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ELECTRON SPIN RESONANCE STUDIES ON THE DEGRADATION OF HYDROPEROXIDES BY RAT LIVER CYTOSOL

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Incubation of ^{t}BuOOH (in the concentration range 200 μM to 20 mM) with rat liver post-microsomal supernatant in the presence of the spin trap DMPO gives three radical species, which can be observed by electron spin resonance spectroscopy. The first of these is the ascorbyl radical (which decreases in concentration with time), the other two are identified as spin adducts of alkoxyl and carbon-centred radicals; these latter species increase in concentration with time. Addition of NADH, but not NADPH, led to an increase in concentration of the alkoxyl and carbon-centred radical adducts and a decrease in the concentration of the ascorbyl radical. Results obtained in the presence of iron chelators and other ligands suggest that the generating system is an NADH-dependent enzyme that reduces ^{t}BuOOH by one-electron to give initially the ^{t}BuO· radical. Results from experiments carried out on dialysed cytosol samples lend support to this conclusion.

KEY WORDS: Electron spin resonance, radicals, hydroperoxides, spin trapping, alkoxyl.

ABBREVIATIONS: ESR, electron spin resonance: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

INTRODUCTION

The breakdown of hydroperoxides by metal-ion complexes is known to yield free radicals; the significance of this type of process in biological systems has been and still is the subject of considerable attention. Whilst information has been obtained about the role of metal-ion chelates (particularly those of an endogenous nature such as haem-proteins, iron-storage and iron-transport proteins) as catalysts in the breakdown of hydrogen peroxide[1-7] and alkyl hydroperoxides[8-14] in *in vitro* systems, very much less is known concerning their role in more complex, heterogenous, biological systems.

It has been known for a number of years that rat hepatocytes metabolize simple alkyl hydroperoxides such as ^{t}BuOOH mainly through a two-electron (non-radical) reduction process mediated by glutathione peroxidase.[15-17] However the observation that hepatocytes are protected from damage induced by relatively high concentrations of ^{t}BuOOH by radical-scavenging antioxidants such as catechol[18] suggest that under certain circumstances (possibly through over-loading of the glutathione peroxidase pathway or depletion of reducing equivalents) radical production can be a significant factor in the cytotoxicity of such hydroperoxides.

One possible source of catalytically-active iron in rat hepatocytes is the haem-proteins of the endoplasmic reticulum and in particular the cytochrome P_{450} system.[19-22] Radical production from ^{t}BuOOH by such systems has recently been directly

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investigated by ESR spin trapping,[23] and it has been shown that radical generation can be a significant process under certain circumstances (particularly when reducing equivalents in the form of NADH or NADPH are provided). Recent studies on the activation of this, and related, hydroperoxides by keratinocytes[24] have however shown, by use of similar techniques, that metabolism occurs mainly in the *cytosolic* fraction, though the actual generating system has not been determined. These studies have prompted an examination of rat liver post-microsomal supernatant (cytosol) to determine whether a similar radical-generating source is present and if so the relative significance of this process relative to other fractions such as the previously examined microsomal system.[23]

MATERIALS AND METHODS

Post-microsomal supernatant samples (material not pelleted after centrifugation of liver homogenates twice at 14,000 × g and twice at 105,000 × g) were prepared from adult male albino rats (200–300 g) maintained on a standard laboratory diet. Protein concentrations were determined by the biuret method.[25] Purified supernatant samples were obtained by dialysis for 12 hours against 2 × 100 volumes of 50 mM pH 7.4 phosphate buffer at 4°C. ^{t}Butyl hydroperoxide (^{t}BuOOH) and all other chemicals were commercial samples of the highest available purity and used as supplied with the exception of DMPO, which was purified before use as described previously.[26] All solutions were prepared in air-saturated double-distilled water.

ESR spectra of incubations contained in a standard aqueous sample cell were recorded at 21°C, at fixed times after mixing, using a Bruker ESP 300 spectrometer equipped with 100 kHz modulation and a Bruker ERO35M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan and compared with previously reported values.[14,32] Percentage changes in signal heights (which are directly proportional to radical concentrations for a given radical) were determined by measurement of peak-to-peak line heights for identical lines of a given adduct on spectra recorded with identical spectrometer settings.

RESULTS AND DISCUSSION

Incubation of post-microsomal supernatant (ca. 4 mg protein/ml, final concentration) with 10 mM ^{t}BuOOH in the presence of 40 mM DMPO in 20 mM phosphate buffer, pH 7.4, resulted in the detection, immediately after mixing, of absorptions from three radical species (Figure 1). The first of these signals, which consists of an intense doublet, has a g value and hyperfine coupling constant identical to those previously reported for the ascorbyl (vitamin C) radical,[27] and is assigned to this species. Supportive evidence for this assignment was obtained by carrying out similar experiments in the absence of DMPO; an identical signal was observed confirming that this species was not an adduct to the spin trap. The other two species, which were not observed in the absence of DMPO, are assigned on the basis of their hyperfine coupling constants, and comparison with previous data,[14,23] to a carbon-centred radical adduct and an alkoxyl radical adduct. As with the microsomal studies,[23] the isotropic nature of these signals, with no noticeable broadening of the high field lines, suggests that the radicals trapped are all relatively small in size and that the spin

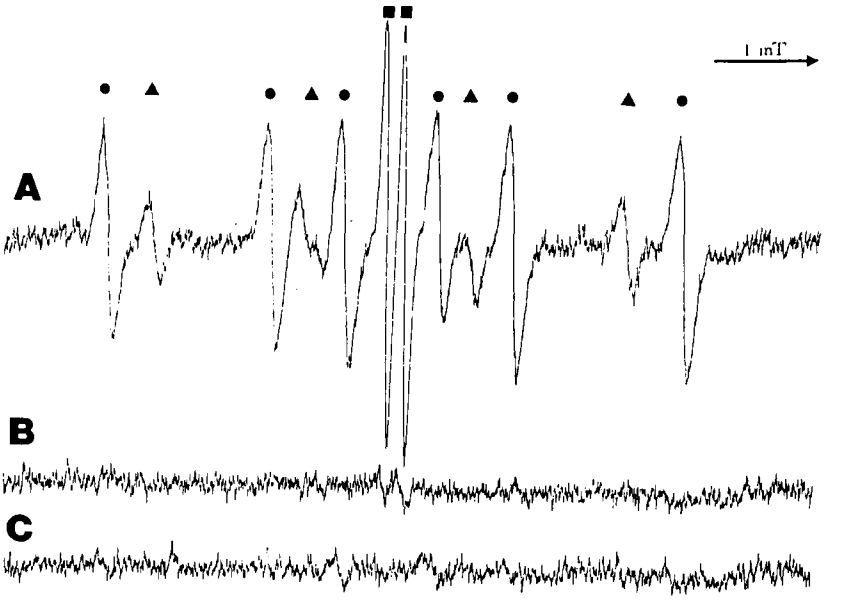


FIGURE 1 ESR spectrum observed on reaction of rat liver post-microsomal supernatant (4 mg protein/ml) with 10 mM ^{t}BuOOH in the presence of 40 mM DMPO in 20 mM phosphate buffer pH 7.4. Spectra recorded 90 s after mixing. (A) Complete system. (B) ^{t}BuOOH omitted. (C) Supernatant omitted. Signals assigned to the ascorbyl radical (■), an alkoxyl radical adduct (▲; a_N 1.488, a_H 1.600 mT), and carbon-centred radical adducts (●; a_N 1.640, a_H 2.352 mT). ESR spectrometer settings: gain 1 × 10,^{6} modulation amplitude 0.1 mT, time constant 320 ms, scan time 300 s, field 347.5 mT, scan 8 mT, power 10 mW, frequency 9.769 GHz.

adducts are undergoing rapid tumbling, i.e. are free in solution. These observations would suggest that the alkoxyl radical that is trapped is the ^{t}BuO· radical. The identity of the carbon-centred species that is trapped cannot be determined with any degree of certainty; it may be a composite signal arising from several small radicals as well as the methyl radical produced from the ^{t}BuO· radical by β-fragmentation. All components of the system were necessary for the observation of the carbon and alkoxyl radical adducts. The signal heights (radical concentrations) of all three species were dependent on the amount of cytosol used (as measured by protein-concentration), and, to a certain extent, the concentration of ^{t}BuOOH. In the absence of added ^{t}BuOOH very weak signals from the ascorbyl radical were still observed; at all other concentrations of hydroperoxide investigated (200 μM-20 mM) all three signals were detected with only a slight increase in the intensity of the signals observed on increasing the hydroperoxide concentration within this range.

In experiments where 10 mM ^{t}BuOOH, 40 mM DMPO and cytosol (ca. 4 mg. protein/ml, final concentrations) were employed, successive scans of the samples showed that the intensity (concentration) of both the carbon-centred and alkoxyl radical adducts increased with time (see Figure 2), demonstrating that the system that generates these species continues to do so for some considerable period of time; this would suggest either a slow stoichiometric reaction or a catalytic cycle that is not readily inactivated. In contrast the intensity of the ascorbyl radical signal decreased

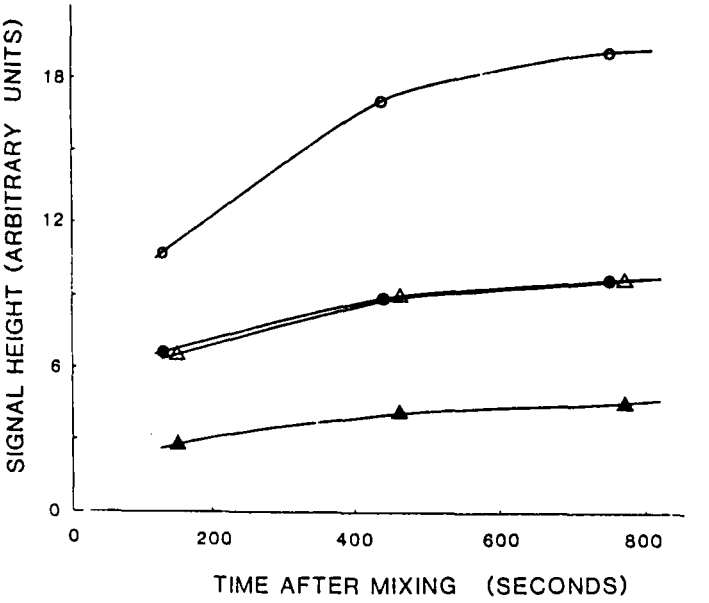


FIGURE 2 Effect of time and NADH on the relative radical concentrations (as measured by peak heights of e.s.r. signals) for a ^{t}BuOOH (10 mM), DMPO (40 mM), rat liver post-microsomal supernatant (4 mg protein/ml) system. Filled symbols in absence of added NADH, open symbols in presence of added NADH (200 μM). (▲, △) alkoxyl radical adduct, (●, ○) carbon-centred radical adduct. ESR spectrometer settings as Figure 1.

with time which would be in accord with the ascorbate acting as a radical scavenger, which is ultimately used up.

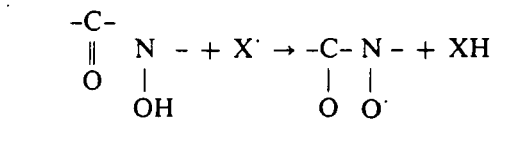
In an effort to further characterize the source of these radicals the effects of a number of possible co-factors, inhibitors, antioxidants and chelators were examined.

Inclusion of reducing equivalents in the form of NADH (either 80 μM or 200 μM final concentration) resulted in the detection of identical species though there was a significant increase in the rate of build-up of both the carbon-centred and alkoxyl radical adducts and a more rapid disappearance of the ascorbyl radical signal with time (see Figure 2). No other radical species were observed. Substitution of NADPH for NADH at similar concentrations again gave identical radical signals, but at a slightly lower initial intensity (ca. 30% inhibition relative to controls). No stimulation of the build-up of the carbon-centred and alkoxyl radical adducts and no increase in the rate of decrease of the ascorbyl radical signal were observed. This slight inhibition of radical production may be due to stimulation of two-electron (non-radical) reduction of the hydroperoxide by glutathione peroxidase via provision of reducing equivalents for glutathione reductase, which regenerates reduced glutathione (an essential co-factor for glutathione peroxidase).[15,16]

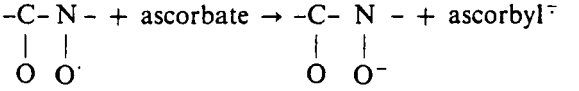
These results are consistent with the carbon-centred and alkoxyl radicals being generated from the hydroperoxide via a reductive process mediated by an enzyme that is specific for NADH.

Further evidence for the production of the R· and RO· radicals via an enzymatic pathway rather than by a low-molecular weight iron catalyst was obtained from experiments where the effects of superoxide dismutase (SOD) and iron chelators were

examined. Inclusion of SOD (0.1 mg/ml final concentration) in a standard cytosol (ca. 4 mg protein/ml), ^{t}BuOOH (10 mM) and DMPO (40 mM, all final concentrations) system did not produce any significant alterations in either the species observed or their rate of build-up or decay. This behaviour is inconsistent with the radical generation occurring via a superoxide-driven Fenton reaction involving a low-molecular weight iron chelate.[28] Pre-incubation of the cytosol fraction with either desferrioxamine (Desferal) or rhodotorulic acid (100 μM final concentration in each case) for 5 minutes before addition of the DMPO and ^{t}BuOOH resulted in a slight decrease in the initial concentration of both the R· and RO· adduct signals (by $\simeq$ 30% in each case) and a minor degree of inhibition of the subsequent build-up of these adducts. The intensity of the ascorbyl radical signal was also marginally decreased in intensity at all time points studied. The behaviour of these two compounds, which can act as both extremely potent chelators of "free" iron (but not iron in prosthetic groups within enzymes) and as free radical scavengers, is consistent with the latter mode of action rather than the former, because a chelating action would be expected to have a much more dramatic effect on both the initial radical concentrations and the subsequent build-up of the adducts. In several previous cases where desferrioxamine and rhodotorulic acid have been shown to act as radical scavengers the nitroxide radical that results from hydrogen-abstraction at the hydroxamic acid groups present in these compounds (reaction 1) has been observed[29,30] [M.J. Davies unpublished experimental observations]. Such species are not observed in this particular case presumably due to the extremely rapid reduction of these nitroxide radicals by ascorbate,[29] which is present in these reaction mixtures (reaction 2).



(1)



(2)

The effect of a number of inhibitors on this enzymatic radical-generation system have been investigated. Pre-incubation of the cytosol fraction with KCN (10 mM final concentration) for 5 minutes before addition of DMPO (40 mM) and ^{t}BuOOH (10 mM) resulted in a marked *increase* in the initial concentration of both the R· and RO· adducts (by 300% and 216% respectively versus control samples). At longer time points the adduct signals increased in intensity at approximately the same rate as in the absence of CN^-. That the observed increase in signal intensities is not due to the trapping of ·CN produced by scavenging of the initially produced radicals by CN^- (reaction 3) is shown by the fact that the signals assigned to both the carbon-centred radical *and* the alkoxyl radical increase in intensity and that the parameters of these signals are inconsistent with those previously reported for the trapping of this radical by DMPO.[31]

$$X\cdot + CN^- \rightarrow X^- + \cdot CN \qquad (3)$$

Inclusion of N_3^- (10 mM, final concentration) in place of CN^- produced somewhat different results in that slight decreases (ca. 30%) in the intensity of all the species were observed when compared to control incubations. This relatively minor effect is

attributed to scavenging of the initial radicals by N_3^-, though no evidence for the trapping of $N_3\cdot$ radicals, which would be produced by this process (analogous to reaction 3), was obtained, though it is known that under certain conditions $N_3\cdot$ radical adducts to DMPO can be observed.[32] The effects observed on inclusion of these two ligands suggests that the enzyme(s) which generate the observed hydroperoxide-derived radicals do not contain a haem prosthetic group as it is known that most haem-containing enzymes and proteins are inhibited by one or both of these species (c.f. results obtained in corresponding studies on rat liver microsomal fractions[23] and isolated haem-proteins[14]). The apparent *stimulation* of radical production by CN^-, which is somewhat surprising, may be due to an inhibitory effect of this ligand on another competing non-radical pathway(s), which is also removing ^{t}BuOOH and hence altering the effective concentration of the substrate. One such possibility is inhibition of glutathione peroxidase which would lower the concentration of ^{t}BuOOH by two-electron reduction; such a process has been previously observed both with the highly purified enzyme and crude cytosol fractions.[33]

If the analysis of the above experiments is correct, in that the generating system is enzymatic and requires NADH as a co-factor, then dialysis of the cytosol fraction to remove endogenous NADH and ascorbate should result in a marked decrease in the concentration of each of the radical species observed, and the production of the R· and RO· should be restored on replacement of NADH. This proved to be the case. Purified cytosol (dialysed for 12 hours against 50 mM pH 7.4 phosphate buffer at 4°C) incubated, at similar protein concentrations to the initial experiments, with 40 mM DMPO and 10 mM ^{t}BuOOH gave weak residual signals from the R· and RO· adducts together with very weak signals from a third species, which are assigned to a ROO· adduct by comparison with previous data.[14,23] No signals due to the ascorbyl radical were observed. The intensity of each of the adduct signals built up at a very slow rate over successive scans. Omission of any of the components of the system resulted in the loss of all signals. Addition of NADH (either 80 μM or 200 μM final concentrations) to the above incubation mixture produced a greater initial concentration of R· and RO· adducts and the intensity of these signals built up with time. The overall radical concentration (as measured by signal heights) was somewhat lower with the dialysed samples than with the undialysed samples at any particular time: this is presumably due to enzyme inactivation during the dialysis period. Inclusion of NADPH in place of NADH at identical concentrations did not have any stimulatory or inhibitory effects.

CONCLUSIONS

The evidence presented above suggests that there is a NADH-dependent enzyme present in rat liver post-microsomal supernatant that can reduce ^{t}BuOOH by one-electron, resulting in the production of both alkoxyl (believed to be ^{t}BuO·) and carbon-centred radicals, which have been trapped with the spin trap DMPO and identified by e.s.r. spectroscopy. These radicals are believed to arise via reactions 4–6.

$$^t\text{BuOOH} + e^- \rightarrow {}^t\text{BuO}\cdot + HO^- \quad (4)$$

$$^t\text{BuO}\cdot \rightarrow CH_3\cdot + CH_3COCH_3 \quad (5)$$

$$^t\text{BuO}\cdot + RH \rightarrow {}^t\text{BuOH} + R\cdot \quad (6)$$

The observation of these radicals is dependent on both the protein concentration and the presence of ^tBuOOH. In the absence of the spin trap, only the ascorbyl (vitamin C) radical is observed. This is believed primarily to be produced by scavenging of the $^tBuO\cdot$ and/or $R\cdot$ radicals (reaction 7).

$$^tBuO\cdot/R\cdot + \text{ascorbate} \rightarrow {}^tBuO^-/R^- + \text{ascorbyl}^-\cdot \quad (7)$$

The relatively minor effects of the iron chelators and the fact that radical production is still observed after dialysis demonstrates that the radical generating system does not involve a low-molecular weight iron chelate, and the lack of inhibition by CN^- and N_3^- suggests that the catalytic centre is not a haem prosthetic group. Further experiments to characterize this radical-generating system are being carried out.

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