# DNA damage *via* intercalation of copper complexes and activation by ascorbate and peroxides: direct EPR evidence for hydroxyl radical formation and reaction

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EPR spectroscopy provides direct evidence for the intercalation of copper(II) complexes of 1,10-phenanthroline and 2,2′-bipyridine with DNA. Reduction of these complexes to copper(I) by glutathione is evidently facile, whereas ascorbate reacts more slowly. Reoxidation of copper(I) with $^t$BuOOH (to give $^t$BuO$^\bullet$) is rapid in both systems, as judged by EPR spin-trapping results. Reaction of copper(I)–DNA with $H_2O_2$ in the presence of ascorbate leads to the generation of HO$^\bullet$ and to the trapping of DNA-derived radical adducts. The role of 1,10-phenanthroline and ascorbate, especially, and the relevance to DNA scission, are discussed.

## Introduction

Copper is a natural constituent of cell nuclei[1] and has, for example, been suggested to play a key role in the structural organization[2] and function of chromosomes.[3] However, copper may also be toxic in biological systems,[4] especially in the presence of hydrogen peroxide and when activated by cellular reductants, including thiols (see *e.g.* ref. 5). For example, ligands such as 1,10-phenanthroline are known to bring about the degradation of DNA in the presence of copper(I) and hydrogen peroxide[6,7] and have been investigated as possible drug analogues.[8] It has also been suggested on the basis of EPR spin-trapping experiments that the hydroxyl radical is formed in the presence of certain ligands and reductants and that DNA strand scission can be achieved under similar conditions.[9]

We have previously used EPR spectroscopy (to monitor $Cu^{II}$ directly and to monitor free radicals formed in spin-trapping experiments) to explore the ready oxidation of thiols by $Cu^{II}$ [reaction (1)] and the subsequent reoxidation of $Cu^{I}$ by

$$Cu^{II} + 2RSH \longrightarrow Cu^{I}SR + \tfrac{1}{2}RSSR \qquad (1)$$

peroxides [*e.g.* reaction (2)].[10] We have recently[11] contrasted the

$$Cu^{I} + ROOH \longrightarrow Cu^{II} + RO^\bullet + HO^- \qquad (2)$$

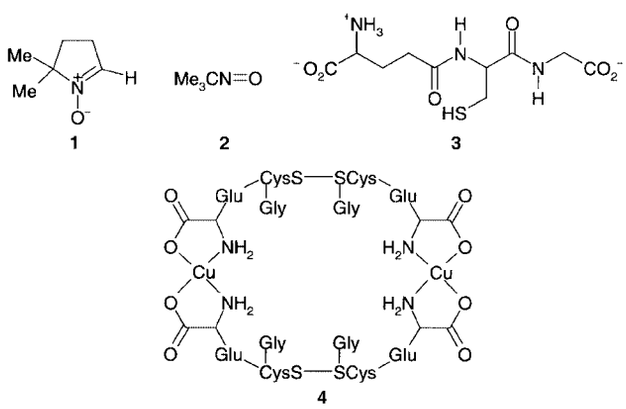
free-radical reaction of $Cu^{I}$–SR [from copper(II) sulfate with glutathione (GSH) and other thiols] with $^t$BuOOH [reaction (2)] and the predominantly non-radical oxidation with $H_2O_2$ [reaction (3)]. In the experiments described here, we explored

$$Cu^{I}SR + H_2O_2 \longrightarrow Cu^{II} + \tfrac{1}{2}RSSR + 2HO^- \qquad (3)$$

the effect of the addition of DNA, together with potential ligands for copper which might intercalate within the nucleic acid, on both the one-electron reduction of copper, with glutathione and with vitamin C, and on the subsequent reoxidation [*cf.* reactions (1)–(3)].

## Results and discussion

Experiments typically involved the recording of aqueous-phase EPR spectra during reduction of $Cu^{II}$ to $Cu^{I}$ and reoxidation to $Cu^{II}$, both in the presence and absence of the spin traps 5,5-dimethylpyrroline *N*-oxide (DMPO) (**1**) and 2-methyl-2-nitrosopropane (MNP) (**2**), the radical adducts of which are expected to have relatively long life-times (from minutes to hours), with EPR spectra which should provide information on the nature of any free radicals formed.[11]




### Reaction of $Cu^{II}$ with glutathione (3) or ascorbate in the presence of DNA: reoxidation reactions with peroxides

**EPR results.** Mixing of copper(II) sulfate ($10^{-3}$ mol dm$^{-3}$) with an unbuffered solution of DNA (type XIV, from herring testes, as the sodium salt, 12.5 mg cm$^{-3}$) resulted in the detection of the broad $Cu^{II}$ EPR signal shown in Fig. 1(a). This is quite distinct from the characteristic signals from aqueous $Cu^{II}$ and is presumably due to complexation of copper with DNA. Addition of glutathione (GSH, **3**, at an equivalent concentration of $10^{-3}$ mol dm$^{-3}$), adjusted to pH 7 with sodium hydroxide, led to a reduction in intensity of the $Cu^{II}$–DNA signal and its replacement by that from a $Cu^{II}$–GSSG complex (**4**), with *g* 2.10 [see Fig. 1(b)].[12,13] This spectrum is identical to that recently described and analysed by Pedersen and co-workers[14] and for which anisotropic parameters, from frozen solutions, indicate a square-planar geometry with two oxygen and two nitrogen ligands in the binding site.[15,16] (This is assigned[14] to a 1 : 1 complex $Cu^{II}$–GSSG with evidence, *via* the occurrence of substantial line-broadening in aqueous solution, for a dimeric structure, see **4**.)

Addition of excess GSH ($5 \times 10^{-3}$ mol dm$^{-3}$) led to the removal of the signals. Subsequent addition of either $H_2O_2$ or $^t$BuOOH ($10^{-2}$ mol dm$^{-3}$) led to the regeneration of copper(II),

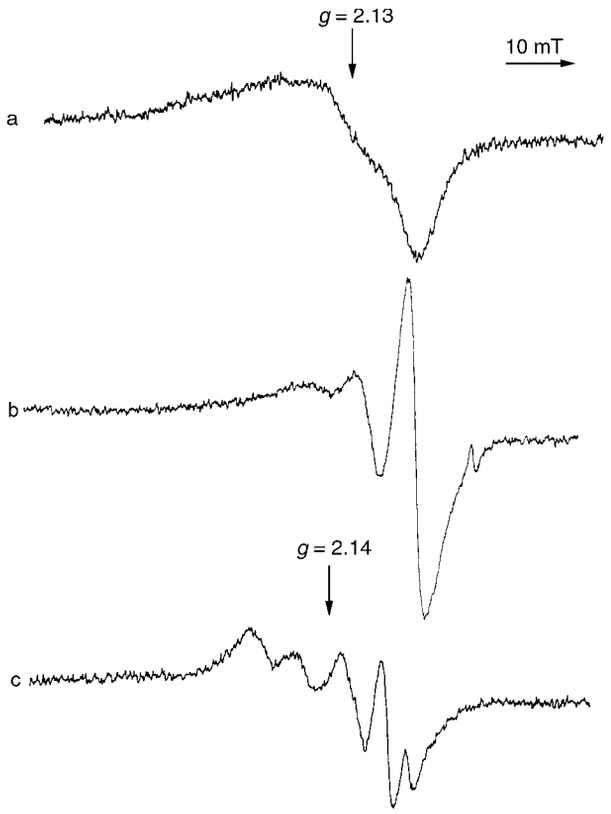
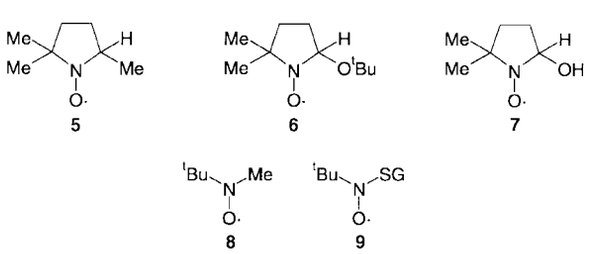



**Fig. 1** EPR spectra of copper(II) complexes: (a) copper(II) sulfate (1 mM) with DNA (12.5 mg $ml^{-1}$); (b) copper(II)–glutathione disulfide formed in the reaction of $CuSO_4$ (1 mM), GSH (5 mM) and $H_2O_2$ (10 mM) in the presence of DNA (12.5 mg $ml^{-1}$); (c) copper(II)–ascorbate complex formed in the reaction of $CuSO_4$ (1 mM), ascorbate (5 mM) and $H_2O_2$ (10 mM) in the presence of DNA (12.5 mg $ml^{-1}$).

as the EPR-detectable $Cu^{II}$–GSSG complex, over a period of about 5 min (noticeably slower than in the absence of DNA).[11]

In analogous experiments with copper(II) sulfate ($10^{-3}$ mol $dm^{-3}$) and ascorbate (added as the sodium salt) at pH *ca.* 4–5, addition of an equivalent concentration of the latter completely removed the EPR signal. Addition of $H_2O_2$ or $^t$BuOOH, as above, led to the regeneration of $Cu^{II}$ over a period of *ca.* 10 min; the resulting spectra [see *e.g.* Fig. 1(c)] have *g* 2.14 and $a_{Cu}$ 6.5 mT, typical of copper–ascorbate complexes (see ref. 13). The structure and spectra (characteristic of copper with four oxygen ligands[16]) have not been investigated further.

When experiments with $CuSO_4$, DNA, $^t$BuOOH and either glutathione or ascorbate were repeated in the presence of DMPO ($10^{-2}$ mol $dm^{-3}$), strong signals were detected from the methyl radical and $^t$BuO$^\bullet$ adducts **5** and **6** with hyperfine




parameters $a_N$ 1.64, $a_H$ 2.35 mT and $a_N$ 1.48, $a_H$ 1.66 mT, respectively (see *e.g.* Fig. 2):[11–13] with ascorbate, signals from the ascorbyl radical (*g* 2.0057, $a_H$ 0.18 mT) were also observed. Similar experiments with $H_2O_2$ gave only an extremely weak signal from the characteristic HO$^\bullet$ adduct (**7**, $a_N$ 1.49, $a_H$ 1.49 mT) with both reductants; in the presence of additional DMSO (as scavenger for HO$^\bullet$) very weak methyl-radical adduct signals

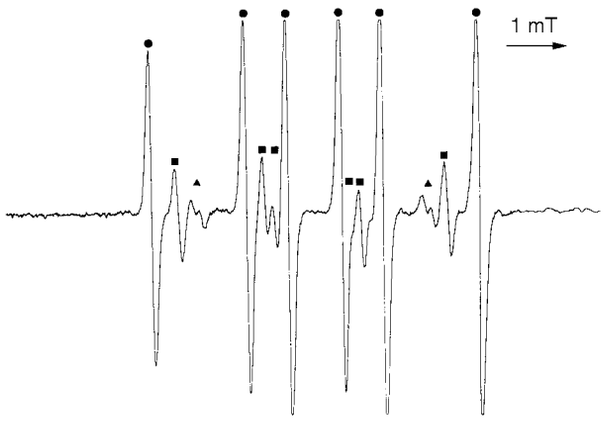



**Fig. 2** EPR spectrum of DMPO spin adducts of Me$^\bullet$ (●)and $^t$BuO$^\bullet$ (■) formed in the reaction of $CuSO_4$ (1 mM), GSH (2 mM), DNA (12.5 mg $ml^{-1}$) and $^t$BuOOH (2 mM) in the presence of DMPO (10 mM). Traces of an ROO$^\bullet$ adduct are also indicated (▲).

were detected, indicating the very much slower formation of HO$^\bullet$ and its reaction to give Me$^\bullet$ (see later).

Related experiments with MNP as spin trap led to the detection of signals from the methyl adduct **8** ($a_N$ 1.73, $a_{3H}$ 1.42 mT),[11] from $^t$BuOOH, and the thiyl adduct **9** (with $a_N$ 1.82 mT)[10] in glutathione reactions involving $^t$BuOOH. A very weak but anisotropic spectrum (characteristic of a DNA adduct) was detected in ascorbate/$H_2O_2$ reactions (the spectrum is discussed later); glutathione and $H_2O_2$ gave a weak spectrum from the thiyl adduct.

**Discussion.** These results strongly suggest that the $Cu^I$ species formed by each reductant reacts with $^t$BuOOH *via* a one-electron transfer reaction to give $^t$BuO$^\bullet$ and hence methyl radical. The absence of DNA adducts suggests that, under these conditions, DNA is not readily susceptible to oxidative damage by Me$^\bullet$ (or, possibly, $^t$BuO$^\bullet$). EPR observations made when GSH is added to $Cu^{II}$ systems in the presence of DNA indicate that GSSG is readily formed and then, as expected, it binds copper(II) more strongly than does DNA. We propose the occurrence of reactions (4) and (5). Subsequent reactions of

$$Cu^{II}(DNA) + 2\,GSH \longrightarrow Cu^{I}(DNA)(SG) + \tfrac{1}{2}\,GSSG + 2\,H^+ \quad (4)$$

$$Cu^{II}(DNA) + GSSG \longrightarrow Cu^{II}(GSSG) + DNA \quad (5)$$

$^t$BuOOH with $Cu^I$, presumed to be present as a $Cu^I$(DNA)(SG) complex (see ref. 17), are indicated by reactions (6)–(8). With

$$Cu^{I}(DNA)(SG) + {}^t\text{BuOOH} \xrightarrow{\text{fast}} Cu^{II}(DNA) + {}^t\text{BuO}^\bullet + \tfrac{1}{2}\,GSSG \quad (6)$$

$$^t\text{BuO}^\bullet \longrightarrow Me^\bullet + Me_2C{=}O \quad (7)$$

$$Me^\bullet/{}^t\text{BuO}^\bullet + GSH \longrightarrow MeH/{}^t\text{BuOH} + GS^\bullet \quad (8)$$

ascorbate, reactions (9) and (10) are correspondingly inferred:

$$Cu^{II}(DNA) + Asc^- \longrightarrow Cu^{I}(DNA) + Asc^\bullet \quad (9)$$

$$Cu^{I}(DNA) + {}^t\text{BuOOH} \xrightarrow{\text{fast}} Cu^{II}(DNA) + {}^t\text{BuO}^\bullet + HO^- \quad (10)$$

we believe that the $Cu^I$(DNA) complex is formed and that it reacts with $^t$BuOOH to give $^t$BuO$^\bullet$.

In the corresponding reactions with $H_2O_2$, $Cu^I$–DNA (in the presence or absence of thiol) generates HO$^\bullet$ slowly [see reactions (11) and (12)]. We note that hydroxyl radicals have

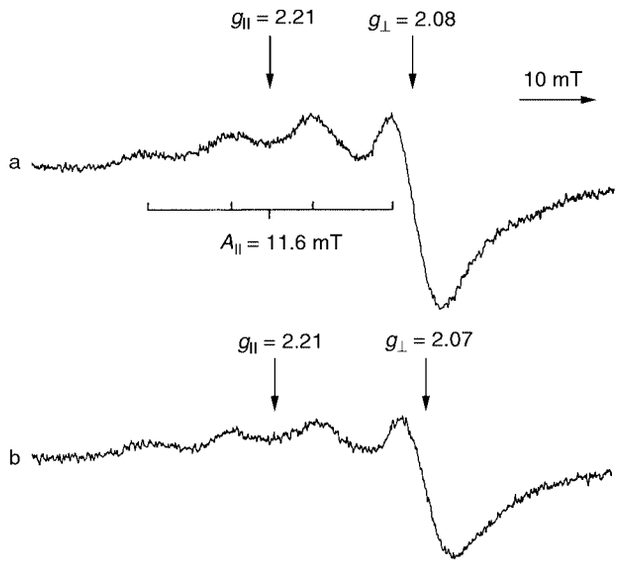



**Fig. 3** Anisotropic EPR spectra of copper(II) complexes (1 mM) in the presence of DNA (12.5 mg $ml^{-1}$) at room temperature in water: (a) $Cu^{II}(phen)_2$–DNA, $g_{\|}$ 2.21, $g_{\perp}$ 2.08, $A(Cu)_{\|}$ 11.6 mT; (b) $Cu^{II}(bipy)_2$–DNA, $g_{\|}$ 2.21, $g_{\perp}$ 2.07, $A(Cu)_{\|}$ 11.7 mT.

$$Cu^{I}(DNA)(SG) + H_2O_2 \xrightarrow{slow} Cu^{II}(DNA) + \tfrac{1}{2}GSSG + 2\,HO^- \quad (11)$$

$$Cu^{I}(DNA) + H_2O_2 \xrightarrow{slow} Cu^{II}(DNA) + HO^{\bullet} + HO^- \quad (12)$$

also been trapped in related experiments[9] with acyclic nitrone traps in the absence of DNA.

### Reactions of other copper complexes with DNA, one-electron reductants and peroxides

**EPR results.** Related experiments were carried out with the bis(ethylenediamine), bis(2,2′-bipyridine) and bis(1,10-phenanthroline) complexes of copper. With copper(II) bis(ethylenediamine) the results were broadly similar to those described above and will not be repeated here. However, markedly different behaviour was observed for the other two ligands, as outlined below.

Firstly, the spectra obtained on mixing the copper complexes ($10^{-3}$ mol $dm^{-3}$) with double-stranded DNA (12.5 mg $ml^{-1}$) in unbuffered aqueous solution exhibit anisotropic features (see Fig. 3, which also gives the appropriate anisotropic $g$ and $A_{Cu}$ parameters) which indicate that copper(II) must now be rigidly held and hence tumbling slowly in solution. The spectrum parameters are similar to those reported for a range of copper(II) complexes involving related ligands including bis(1,10-phenanthroline) (see *e.g.* refs. 18–20) and bis(2,2′-bipyridine) (*e.g.* refs. 21–23). For example, Ogawa and colleagues[18] have described the spectrum from $Cu^{II}(phen)_2$ itself in a rigid matrix at 77 K ($g_{\|}$ 2.24, $g_{\perp}$ 2.045, $A_{\|}$ 16.0 mT), with parameters which indicate a pseudo-square-planar complex[16,19] (see also ref. 20). The lower copper splitting ($A_{\|}$) observed here may indicate some tetrahedral distortion (see *e.g.* ref. 16). The parameters we have observed for the bipyridyl analogue are close to those reported for copper(II) bis(2,2′-bipyridyl) bis(hexafluorophosphate) ($g_{\|}$ 2.253, $g_{\perp}$ 2.060, $A_{\|}$ 1.75 mT) as studied by EPR and X-ray crystallography,[21] which has been characterised as a $CuN_4$ chromophore with compressed tetrahedral structure (as also noted by Siddiqui and Shepherd[22]). As pointed out by the latter authors, the structure is distorted towards *cis* geometry, which was also noted in EPR studies by Marov *et al.*[23] who have distinguished *trans* and *cis* $Cu(bipy)_2$ isomers in aqueous buffers and mixed solvents through their $A_{Cu}$ values (typically *ca.* 16.0 and 12.0 mT, respectively). We conclude that incorporation of the $Cu^{II}(phen)_2$ and $Cu^{II}(bipy)_2$ complexes into DNA is accompanied by a certain amount of (tetrahedral) distortion; this distortion would also explain the lack of observable nitrogen hyperfine splitting.[22]

We also note that intercalation is anticipated since the hydrophobic aromatic ligands would be expected to bind efficiently with the purine and pyrimidine bases in DNA; viscometry, electrochemical and UV spectroscopic studies also provide evidence that interaction with DNA occurs *via* partial intercalation of the middle ring of phenanthroline.[24]

Addition of glutathione [$(1–5) \times 10^{-3}$ mol $dm^{-3}$] brought about a reduction in intensity of the $Cu^{II}$ EPR signal, with complete removal at the higher concentrations of GSH; at intermediate [GSH] the shape of the copper signal was altered, reflecting competition of GSSG to ligate the metal. We believe that GSSG is formed from $Cu^{II}(SG)_2$, probably with the copper removed from the 1,10-phenanthroline/DNA environment prior to reduction [see reactions (13) and (14)]. The rate of

$$Cu^{II}(phen)_2(DNA) + 2\,GSH \xrightarrow{fast} Cu^{II}(SG)_2 + 2\,phen + DNA + 2\,H^+ \quad (13)$$

$$Cu^{II}(SG)_2 \xrightarrow{fast} Cu^{I}(SG) + \tfrac{1}{2}GSSG \quad (14)$$

reduction was notably slower than for the copper complexes of 2,2′-bipyridine and 1,10-phenanthroline themselves. Subsequent addition of either $H_2O_2$ or $^t$BuOOH resulted in the fairly rapid (*ca.* 2 min) regeneration of a $Cu^{II}$ EPR signal [$Cu^{II}$-(GSSG) for bis(2,2′-bipyridine), a mixture of $Cu^{II}$(GSSG) and $Cu^{II}(phen)_2$(DNA) for bis(1,10-phenanthroline)]; in the latter case at least, $Cu^{I}$ is evidently complexed to DNA and the ligand [reaction (15)].

$$Cu^{I}(SG) + 2\,phen + DNA \rightleftharpoons Cu^{I}(phen)_2(DNA) + GS^- \quad (15)$$

In the corresponding spin-trapping experiment with $^t$BuOOH and DMPO, strong signals were obtained for the $Me^{\bullet}$ adduct (and weaker signals from the $^t$BuO$^{\bullet}$ adduct); with MNP, $Me^{\bullet}$ was also detected. We conclude that $Cu^{I}(phen)_2$(DNA) undergoes rapid electron-transfer [reaction (16)]. With $H_2O_2$,

$$Cu^{I}(phen)_2(DNA) + {}^t\!BuOOH \longrightarrow Cu^{II}(phen)_2(DNA) + {}^t\!BuO^{\bullet} + HO^- \quad (16)$$

very weak $HO^{\bullet}$ adduct signals were detected, and traces of a DNA adduct with MNP (see below).

Addition of ascorbate ($5 \times 10^{-3}$ mol $dm^{-3}$) to $Cu^{II}$–bis(1,10-phenanthroline) or $Cu^{II}$–bis(2,2′-bipyridine) in the presence of DNA led to the very slow decay of the anisotropic $Cu^{II}$ signal (with no change in shape). The solution became vividly coloured (purple with 1,10-phenanthroline, dark red with 2,2′-bipyridine), as previously noted[25] for the formation of the appropriate copper(I) complexes. Subsequent addition of either $^t$BuOOH or $H_2O_2$ regenerated $Cu^{II}$ very quickly, with fading of the colour; the EPR spectra comprised a mixture of the anisotropic spectrum and an isotropic spectrum (see *e.g.* Fig. 4) with $g$ 2.12, $a_{Cu}$ 8.44, $a_N$ 2.1 mT. These parameters are characteristic of a four-coordinate (pseudo-square-planar) complex of copper with two oxygens and two nitrogen ligands,[16] presumably from ascorbate and the ligand (phen or bipy). These complexes have not been studied further at this stage.

Parallel spin-trapping experiments with $^t$BuOOH and DMPO gave relatively strong signals from $Me^{\bullet}$ and $^t$BuO$^{\bullet}$, as above. Experiments with $H_2O_2$ gave some broader anisotropic signals (more prominent at high modulation amplitude) attributed to adducts of DNA-derived species (see later). Ascorbate-derived radicals were also trapped.[13] In the presence of DMSO

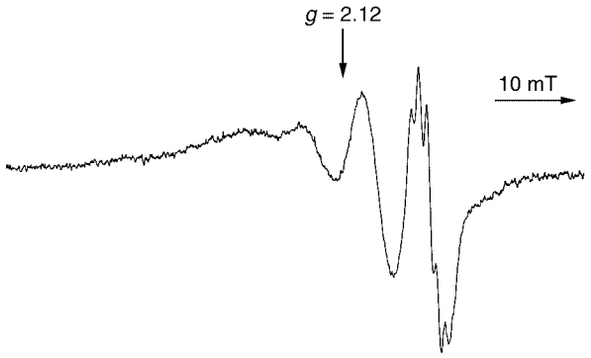



**Fig. 4** EPR spectrum assigned to a copper–bipy–ascorbate complex formed in the reaction of $Cu^{II}(bipy)_2$ (1 mM), ascorbate (5 mM) and $^tBuOOH$ (10 mM) in the presence of DNA (12.5 mg $ml^{-1}$).

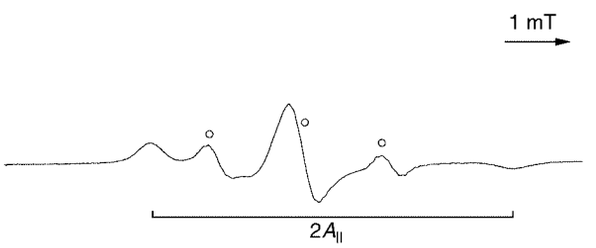



**Fig. 5** EPR spectrum of spin-trapped DNA–radical adduct formed from reaction of $Cu^{II}(phen)_2$ (1 mM), ascorbate (1 mM) and $H_2O_2$ (2 mM) in the presence of DNA (12.5 mg $ml^{-1}$) and MNP (4 mM). Peaks marked (○) indicate a contribution from trapped ascorbate-derived radical (see text).

(1.0 mol $dm^{-3}$), the methyl radical was trapped (in low concentration), suggesting the slow generation of the hydroxyl radical in solution.

More conclusive evidence for radical attack on DNA was obtained in experiments with $Cu^{II}$–bis(1,10-phenanthroline) [or $Cu^{II}$–bis(2,2′-bipyridine)], DNA, ascorbate and $H_2O_2$ in the presence of spin-trap MNP. The spectra (see Fig. 5) provide evidence for relatively high concentrations of highly-immobilized DNA adducts (with $2A_{||}$ 6.20 mT), together with ascorbate-derived species. These signals are noticeably more intense than corresponding weak signals observed with glutathione.

**Discussion.** The relatively slow reduction of $Cu^{II}$ by ascorbate in the presence of DNA and the ligands 1,10-phenanthroline and 2,2′-bipyridine [reaction (17)] compared to that in the

$$Cu^{II}(phen)_2(DNA) + Asc^- \longrightarrow Cu^{I}(phen)_2(DNA) + Asc^{\bullet} \quad (17)$$

absence of DNA clearly indicates that intercalation retards (one-electron) reduction. This may reflect the enforcement of a pseudo-square-planar geometry upon the copper(I) complexes (which prefer a tetrahedral geometry).[25] It is notable that the reduction with GSH is much faster, which suggests the occurrence of a different mechanism, presumably involving a thiolate complex and possible re-insertion into DNA as suggested above. On the other hand, the copper(II) complexes of bis(phenanthroline) and bis(bipyridine) in the presence of ascorbate appear to be particularly damaging towards DNA as indicated by the detection of DNA–radical adducts. These are believed to be formed as shown in reactions (18) and (19) in which the

$$Cu^{I}(phen)_2(DNA) + H_2O_2 \longrightarrow Cu^{II}(phen)_2(DNA) + HO^{\bullet} + HO^- \quad (18)$$

$$HO^{\bullet} + DNA \longrightarrow DNA^{\bullet} \quad (19)$$

hydroxyl radical is generated initially upon reaction of hydrogen peroxide with copper(I); the close association of copper with DNA ensures that this highly-oxidizing radical is generated in close proximity to the DNA. However, it is also notable that addition of DMSO, a known hydroxyl radical scavenger, prevents the formation of detectable DNA–radical adducts, which suggests that this site-specific copper(I)-mediated DNA damage may be intercepted by suitable radio-protectors.

In contrast, reactions of $Cu^I$ with $H_2O_2$ in the presence of glutathione are evidently less damaging. We believe that this reflects the occurrence of reactions of $Cu^I$–SG which, as noted before,[11] are relatively slow and have been shown to proceed predominantly without the release of hydroxyl radicals. Reaction of $Cu^I$–L–DNA, in either system, with $^tBuOOH$ also gives free radicals ($^tBuO^{\bullet}$, $Me^{\bullet}$) but these are evidently formed more slowly and with less damaging consequences, at least as judged by the spin-trapping results.

## Conclusions

Copper(II) complexes of 1,10-phenanthroline and 2,2′-bipyridine are strongly intercalated to DNA at pH *ca*. 7. Reduction of these complexes by glutathione to yield copper(I) is found to be a facile reaction whilst reduction by ascorbate is markedly slower, which may reflect a difference in mechanism whereby copper(II) is sequestered and then reduced by glutathione whilst ascorbate reacts *via* electron-transfer with copper–DNA *in situ*.

*tert*-Butyl hydroperoxide and hydrogen peroxide readily react with the intercalated copper(I) regenerating copper(II) and yielding free radicals in most of the systems investigated. The *tert*-butoxyl radical is efficiently generated in each ligand–reductant–*tert*-butyl hydroperoxide system, but little evidence for DNA damage is revealed by EPR. In contrast, hydrogen peroxide reactions in the presence of ascorbate and appropriate chelators were noted to be potentially damaging, as indicated by the appearance of DNA–radical adducts.

Glutathione, in sequestering copper(II) from DNA complexes [and reducing it to form a copper(I)–thiolate complex], largely prevents the liberation of potentially damaging free radicals. However, in the presence of ligands such as 1,10-phenanthroline, copper(I) evidently re-complexes these ligands, which are closely associated to DNA, and becomes more redox active. The combined effect of vitamin C and 1,10-phenanthroline in producing $HO^{\bullet}$ in these systems is particularly striking. Such systems evidently produce considerable oxidative stress, and the observed nuclease activity of copper(II)–bis(1,10-phenanthroline), in the presence of a reductant and hydrogen peroxide, can be readily understood.[6]

## Experimental

EPR spectra were recorded on either a JEOL JES-RE1X or a Bruker ESP300. Splitting constants were determined to within ±0.01 mT using the spectrometer field scan and *g*-values (for the copper species) to within ±0.005.

All chemicals were obtained from either Aldrich or Sigma and were used as supplied, except for DMPO which was further purified by stirring with activated charcoal for 30 min and then filtering. DNA (type XIV, from herring testes, as the sodium salt) was purchased from Sigma. Peroxide concentrations were accurately determined by iodometric titration. Complexes of copper(II) were simply prepared by mixing the relative amounts of $CuSO_4$ and ligand—a slight excess of ligand over copper was used to ensure total complexation, *i.e.* $Cu^{II}(phen)_2$ solutions contained copper : phen in the ratio 1 : 2.2.

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