bullet}R^- + H^+(B) \qquad (3)$$
(B is any local base material)

Effect of Oxygen.—In addition to the normal $RO_2^{\bullet}$ signals which formed at *ca.* 190 K, but to a diminished extent, clear features at *ca.* 2.025 (g_x) and 2.017 (g_y) were observed rather than $RSS^{\bullet}R^-$ features [Figure 3(c)]. These are similar to the g_x and g_y features assigned by Copeland to $RSO_2^{\bullet}$ radicals.[48] Our own work on the addition of oxygen to the $RS^{\bullet}$ radicals confirms this assignment (Table).[49] These signals were also lost by *ca.* 210 K.

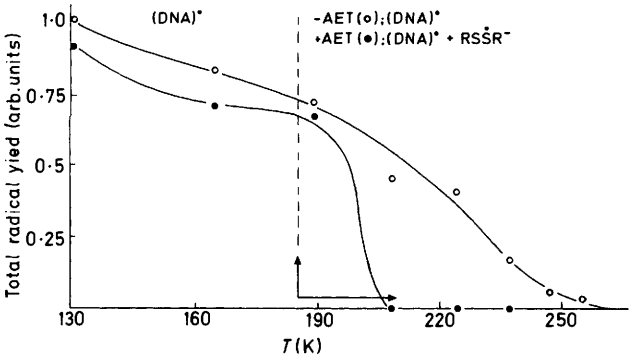


Figure 4. The temperature dependence of the total radical yield of anoxic DNA solutions (~50 mg ml^{-1}) with (●) and without (○) 2-aminoethanethiol (40 mM). At temperatures < 130 K, both the DNA-located (DNA)• and the H_2O-located free radicals $(H_2O)^•$ contribute to the total radical yield. At temperatures ≥ 130 K all $(H_2O)^•$ radicals have already reacted and the total yield corresponds to the yield of (DNA) ± $RSS^•R^-$. Temperatures for the onset and duration of $RSS^-R^•$ radicals existence are shown (↾⇂)

Strand-break Analysis.—The presence of 2-aminoethanethiol (40 mM) was demonstrated to protect plasmid DNA (pBR 322) against both single and double strand-breaks arising as a result of irradiation at 77 K. The degree of damage and the extent of protection by 2-aminoethanethiol (40 mM) was largely unaffected by the presence of oxygen, despite the marked changes in the e.s.r. spectra [Figures 5(a) and (b)]. At lower concentrations of 2-aminoethanethiol (5 mM) the extent of protection under anoxic conditions was reduced [Figures 6(a) and (b)] and this was further reduced in the presence of oxygen, suggesting opposing, competitive reactions of thiols and of oxygen at the lower thiol concentrations.

It is noteworthy that even at high relative [RSH] a significant number of strand-breaks were detected [Figures 5(a) and (b)]. We suggest that this correlates with the results shown in Figure 3 which show a gradual loss of radicals as the temperature increases from 130—200 K; that is, prior to the onset of reaction with the thiol molecules. This is an artefact of our system and reflects the unreactivity of RSH below *ca.* 200 K.

Mechanism of Thiol Action.—That thiols are acting as efficient repair agents, in our system, is shown by the dramatic and sudden loss of DNA radicals at temperatues well below those at which they normally decay, and the dependence of such action on the presence of an –SH functionality (see below) indicates that thiols are acting as efficient hydrogen-atom donors toward the DNA base radicals, *i.e.* reactions (1)—(3).

However, there are important liquid-phase results which suggest that whilst these reactions do occur under most circumstances for DNA, in the particular case of $G^{•+}$ formation, electron-transfer dominates (4), with the thiolate anion being the active species.[50]

$$G^{•+} + RS^- \longrightarrow G + RS^• \quad (4)$$

It is unlikely that this type of reaction can occur with RSH rather than RS^-. However, to check this, we studied the effect of RSMe (*S*-methyl-L-cysteine) which is likely to be a better electron donor than RSH. In fact, this compound had little effect on the course of DNA damage, and no $RS^•Me^+$ radicals were detected.[51]

We therefore tried to detect process (4) by raising the pH of a DNA–RSH sample to generate RS^-. Unfortunately, the high pHs involved modify the DNA and the course of damage.[31] However, we could conclude that there was a small decrease in the yield of $G^{•+}$ and a small growth in $RSS^•R^-$ at temperatures well below the onset of thiol action noted in neutral pH systems, *i.e.* over the range 130—145 K. So in a limited sense these results support (4). At higher temperatures $G^{•+}$ converts into GN• and charge-transfer is no longer expected.

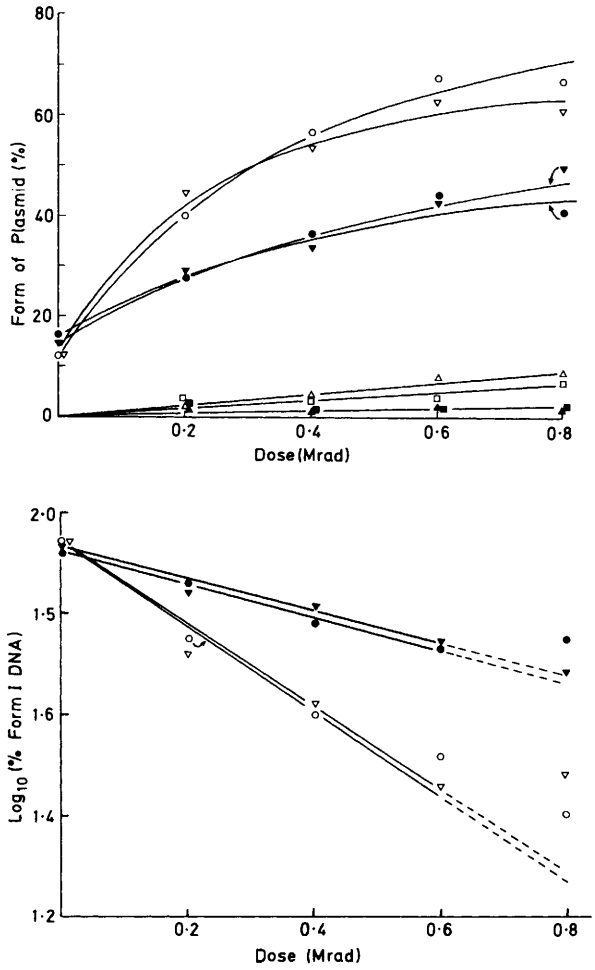


Figure 5. (a) The effect of 2-aminoethanethiol (40 mM) on strand-breaks induced by γ-irradiation of plasmid (pBR 322) at 77 K under oxygenated and deoxygenated atmosphere. The percentage of Form II indicates single strand-breaks produced: ▼ + AET + O_2, ● + AET − O_2, ▽ + O_2 and ○ − O_2. Double strand-breaks formed are indicated by Form III; ▲ + AET + O_2, ■ + AET − O_2, △ + O_2, and □ − O_2. (b) Semi-log plot of single strand-breaks induced in plasmid DNA (pBr 322) following irradiation as in (a); ▼ + AET + O_2, ● + AET − O_2, ▽ + O_2, and ○ − O_2

However, since no such low-temperature action was noted in our neutral systems, coupled with the fact that at pH 6.5 RS^- cannot constitute more than *ca.* ~2.5% of the total thiol present, we conclude that thiol-mediated electron-transfer does not have a major rôle to play in the repair of DNA direct damage in frozen aqueous systems. This, together with the evidence that a compound of the type RSMe does not influence the DNA radical decay to anything like the extent for that noted with free thiols, substantiates the claim that RSH is essential to the efficient repair of directly induced DNA damage.

The most dramatic effects on DNA radicals in our frozen aqueous systems have been observed at neutral pHs in the presence of free thiols. We believe that this observation is easily understood in terms of hydrogen-atom-transfer reactions occurring between free thiols and the DNA base radicals. Hydrogen-atom abstraction by the base radicals from the thiols

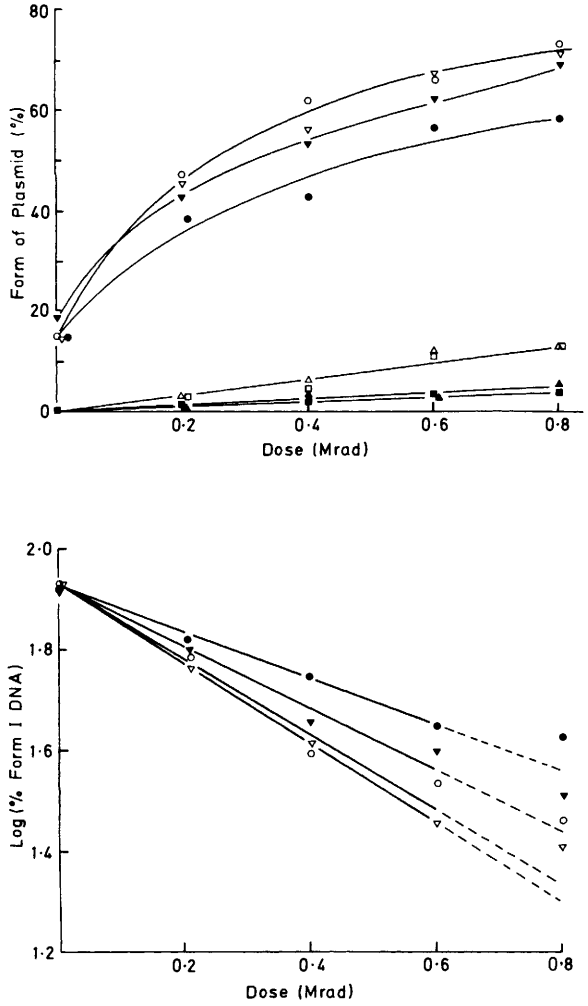
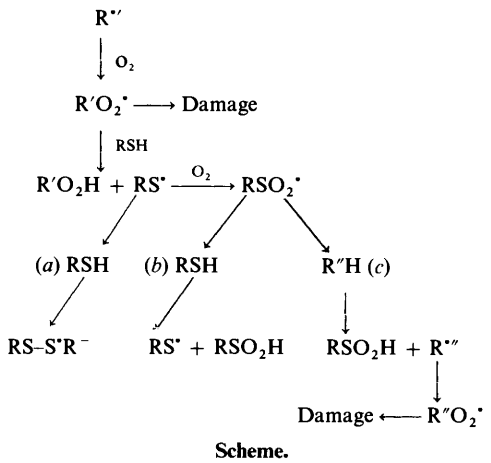


Figure 6. (a) The effect of 2-aminoethanethiol (5 mM) on strand-breaks induced by γ-irradiation of plasmid (pBR 322) at 77 K under oxygenated and deoxygenated atmosphere. The percentage of Form II indicates single strand-breaks produced; ▼ + AET + O_2, ● + AET − O_2, ▽ + O_2, and ○ − O_2. Double strand-breaks formed are indicated by Form III; ▲ + AET + O_2, ■ + AET − O_2, △ + O_2, and □ − O_2.(b) Semi-log plot of single strand-breaks induced in plasmid DNA (pBR 322) following irradiation as in (a); ▼ + AET + O_2, ● + AET−O_2, ▽ + O_2, and ○ − O_2

clearly competes favourably with the intramolecular hydrogen-atom abstraction from neighbouring deoxyribose moieties, postulated above. Since the latter reaction is thought to give rise to strand-breaks, thiols should protect DNA against strand-breaks under these conditions of direct damage and, as discussed above, this is indeed the case.

Under oxic conditions and low thiol concentrations the principal sulphur-containing radical detected by e.s.r. spectroscopy is $RSO_2^{\bullet}$ which arises from addition of O_2 to the thiyl radical RS$^{\bullet}$. As noted above, at lower concentrations of RSH, the effect of RSH on strand-breaks is competitive with the influence of oxygen. These observations are consistent with the reactions shown in the Scheme.

According to this Scheme, hydrogen-atom donation by RSH to the base peroxyl radicals initially occurs to give RS$^{\bullet}$ radicals. These add oxygen to give $RSO_2^{\bullet}$, rather than giving $RSS^{\bullet}R^-$. As the relative [RSH] increases pathway (*b*) begins to dominate, oxygen is consumed, and DNA is protected. Reaction to give $RSS^{\bullet}R^-$ will also occur as [RSH] increases [pathway (*a*)].



Scheme.

At low concentrations of thiol, however, the thiol is effectively consumed and, therefore, pathways (*a*) and (*b*) are prevented. The alternative pathway (*c*) in which the $RSO_2^{\bullet}$ radical abstracts a hydrogen atom from DNA helps to account for the attenuation, by oxygen, of the protection afforded by thiols at low concentrations.

The precise chemical nature of the DNA 'repair' product is of major importance in relation to biological function, which may depend on further enzymatic repair. Under anoxic conditions, the quenching of TH$^{\bullet}$ by thiols leads to TH_2 (reduced thymine) rather than the restoration of the thymine base. That TH_2 is a major identifiable product under conditions of direct irradiation supports our proposals.[52] We have less direct evidence concerning $G^{\bullet+}$. However, if the principal decay pathway for $G^{\bullet+}$ is by loss of a proton to give G(N$^{\bullet}$) as proposed by Hüttermann,[35] then hydrogen-atom donation to G(N$^{\bullet}$) by RSH would fully restore the guanine base. Under oxic conditions these reactions lead to organic peroxides (RO_2H). The reactive nature of these latter products must represent a further danger to the integrity of the DNA.

Dialkyl Disulphides.—Incidental to our primary study of sulphydryl derivatives, we made a brief study of the effect of disulphides on DNA damage.

As expected, these molecules proved to be efficient electron scavengers, features due to $RS^{\bullet}$–SR^- anions being clearly present after irradiation, at the expense of $T^{\bullet-}$ (TH$^{\bullet}$) formation. In this respect, these RSSR molecules protect DNA from electron damage, but there was no suppression of $G^{\bullet+}$ formation, and no appearance of e.s.r. features for $RSS^{\bullet}R^+$ π-radical cations.[51]

Conclusions

We conclude that, under our conditions, thiols act as efficient repair agents. Although this might be seen to be an expected, and therefore trivial, result, there are several reasons why we consider it to be significant:

(i) Most previous studies on pure DNA have involved working with dilute fluid solutions under which conditions attack on DNA is largely by $^{\bullet}OH$ radicals. Our work shows that repair by hydrogen-atom transfer is remarkably effective under conditions of direct damage and involves the base radicals. This leads to a significant reduction of directly induced biologically significant lesions.

(ii) The results support our previous contention that our methods of study can reveal in considerable detail, aspects of the reactions of DNA that are thought to be biologically important.

(iii) The results confirm that, in the absence of oxygen, RS$^{\bullet}$

radicals do not attack DNA but, even at temperatures well below the freezing point of water, are able to find, and react selectively with, other RSH molecules, and that these adducts are efficiently deprotonated to give stable $RS^{\bullet}$–SR^{-} radical-anions.

(iv) In the presence of oxygen the situation is more involved but out results support the contentious idea that RS• radicals can add oxygen to give the peroxide, $RSO_2^{\bullet}$ [48,49,53–55] and that these can lead to further damage, or themselves be repaired.

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