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Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with rat liver microsomal fractions

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E.s.r. spin trapping using the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used to detect peroxyl, alkoxyl and carbon-centred radicals produced by reaction of t-butyl hydroperoxide (^{t}BuOOH) with rat liver microsomal fraction. The similarity of the hyperfine coupling constants of the peroxyl and alkoxyl radical adducts to those obtained previously with isolated enzymes suggests that these species are the ^{t}BuOO$^•$ and ^{t}BuO$^•$ adducts. The effects of metal-ion chelators, heat denaturation, enzyme inhibitors and reducing equivalents demonstrate that these species arise from reaction of ^{t}BuOOH with a haem enzyme such as cytochrome *P*-450 or cytochrome b_5. In the absence of NADPH or NADH the previously undetected peroxyl radical adduct is the major species observed. In the presence of these reducing equivalents the alkoxyl and carbon-centred radical adducts predominate, which is in accord with product studies on similar systems. These results demonstrate that both reductive and oxidative decomposition of ^{t}BuOOH can occur in rat liver microsomal fraction with the reductive pathway favoured in the presence of NADH or NADPH.

INTRODUCTION

The breakdown of hydroperoxides by metal-ion complexes is thought to be of considerable biological importance. Controversy exists as to the mechanism(s) of degradation and the identity of the species produced both *in vivo* and *in vitro*. Product studies have suggested that alkoxyl-radical (RO$^•$) formation is of considerable importance, as alcohols (ROH) and alkoxyl-radical fragmentation products (ketones and aldehydes) are the major metabolites detected (McCarthy & White, 1983; Lee & Bruice, 1985; Thompson & Wand, 1985; Larroque & Van Leir, 1986; Weiss & Estabrook, 1986; Vaz & Coon, 1987). Spectroscopic studies (mainly e.s.r.) on the intermediates formed in model systems have, however, also detected peroxyl radicals (ROO$^•$), as well as RO$^•$ and R$^•$ species, suggesting that the overall mechanism may be considerably more complex (Rosen & Rauckman, 1980; Kalyanaraman *et al.*, 1983; Thornalley *et al.*, 1983; Davies, 1988). E.s.r. studies using the cyclic nitrone DMPO have shown that this compound can trap, and hence allow detection of, ROO$^•$, RO$^•$ and R$^•$ radicals arising from decomposition of hydroperoxides by both model porphyrins and haem enzymes (Rosen & Rauckman, 1980; Kalyanaraman *et al.*, 1983; Thornalley *et al.*, 1983; Davies & Slater, 1987; Taffe *et al.*, 1987; Davies, 1988); the present study was carried out to determine whether it is possible to detect similar species in microsomal fractions (referred to below simply as microsomes) and hence allow further information on the mechanism(s) of hydroperoxide decomposition in biological systems to be obtained.

EXPERIMENTAL

Methods

Washed liver microsomes were prepared from adult male albino rats (200–300 g) maintained on a standard laboratory diet as described previously (Slater & Sawyer, 1971). Microsome pellets were resuspended before use in 0.15 M-KCl such that 1 ml of suspension was equivalent to 1 g wet wt. of liver. Protein concentrations were determined by the biuret method (Gornall *et al.*, 1949). 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 (Beuttner & Oberley, 1978). All solutions were prepared in air-saturated double-distilled water.

E.s.r. studies

Spectra of incubations (22 °C) contained in an aqueous-sample cell were recorded 90 s after mixing with a Bruker ESP 300 spectrometer equipped with 100 kHz modulation and a Bruker ER035M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan and compared with previously reported values (Davies, 1988). Where necessary, spectra were scanned repeatedly with 90 s intervals. Percentage changes in signal intensities were determined by measurement of peak-to-peak line heights for the relevant adducts on spectra recorded with the use of identical spectrometer settings at similar times after mixing.

RESULTS

Incubation of microsomes (8 mg of 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 observation of three radical species (Fig. 1). The hyperfine coupling constants of the signals identify these species as being due to the DMPO adducts of a carbon-centred radical, an alkoxyl radical and peroxyl radical by comparison with data obtained from model systems (Davies, 1988). The isotropic nature of the spectra from all these adducts suggests that the radicals

Abbreviation used: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

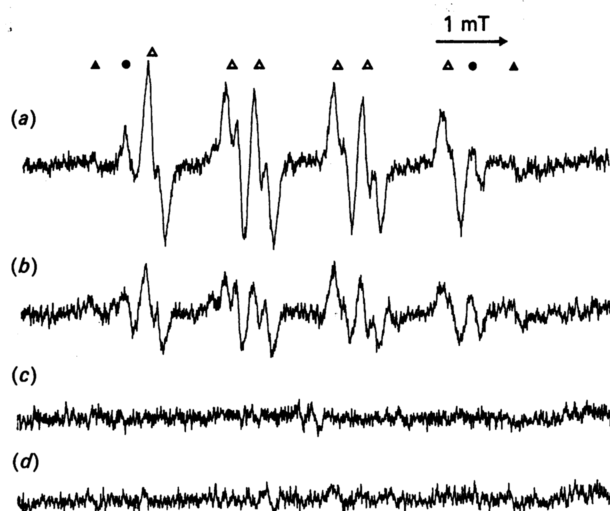


Fig. 1. E.s.r. spectra observed on reaction of rat liver microsomes (8 mg of protein/ml) with 10 mM-^{t}BuOOH in the presence of 40 mM-DMPO

Spectra of incubations (22 °C, air-saturated, pH 7.4) were recorded 90 s after mixing with the exception of (*b*), which was recorded 9 min after mixing. (*a*) and (*b*) Complete system. (*c*) ^{t}BuOOH omitted. (*d*) Microsomes omitted. Spectra are assigned to a mixture of peroxyl (△), alkoxyl (●, only outermost lines indicated) and carbon-centred (▲, only outermost lines indicated) radical adducts.

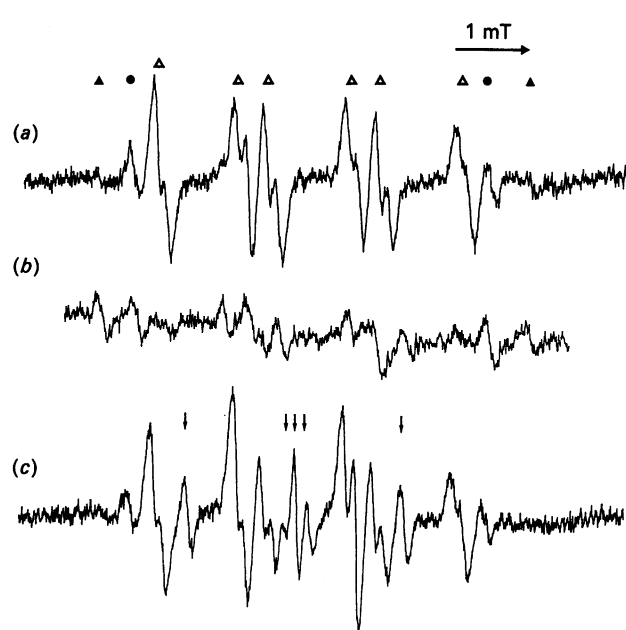


Fig. 2. Effect of heat denaturation and desferrioxamine on spin adduct concentrations

(*a*) Complete system: concentrations, conditions and assignments were as indicated for Fig. 1(*a*). (*b*) As (*a*) except that microsomes were heat-denatured at 80 °C for 10 min. (*c*) As (*a*) except that 250 μM-desferrioxamine was added. Arrowed lines are assigned to the desferrioxamine nitroxide radical.

that have been trapped are all relatively small species; larger molecules would produce anisotropic spectra (with broadening of the high-field lines) as a result of slow tumbling. The alkoxyl and peroxyl species that have been trapped are therefore believed to be the ^{t}BuO$^•$ and ^{t}BuOO$^•$ radicals respectively; identification of the carbon-centred species is more problematic, and the observed signal may be a composite of signals from the adducts of the methyl radical (produced from fragmentation of the alkoxyl radical) and other carbon-centred radicals. The relative concentrations of these three adducts were peroxyl ≫ alkoxyl > carbon-centred. All components of the system were necessary for the observation of these signals (Fig. 1), and the adduct concentrations were dependent on both [^{t}BuOOH] and [microsomes]. In experiments where the samples were scanned consecutively these signals decayed rapidly, showing that the system(s) that generate these species are rapidly deactivated (Fig. 1). Heat inactivation of the microsomes at 80 °C for 10 min led to a dramatic decrease in the intensity of all three radical adducts (Fig. 2).

Effect of inhibitors

In order to obtain information as to mechanism of generation of these radicals, experiments were carried out with a number of enzyme inhibitors and metal-ion chelators. Separate additions of 3 mM-KCN, 10 mM-NaN_3 and 10 mM-imidazole all resulted in over 90% inhibition of all signals, whereas 100 μM-metyrapone gave approx. 50% inhibition.

Inclusion of the potent 'free' Fe^{3+}-chelator desferrioxamine at a final concentration of 250 μM produced only weak (less than 20%) inhibition of the peroxyl, alkoxyl and carbon-centred adducts; an additional signal was also observed with parameters identical with those previously assigned (Davies *et al.*, 1987; Morehouse *et al.*, 1987) to the desferrioxamine nitroxide radical (Fig. 2). This nitroxide radical was also produced, though at a much lower rate, in the absence of microsomes; this is believed to be due to molecular oxidation by ^{t}BuOOH, as observed previously (Davies *et al.*, 1987). Addition of the enzyme superoxide dismutase (480 units/ml) had no significant effect on the observed DMPO adducts, suggesting that the observed radicals are not produced in a superoxide-dependent process.

Effect of enzyme cofactors

Addition of 1 mM-NADH or -NADPH to a standard incubation system consisting of microsomes (8 mg of protein/ml), 10 mM-^{t}BuOOH and 40 mM-DMPO in 20 mM-phosphate buffer, pH 7.4, produced dramatic increases in the intensities of the signals from the alkoxyl and carbon-centred radical adducts, and a decrease in the intensity of the $RO_2^•$ adduct (Fig. 3). This effect was more marked with NADPH (1100% and 1200% increases for the alkoxyl and carbon-centred radical adducts respectively and a 20% decrease for the peroxyl adduct) than with NADH (150% and 300% increases and a 500% decrease for the alkoxyl, carbon-centred and peroxyl radical adducts respectively). Similar effects were also observed at lower concentrations of NADH and NADPH. In contrast with systems where these cofactors were omitted, the intensities of the alkoxyl and carbon-centred radical adducts initially increase with time before decaying away.

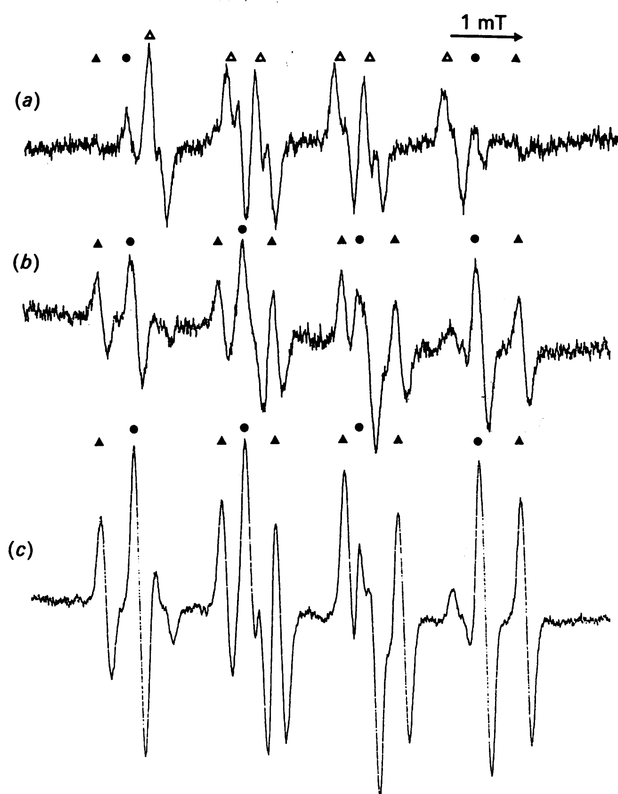


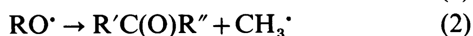
Fig. 3. Effect of NADH and NADPH on spin adduct concentrations

(*a*) Complete system: concentrations, conditions and assignments were as indicated for Fig. 1(*a*). (*b*) As (*a*) except that 1 mM-NADH was added. (*c*) As (*a*) except that 1 mM-NADPH was added and spectrometer gain was ×0.5.

DISCUSSION

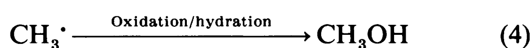
Previous e.s.r. spin-trapping experiments have shown that $^{t}BuO^{\bullet}$ and methyl radicals are produced on metabolism of $^{t}BuOOH$ by erythrocytes (Thornalley *et al.*, 1983) and keratinocytes (Taffe *et al.*, 1987), suggesting that reductive cleavage of the –O–O– bond in the hydroperoxide (reaction 1) is a major metabolic pathway. Product studies have detected acetone and methane on metabolism of $^{t}BuOOH$ by a reconstituted cytochrome *P*-450 system (Vaz & Coon, 1987) and ROH and acetophenone on metabolism of cumene hydroperoxide by rat liver microsomes (Thompson & Wand, 1985; Weiss & Estabrook, 1986; Vaz & Coon, 1987); these products are consistent with the occurrence of reactions (1)–(4):

$$ROOH \xrightarrow{e^-} HO^- + RO^{\bullet} \quad (1)$$



$$RO^{\bullet} \rightarrow R'C(O)R'' + CH_3^{\bullet} \quad (2)$$

$$CH_3^{\bullet} \xrightarrow{XH} CH_4 \quad (3)$$



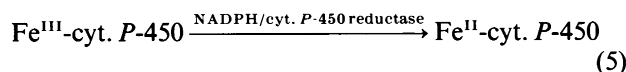
$$CH_3^{\bullet} \xrightarrow{\text{Oxidation/hydration}} CH_3OH \quad (4)$$

In this study the detection (for the first time in a biological experimental system) of peroxyl radical adducts (which are believed to be from $^{t}BuO_2^{\bullet}$), in addition to alkoxyl and carbon-centred radical adducts, suggests that a second decomposition pathway also exists. These radical adducts, which are only observed when all components of the reaction system are present and are dependent on both the microsome and $^{t}BuOOH$ concentrations, are transient in nature, which would suggest that they are formed by a system that becomes rapidly inactivated.

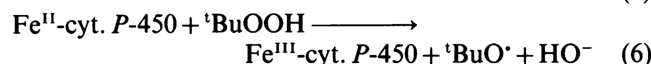
The marked inhibition of the observed signals by heat treatment, the fact that various inhibitors that are known to affect enzyme reactions modulate the signals and the observation that enzymic cofactors such as NADH and NADPH increase radical production suggest that these radicals are produced via an enzymic mechanism rather than through reactions of adventitious metal ions. Further evidence for this suggestion is provided by the relatively minor effects the potent Fe^{3+}-chelator desferrioxamine has on radical production; this molecule is known to inhibit almost completely the reaction of simple metal ions with hydroperoxides, but not to affect reactions of porphyrins. The weak inhibition produced by this compound is believed to be due to direct scavenging of the oxygen-centred radicals (resulting in the formation of the observed nitroxide radical) rather than a chelating effect on the generating system. The inhibition observed with CN^-, imidazole and N_3^-, which are known to affect haem enzymes (Hrycay & O'Brien, 1971), suggests that the observed radicals are produced at a haem prosthetic group, with the most likely candidates being cytochrome *P*-450 or cytochrome b_5, which are the predominant haem enzymes in rat liver microsomes (Omura & Sato, 1964; Ichikawa & Yamano, 1967). The inhibition observed with metyrapone, which is a type II inhibitor of cytochrome *P*-450 (Horie, 1978), suggests that at least some of the hydroperoxide activation is carried out by this complex.

The behaviour observed in the absence of NADPH and NADH is very similar to that found with purified enzymes and model porphyrins in the presence of $^{t}BuOOH$ and DMPO; the production of $RO_2^{\bullet}$ radicals in these systems has been suggested to occur via the production of high-oxidation-state iron species such as ferryl or perferryl ions (Davies, 1988). A similar reaction mechanism may be occurring in this situation.

The increase in $R^{\bullet}$ and $RO^{\bullet}$ adduct concentration in the presence of both NADPH and, to a lesser extent, NADH suggests that a pathway that results in overall one-electron reduction of $^{t}BuOOH$ (which would give $RO^{\bullet}$ and hence $R^{\bullet}$ by fragmentation; reactions 1–4) can occur, and that this is the preferred pathway in the presence of reducing equivalentss. These observations are consistent with cytochrome *P*-450 being the site of activation, as it is known that NADPH is preferred over NADH as the source of reducing equivalents for cytochrome *P*-450 reductase and hence cytochrome *P*-450 (Cohen & Estabrook, 1971). This preference for overall reductive cleavage of $^{t}BuOOH$, presumably via the production of an Fe^{II} intermediate (reactions 5 and 6), is also consistent with previous product studies (Thompson & Wand, 1985; Weiss & Estabrook, 1986; Vaz & Coon, 1987):



$$Fe^{III}\text{-cyt. }P\text{-450} \xrightarrow{\text{NADPH/cyt. }P\text{-450 reductase}} Fe^{II}\text{-cyt. }P\text{-450} \quad (5)$$



$$Fe^{II}\text{-cyt. }P\text{-450} + {}^{t}BuOOH \longrightarrow Fe^{III}\text{-cyt. }P\text{-450} + {}^{t}BuO^{\bullet} + HO^- \quad (6)$$

^tBuOOH is metabolized in rat hepatocytes mainly by two-electron reduction to the alcohol by the glutathione peroxidase system with the concomitant oxidation of GSH to GSSG; the GSSG produced is subsequently reduced by glutathione reductase at the expense of NADPH (Sies *et al.*, 1972; Lotscher *et al.*, 1979, 1980). This depletion of GSH and nicotinamide nucleotides has been associated with the stimulation of the pentose phosphate pathway, impairment of Ca^{2+} sequestration (by the endoplasmic reticulum and mitochondria), an increase in cytosolic free Ca^{2+} and the disruption of cellular membranes (e.g. cell blebbing) (Bellomo *et al.*, 1982, 1984; Thor *et al.*, 1984; Rush *et al.*, 1985). However, it has also been shown that significant hydroperoxide-mediated oxidation of haemoproteins (such as cytochrome b_5; Sies & Grosskopf, 1975) occurs, and this finding, together with the observation that hepatocytes are protected from hydroperoxide-induced damage by radical-scavenging antioxidants such as catechol (Rush *et al.*, 1986), suggests that the haem-dependent free-radical formation observed in this study, both in the presence and in the absence of NADPH/NADH, may be a significant factor in the cytotoxic action of hydroperoxides.

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