Current Topics in Biophysics 2002, 26(1), 137-144

COMPARISON OF 5,5-DIMETHYL-1-PYRROLINE-N-OXIDE (DMPO) AND A NEW CYCLIC NITRONE SPIN TRAP - 5-TERT-BUTOXYCARBONYL-5-METHYL-1-PYRROLINE-N-OXIDE (BMPO) FOR THE TRAPPING OF NITROGEN-CENTRED RADICALS

CLARE L. HAWKINS, MICHAEL J. DAVIES

The EPR Group, Heart Research Institute, Camperdown, Sydney, New South Wales 2050, Australia.

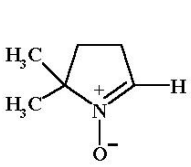
The ability of the commonly used spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and a new cyclic nitrone spin trap, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (BMPO), to give stable, readily detectable, spin adducts with nitrogen-centred radicals, formed on decomposition of nucleoside, peptide, protein and cellular chloramines, has been investigated. It is shown that BMPO gives readily detectable adducts with this type of radical and that the signal-to-noise ratio (i.e. intensity) of the EPR spectra is greater than that of the analogous adducts formed with identical concentrations of DMPO for all substrates studied. This enhanced signal intensity is at least partly due to the longer half-lives of the adducts, which results in a greater accumulation of these species over time. An increased rate of trapping of the initial radicals may also contribute to this enhanced signal intensity. In addition, BMPO is shown to be less prone to the formation of artefactual radical signals (e.g. those from direct oxidation of the spin trap) compared to DMPO. Thus, there are definite advantages in the use of this new cyclic spin trap over DMPO to trap nitrogen-centred radicals in biological systems..

INTRODUCTION

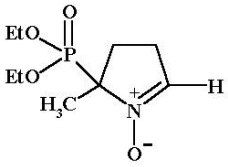
The role of free radicals in biological systems has been investigated extensively using the method of EPR spectroscopy and spin trapping. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO, 1) is probably the most widely utilised spin trap, but has been shown to have several disadvantages. One major limitation of use of DMPO in biological systems is the rapid decay of the DMPO-superoxide adduct to the DMPO-hydroxyl adduct (Finkelstein, Rosen & Rauckman, 1982); this can make the analysis of biological superoxide radical formation difficult. Other problems associated with the use of DMPO as a spin trap are: (a) that the parameters of the hydroxyl radical adduct are very similar to those of the glutathiyl radical adduct, (b) that commercial samples often contain adventitious nitroxides which are difficult to remove, (c) that DMPO is a very hydroscopic low-melting-point solid, (d) that DMPO is readily oxidised to artefactual species such as DMPOX, (e) that many of the adducts formed with heteroatom-centred radicals are relatively short-lived (reviewed in Davies & Timmins, 1996, Mason, 1996).

The development of new substituted traps such as 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO, 2) (Frejaville, Karoui, Tuccio, Le Moigne, Culcasi, Pietri, Lauricella & Tordo, 1994; Frejaville, Karoui, Tuccio, Le Moigne, Culcasi, Pietri, Lauricella & Tordo, 1995; Roubaud, Sankarapandi, Kuppusamy, Tordo & Zweier, 1997b; Clement, Gilbert, Ho, Jackson, Newton, Silveater, Timmins, Tordo & Whitwood, 1998), 5-ethoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (EMPO, 3) (Olive, Mercier, Le Moigne, Rockenbauer & Tordo, 2000), and related species (Roubaud, Mercier, Olive, Le Moigne & Tordo, 1997a; Stolze, Udilova & Nohl., 2000) have helped to (partially) overcome these problems. Thus it has been shown that the superoxide adducts generated from these traps are significantly more stable than the corresponding DMPO species and that these adducts do not decay to give the corresponding hydroxyl radical adducts. However, some of the commercially available preparations of DEPMPO have been reported to be contaminated with nitroxide impurities and therefore require purification before use. Commercial samples of EMPO appear to have fewer contaminating impurities, but this material is a liquid rather than a solid, and is therefore more difficult to handle and

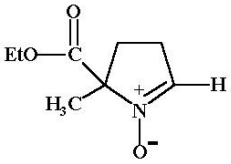
purify. In addition, artefact signals can be observed on extended storage of many spin traps in aqueous solution due to slow hydrolysis reactions (Janzen, 1980, Buettner & Mason, 1990).



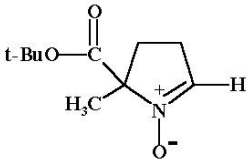
DMPO (1)



DEPMPO (2)



EMPO (3)



BMPO (4)

The synthesis of a new, butoxylated trap, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (BMPO, 4) has been recently described (Zhao, Joseph, Zhang, Karoui & Kalyanaraman, 2001). This trap is a water-soluble, crystalline solid that can be readily purified by recrystallisation. Initial studies with this trap have shown that BMPO can successfully trap the superoxide radical and that this adduct does not decompose to give the hydroxyl adduct. In addition, it was shown that BMPO could trap both the glutathiyl radical and the hydroxyl radical forming more persistent adducts with improved resolution and distinctly different spectral parameters (Zhao *et al*., 2001).

Reaction of HOCl with the free amine groups present on proteins or DNA results in the formation of chloramine species (Weiss, Lampert & Test, 1983; Prutz, 1996). Chloramines are short-lived intermediates with half-lives ranging from minutes to hours depending on the substrate. Chloramine decomposition has been shown to result in the formation of nitrogen-centred radicals with many different substrates (Hawkins & Davies, 1998a, Hawkins & Davies, 1998b, Hawkins & Davies, 1998c, Hawkins & Davies, 2001). These radicals have so far been characterised by spin trapping with DMPO. However, in some cases, the short lifetime of the trapped nitrogen-centred species makes detection of radical formation difficult. Thus, we have now investigated the reaction of various nitrogen-centred radicals with the new spin trap BMPO. The intensity and stability of the resulting radical adducts have been compared to results obtained in analogous experiments with DMPO.

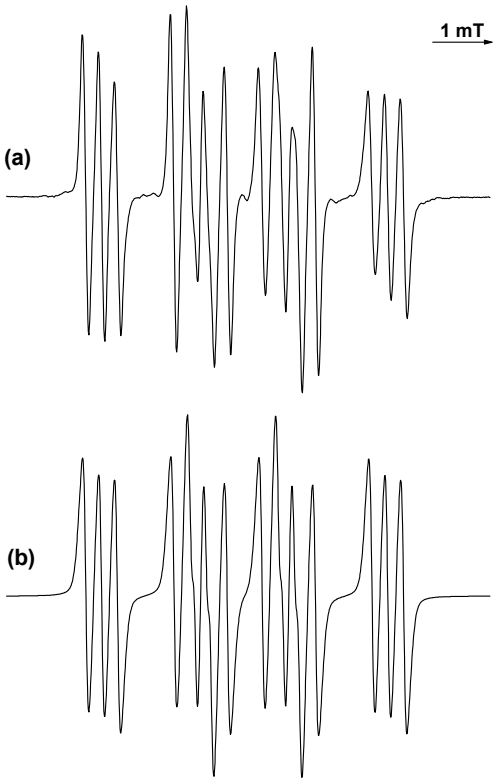


Fig. 1. (a) EPR spectrum observed on reaction of cytidine (25 mM) with HOCl (5 mM) for 2 min at 20 °C before addition of BMPO (9.4 mM), $CuSO_4$ (625 μM) and $TiCl_3$ (500 μM). MA 0.02 mT, gain 2×10^5. (b) Computer simulation of the experimental spectrum using the parameters given in Table 1. Signal assigned to a nitrogen-centred radical formed on the exocyclic NH_2 group of cytidine as a result of decomposition of an initial chloramine

MATERIALS AND METHODS

Materials

Solutions were prepared using water filtered through a four-stage Milli Q system. pH Control was achieved using 0.1 M pH 7.4 phosphate buffer treated with Chelex resin (BioRad, Hercules, CA, USA). All substrates were obtained from Sigma-Aldrich (St Louis, MO, USA). DMPO (ICN, Seven Hills, NSW, Australia) was purified before use by treatment with activated charcoal. BMPO was a gift from Dr. J. Joseph and Prof. B. Kalyanaraman (Biophysics Research Institute, Medical College of Wisconsin, Milwaukee, WI, USA). HOCl solutions were prepared by dilution of a concentrated stock (0.5 M in 0.1 M NaOH) into 0.1 M pH 7.4 phosphate buffer, with the HOCl concentration determined spectro-

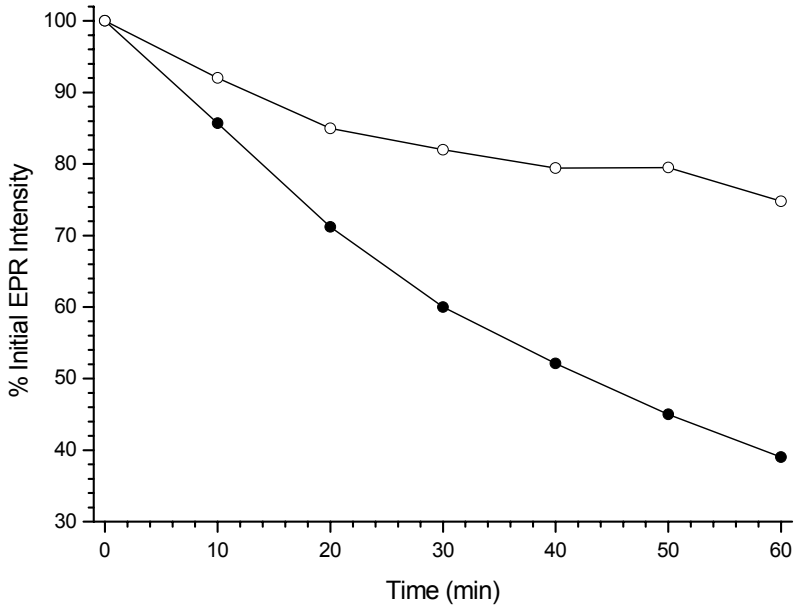


Fig. 2. Stability of the cytidine-derived, nitrogen-centred, radical adducts generated during reaction of cytidine (25 mM) with HOCl (5 mM) for 2 min at 20 °C before addition of either BMPO (o) or DMPO (●) (both 9.4 mM), $CuSO_4$ (625 μM) and $TiCl_3$ (500 μM). Data are expressed as a percentage of the initial radical concentration determined by double integration of the EPR spectra.

photometrically at pH 12 ($\varepsilon_{412\,nm}$ 350 M^{-1} cm^{-1} (Morris, 1966)).

Cell culture

J774 A.1 cells (a murine macrophage-like cell line) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO, USA) containing 10% v/v heat-inactivated fetal calf serum (Gibco BRL, Life Technologies, Melbourne, Australia), 2 mM L-glutamine, 100 units ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA).

Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER4103TM cavity. Samples were contained in a flattened aqueous-sample cell (WG-813-SQ, Wilmad, Buena, NJ, USA) and spectral recording was initiated within 2 min of addition of the spin trap. Hyperfine coupling constants were measured directly from the field scan and confirmed by computer simulation (WINSIM, available at www.epr.niehs.nih.gov). Correlation coefficients between simulated and experimental spectra were > 0.90. Typical EPR spectrometer settings were: gain, $10^5 - 10^6$; modulation amplitude (MA), 0.02-0.1 mT; time constant, 0.16 s; scan time, 84 s; resolution, 1024 points; centre field, 348 mT; field scan, 8 – 10 mT; power 25 mW; frequency, 9.78 GHz; 4 scans averaged.

RESULTS AND DISCUSSION

Nitrogen-centred radicals were generated by either metal-ion catalysed, or thermal, decomposition of chloramines as described in previous studies (Hawkins & Davies, 1998a, Hawkins & Davies, 1998b, Hawkins & Davies, 1998c, Hawkins & Davies, 2001). As BMPO is less soluble in water, or many buffer solutions, than DMPO, lower spin trap concentrations were employed in the current work (ca. 10 mM) than those used in previous studies (where 125–200 mM DMPO was employed (Hawkins & Davies, 1998a, Hawkins & Davies, 1998b, Hawkins & Davies, 1998c, Hawkins & Davies, 2001)), in order to allow a direct comparison to be made between adduct formation by BMPO and DMPO at identical spin trap concentrations.

Spin trapping of nucleoside nitrogen-centred radicals

Initial studies involved reaction of cytidine (25 mM) with HOCl (5 mM) for 2 min at 20°C

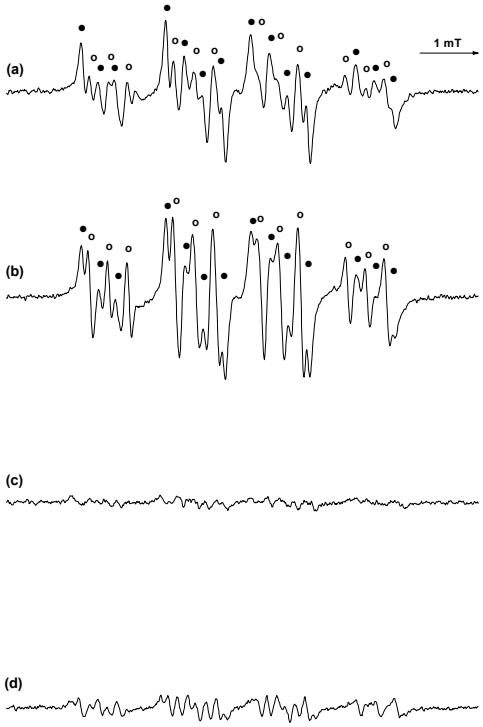


Fig. 3. EPR spectra observed on reaction of guanosine (ca. 4 mM) with HOCl (2.5 mM) for 2 min at 20 °C before addition of the spin trap (9.4 mM) and $CuSO_4$ (625 μM) and $TiCl_3$ (500 μM). (a) with BMPO, t=0; (b) with BMPO, t=10 min after mixing; (c) with DMPO, t=0; and (d) with DMPO, t=10 min after mixing. Signals marked (●) are assigned to a radical centred on a ring NH group, whereas those marked (**o**) are attributed to a radical centred on the exocyclic NH_2 group. These radicals are believed to arise from the decomposition of initial chloramine species at these two sites. Hyperfine coupling constants of the adducts are given in Table 1.

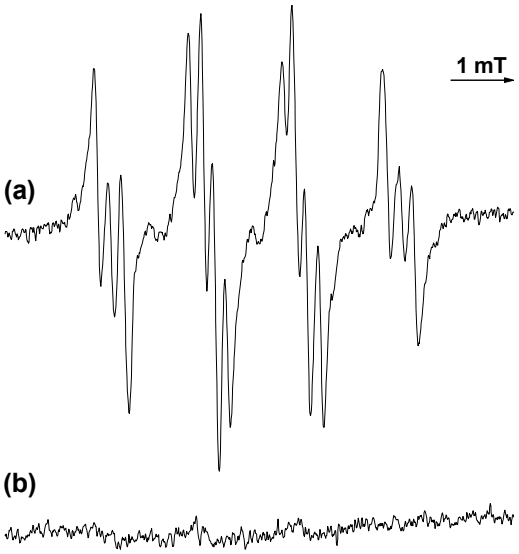


Fig. 4 : EPR spectra observed on reaction of Gly-Gly-Gly (25 mM) with HOCl (5 mM) before addition of (a) BMPO (9.4 mM) and (b) DMPO (9.4 mM) with $CuSO_4$ (625 μM) and $TiCl_3$ (500 μM). Signals observed in (a) are assigned to a nitrogen-centred radical formed on the N-terminal amine group from decomposition of an initial chloramine. Hyperfine coupling constants of the adducts are given in Table 1.

followed by addition of BMPO (9.4 mM) and Cu(I) ions [generated by the sequential addition of Cu(II) (625 μM) and Ti(III) (500 μM)]. This treatment resulted in the detection of intense EPR signals (Fig. 1a). This signal (a_N 1.46, a_H 1.82, a_N 0.26 mT) has been assigned to a nitrogen-centred radical adduct on the basis of the second small 1:1:1 nitrogen-coupling. Omission of any component of the reaction system resulted in the loss of this signal. In the absence of spin trap no signals were detected, whereas omission of the cytidine resulted in the detection of strong signals from BMPO-OH as characterised previously (Olive *et al*., 2000). Nitrogen-centred radical adduct signals were also detected in the corresponding experiments with DMPO, as reported previously (data not shown; Hawkins & Davies, 2001), but these were of reduced intensity (approx. 60% lower than with BMPO). In both systems the observed signals are attributed to the trapping of a nitrogen-centred radical formed on the exocyclic NH_2 group of cytidine as a result of the decomposition of an initial chloramine formed at this position (Hawkins & Davies, 2001). The spectral parameters of the BMPO and DMPO adducts are noticeably different and are given in Table 1.

The stability of the cytidine-derived, nitrogen-centred radicals trapped by BMPO and DMPO was investigated by recording the EPR spectrum at 10 min intervals for 60 min, with the concentration of the adduct (in arbitrary units) calculated by double integration of the spectrum at each time point. The cytidine-derived, nitrogen-centred, BMPO adduct was found to be considerably more stable than the corresponding DMPO adduct (Fig. 2).

Complex EPR signals (Fig. 3a) assigned to two different nitrogen-centred radical adducts (radical 1: a_N 1.44, a_H 1.79, a_N 0.30 mT; radical 2: a_N 1.44, a_H 1.48, a_N 0.33 mT) were observed in analogous experiments with HOCl-treated guanosine (2.5 mM and ca. 4 mM respectively). The relative ratio of radical 2 compared to radical 1 increased at longer incubation times (Fig. 3b). The signals detected with DMPO, which had spectral parameters as reported previously (Hawkins & Davies, 2001), were weaker than

Table 1: Hyperfine coupling constants of nitrogen-centred radical adducts to BMPO and DMPO.

Substrate	Spin trap	Hyperfine coupling constants / mT [a]			Assignment
		a_N	a_H	a_N	
Cytidine	BMPO	1.46	1.82	0.26	Exocyclic NH_2 group
	DMPO	1.55	2.05	0.27	Exocyclic NH_2 group
Guanosine	BMPO	1.44	1.79	0.30	Ring NH group
		1.44	1.48	0.33	Exocyclic NH_2 group
	DMPO	1.53	2.00	0.30	Ring NH group
		1.51	1.68	0.33	Exocyclic NH_2 group
Gly-Gly-Gly	BMPO	1.45	1.65	0.19	N-terminal NH_2 group
	DMPO	1.54	1.79	0.20	N-terminal NH_2 group
BSA + Pronase	BMPO	1.38	1.65	0.27	Lysine side chain NH_2
	DMPO	1.50	1.79	0.29	Lysine side chain NH_2
J774 cells	BMPO [b]	1.38	1.60	0.26	Cell protein-derived
	DMPO	1.46	1.80	0.27	Cell protein-derived

[a] ± 0.01 mT
[b] An additional cell-derived carbon-centred radical adduct with a_N 1.54, a_H 2.12 mT, was also observed.

those detected with BMPO (compare Figs. 3c and d; for parameters see Table 1). With both spin traps, the observed signals are assigned to nitrogen-centred radicals derived from the decomposition of guanosine-derived chloramines with the two species being generated on the ring NH (radical 1) and the exocyclic NH_2 (radical 2) groups as described previously (Hawkins & Davies, 2001).

Spin trapping of peptide and protein nitrogen-centred radicals

Nitrogen-centred radical adducts (a_N 1.45, a_H 1.65, a_N 0.19 mT) were observed on incubation of HOCl-treated Gly-Gly-Gly (5 mM and 25 mM respectively) with Cu(I) ions in the presence of BMPO (Fig. 4a). In contrast, no signals were detected in corresponding experiments with DMPO at an identical spin trap concentration (Fig. 4b). Higher concentrations of DMPO (200 mM) gave rise to nitrogen-centred radical adduct signals (Table 1). This suggests that either the rate of trapping of nitrogen-centred radicals is greater with BMPO than DMPO, or that the adduct formed with BMPO is markedly more stable than that formed with DMPO, which allows this species to accumulate to detectable concentrations.

Reaction of bovine serum albumin (BSA, 250 μM) with a 10-fold molar excess of HOCl (2.5 mM) for 5 min at 20°C followed by addition of BMPO gave broad signals, due to slowly-tumbling adducts with some partially resolved isotropic couplings superimposed (Fig. 5a). The protein-derived adducts were subsequently incubated with the proteolytic enzyme Pronase (5 min at 37°C, 125 μg, 1 unit) to release small, more mobile, fragments from the spin trapped protein and hence give sharper EPR signals. This treatment resulted in the detection of a protein-derived, nitrogen-centred radical adduct (a_N 1.38, a_H 1.65,

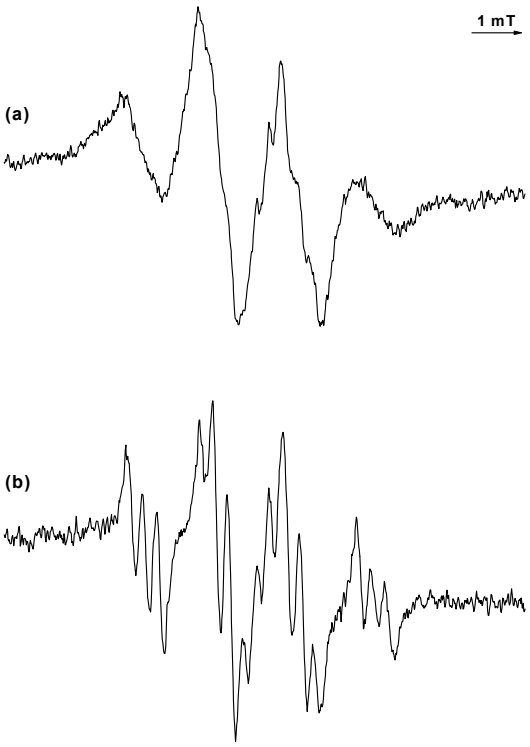


Fig. 5 : (a) EPR spectra observed on reaction of BSA (250 μM) with HOCl (2.5 mM) for 5 min at 20 °C followed by the addition of BMPO (9.4 mM). (b) After treatment of the adducts present in (a) with Pronase (125 μg, 1 unit). The signals observed are assigned to nitrogen-centred radicals formed on lysine side chains as a result of chloramine formation and decomposition. Hyperfine coupling constants of the adducts are given in Table 1.

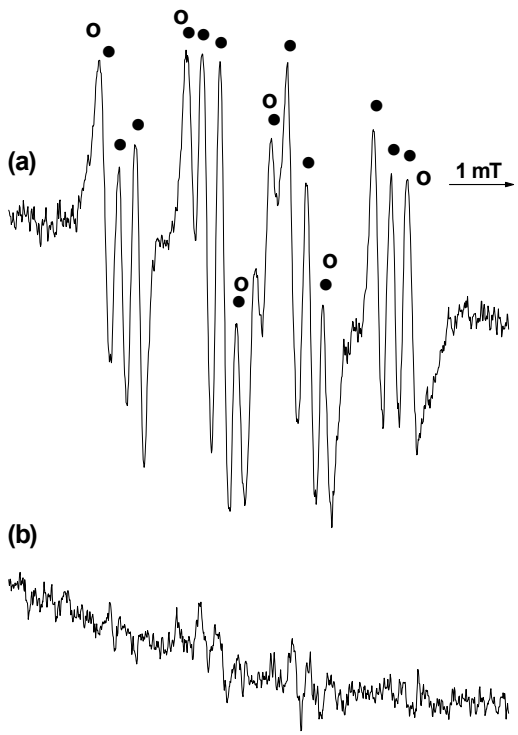


Fig. 6 : EPR spectra observed on treatment of J774 cells (2×10^6 cells/ml) with HOCl (800 nmol/10^6 cells) for 5 min at 20 °C before the addition of (a) BMPO (8.3 mM) and (b) DMPO (8.3 mM). The signals marked (●) are assigned to a cell protein-derived, nitrogen-centred radical. The signals marked (**o**) are attributed to the formation of a cell-derived, carbon-centred radical. Hyperfine coupling constants of the adducts are given in Table 1.

a_N 0.27 mT) (Fig. 5b). Similar, but weaker, signals (approx. 30% lower compared to BMPO) were observed in the corresponding experiments with DMPO (data not shown), with spectral parameters in accord with previous studies (Table 1; Hawkins & Davies, 1998b). These BSA-derived, nitrogen-centred, radicals are believed to arise via the formation and subsequent decomposition of chloramines on lysine side chains as reported previously with DMPO (Hawkins & Davies, 1998b).

Spin trapping of cell-derived nitrogen-centred radicals

The role of radical formation during HOCl-induced cell lysis has recently been investigated using DMPO (Hawkins, Brown & Davies, 2001). Evidence was obtained for the formation of cell protein-derived, nitrogen-centred radicals on addition of HOCl to macrophage-like J774 cells. These cell-derived radical adducts were of low intensity, and the spectral features of the observed radical adducts were significantly distorted by the presence of DMPO-OH. In order to investigate the nature of these radicals in more detail analogous experiments were carried out using BMPO (8.3 mM) as the spin trap. J774 cells (2×10^6 cells/ml) were treated with HOCl (800 nmol/10^6 cells) for 5 min at 20°C and BMPO was subsequently added; this sequence of additions prevents significant direct reaction of HOCl with the spin trap. This treatment resulted in the detection of intense EPR signals (Fig. 6a) consistent with the generation of two different types of cell-derived radical adducts, arising from the trapping of a nitrogen-centred radical, with a_N 1.38, a_H 1.60, a_N 0.26 mT, and a carbon-centred radical with a_N 1.54, a_H 2.12 mT. These species were only observed in the presence of all of the components of the reaction mixture. No signals from BMPO-OH were detected. In contrast when DMPO was employed at identical concentrations only very weak signals were detected (Fig. 6b). Previous studies using higher concentrations of

DMPO (125 mM) gave more intense signals (Hawkins *et al*., 2001), but these were always less intense than those detected with the above (low) concentrations of BMPO. Thus BMPO gives more intense signals than with identical concentrations of DMPO, thereby allowing additional radical adducts to be discerned, and also does not suffer from the presence of distorting features from spin trap-derived oxidation products such as DMPO-OH.

CONCLUSIONS

BMPO has been shown to efficiently trap nitrogen-centred radicals formed on reaction of HOCl with nucleosides, peptides, proteins and cells. In particular, BMPO has been shown to offer major advantages over DMPO in terms of enhanced signal intensity, the detection of additional (previously unobserved) species, and the absence of artefactual signals such as BMPO-OH. Thus in experiments with HOCl-treated J774 cells, BMPO gave considerably more information about the nature of the radical intermediates formed compared to previous experiments carried out with DMPO (Hawkins *et al*., 2001), even when 20-fold lower spin trap concentrations were employed.

The higher radical adduct concentrations detected with BMPO are believed to be due, primarily, to the generation of more stable radical adducts with BMPO (cf. previous data on the increased lifetime of other BMPO adducts when compared to the corresponding DMPO species (Olive *et al*., 2000)), though it is impossible to rule out, in the absence of further data, a contribution from an increased rate of radical trapping. The latter is probably a minor factor, as the steric bulk of the ester function might be expected to hinder the approach of the attacking radical and hence decrease the rate of the radical trapping reaction (cf. data with DEPMPO; (Frejaville *et al*., 1995)). The increased lifetime of the BMPO adducts probably arises from a decreased rate of disproportionation of the radical adducts as a result of the increased steric hindrance afforded by the bulky *tert*-butyl ester substituent at C-5. The spectral parameters of the nitrogen-centred radicals trapped with BMPO are somewhat different to those detected with DMPO, in that the (nitroxide) nitrogen and hydrogen couplings from the trap are respectively approx. 0.1 mT and 0.2 mT smaller with BMPO than DMPO (Table 1). The second nitrogen coupling (from the trapped nitrogen-centred radical) are however similar with both traps. In all of the systems studied, no evidence has been detected for the presence of two stereoisomers that might arise from addition of the attacking radical either *cis* or *trans* to the C-5 t-butyl ester function (Frejaville *et al*., Clement *et al*., 1998). The single isomer detected is presumably that which arises via addition of the attacking radical to the less hindered face of the trap (i.e. trans to the ester group) (Clement *et al*., 1998).

Acknowledgements

The authors are grateful to the Australian Research Council and the Association for International Cancer Research UK for financial support.

REFERENCES

Buettner G. R. & Mason R. P. (1990). Spin-trapping methods for detecting oxygen-radical formation in vitro and in vivo. *Meth. Enzymol.,* **186**, 127-133.

Clement J., Gilbert B. C., Ho, W. F., Jackson, N. D., Newton M. S., Silvester S., Timmins G. S., Tordo P. & Whitwood A. C. (1998). Use of phosphorylated spin trap to discriminate between the hydroxyl radical and other oxidising species. *J. Chem. Soc., Perkin Trans.* **2**, 1715-1717.

Davies, M. J. & Timmins, G. S. (1996). [Chapter title] [In:] Clark, R. J. H. & Hester, R. E. (Eds.), *Biomedical Applications of Spectroscopy* (pp. 217-266), Chichester : John Wiley & Sons Ltd.

Finkelstein E., Rosen G. M. & Rauckman E. J. (1982). Production of hydroxyl radical by decomposition of superoxide spin trapped adducts. *Mol. Pharmacol.,* **21**, 262-265.

Frejaville C., Karoui H., Tuccio B., Le Moigne F., Culcasi M., Pietri S., Lauricella R. & Tordo P. (1994). 5-Diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO): a new phosphorylated nitrone for the efficient in vitro and in vivo spin trapping of oxygen-centred radicals. *J. Chem. Soc. Chem. Comm.*, 1793-1794.

Frejaville C., Karoui H., Tuccio B., Le Moigne F., Culcasi M., Pietri S., Lauricella R. & Tordo P. (1995). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: A new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centred radicals. *J. Med. Chem.*, **38**, 258-265.

Hawkins C. L., Brown B. E. & Davies M. J. (2001). Hypochlorite- and hypobromite mediated radical formation and its role in cell lysis. *Arch. Biochem. Biophys.*, **395**, 137-145.

Hawkins C. L. & Davies M. J. (1998a). Degradation of hyaluronic acid, poly- and mono-saccharides, and model compounds by hypochlorite: evidence for radical intermediates and fragmentation. *Free Radic. Biol. Med.*, **24**, 1396-1410.

Hawkins C. L. & Davies M. J. (1998b). Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. *Biochem. J.*, **332**, 617-625.

Hawkins C. L. & Davies M. J. (1998c). Reaction of HOCl with amino acids and peptides: EPR evidence for rapid rearrangement and fragmentation reactions of nitrogen-centered radicals. *J. Chem. Soc. Perkin Trans. 2*, 1937-1945.

Hawkins C. L. & Davies M. J. (2001). Hypochlorite-induced damage to nucleosides: formation of chloramines and nitrogen-centered radicals. *Chem. Res. Toxicol.*, **14**, 1071-1081.

Janzen E. G. (1980). A critical review of spin trapping in biological systems. [In:] Pryor, W. A (Ed.) *Free radicals in biology*, Vol. IV (pp. 115-154) New York: Academic Press.

Mason R. P. (1996). *In vitro* and *in vivo* detection of free radical metabolites with electron spin resonance. **[**In:] Punchard, N. A. & Kelly, F. J. (Eds.) *Free Radicals. A practical approach* (pp. 11-24) New York: Oxford University Press, New York.

Morris J. C. (1966). The acid ionization constant of HOCl from 5^{o}C to 35^{o}C. *J. Phys. Chem.*, **70**, 3798-3805.

Olive G., Mercier A., Le Moigne F., Rockenbauer A. & Tordo P. (2000). 2-Ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide: evaluation of the spin trapping properties. *Free Radic. Biol. Med.*, **28**, 403-408.

Prutz W. A. (1996). Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Arch. Biochem. Biophys.*, **332**, 110-120.

Roubaud V., Mercier A., Olive G., Le Moigne F. & Tordo P. (1997a). 5-(Diethoxyphosphorylmethyl)-5-methyl-4,5-dihydro-3H-pyrrole N-oxide: synthesis and evaluation of spin trapping properties. *J. Chem. Soc., Perkin Trans.* **2**, 1827-1830.

Roubaud V., Sankarapandi S., Kuppusamy P., Tordo P. & Zweier J. (1997b). Quantitative measurement of superoxide generation using the spin trap 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide. *Anal. Biochem.*, **247**, 404-411.

Stolze K., Udilova N. & Nohl K. (2000). Spin trapping of lipid radicals with DEPMPO-derived spin traps: detection of superoxide, alkyl and alkoxyl radicals in aqueous and lipid phase. *Free Radic. Biol. Med.*, **29**, 1005-1014.

Weiss S. J., Lampert M. B. & Test S. T. (1983). Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science*, **222**, 625-628.

Zhao H., Joseph J., Zhang H., Karoui H. & Kalyanaraman B. (2001). Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals. *Free Radic. Biol. Med.*, **31**, 599-606.