Studies on the metal-ion and lipoxygenase-catalysed breakdown of hydroperoxides using electron-spin-resonance spectroscopy

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The breakdown of cumene hydroperoxide and peroxidized fatty acids by iron is shown, by use of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide, to be sensitive to (a) the oxidation state of the metal and (b) the nature of the chelating ligands. The initial step in the Fe^{2+} -catalysed breakdown is the production of an alkoxyl radical by one-electron reduction, and this type of radical has been successfully trapped from each substrate. Subsequent reactions of this alkoxyl species produce both carbon-centred and peroxy radicals, depending on the concentrations of the reagents present. The use of the same spin trap in microsomal systems undergoing either NADPH-supported or Fe^{2+} -induced peroxidation led to the detection of low concentrations of radical adducts, among which are signals that are believed to be due to lipid alkoxyl radicals. Reaction of polyunsaturated fatty acid hydroperoxides with both Fe^{2+} and lipoxygenase under anaerobic conditions gives rise to signals not only from the alkoxy-radical adduct, but also from a further species which is tentatively identified as being due to an acyl $[\text{RC}(\text{O})^\cdot]$ -radical adduct; chemical studies lend support to this assignment.

INTRODUCTION

The breakdown of lipid hydroperoxides by metal-ion complexes is of considerable biological importance and has been investigated on several occasions (Hryciak & O'Brien, 1971; Gardner, 1975; Griffin & Ramirez, 1981; Kalyanaraman *et al.*, 1983; Dix & Marnett, 1985). Despite this effort, there is controversy as to the mechanism(s) of degradation and the species produced. Alkoxyl ($\text{RO}^\cdot$), peroxy ($\text{RO}_2^\cdot$), and carbon-centred ($\text{R}^\cdot$) radicals, as well as singlet oxygen, have been suggested as intermediates in the breakdown of hydroperoxides, on the basis of product studies, oxygen-uptake measurements and other techniques, though very little *direct* evidence for the occurrence of these species has been obtained (Hawco *et al.*, 1977; Borg & Schaich, 1984; Schaich, 1980; Mason, 1984).

E.s.r. spectroscopy can detect these types of radical under certain circumstances. Although some success has been achieved with this technique, it has been limited either by the need to use unphysiological conditions or by the difficulty in obtaining meaningful data from spin-trapping studies. The latter have been hampered by the use of spin traps which cannot readily distinguish between $\text{R}^\cdot$, $\text{RO}^\cdot$, and $\text{RO}_2^\cdot$ adducts, owing to the similarity of the coupling constants (Ohto *et al.*, 1977; Niki *et al.*, 1983). Recent studies using the spin traps DMPO and MDN in organic solvents have shown that it is possible to distinguish between these types of radical (Niki *et al.*, 1983; Yamada *et al.*, 1984; Davies & Slater, 1986). The present study was designed to apply this knowledge to determine (a) whether DMPO (chosen on the basis of its commercial availability) could still trap and distinguish between these radicals in biological situations, (b) which radicals are involved in the metal-catalysed breakdown of cumene and lipid hydroperoxides and what effect complexing ligands and

oxidation state of the metal ion have on this process and (c) whether similar radicals are observed in enzymatic and microsomal systems undergoing lipid peroxidation.

MATERIALS AND METHODS

Incubations of the required hydroperoxides (cumene, oleic acid, linoleic acid, linolenic acid and arachidonic acid) were carried out anaerobically at 37 °C in the dark in a shaking water bath. The reaction mixture consisted of the hydroperoxide, DMPO and the metal-ion complex. After incubation (1 min), the solution was added to 1 ml of ice-cold toluene, vortex-mixed, then centrifuged (800 g_{av} , 5 min) to separate the two phases. The e.s.r. spectra of the toluene layer was then recorded at –60 °C. This extraction process prolongs the lifetime of the radical adducts. Experiments using lipoxygenase (EC 1.13.11.12, 3 mg/ml, 170 000 units/mg final concn. in 50 mM-borate buffer, pH 8.0) were carried out in a similar manner.

Adult male albino rats (approx. 200 g) maintained on a standard laboratory diet (Labsure Animal Food; Christopher Hill Group, Poole, Dorset, U.K.) were killed by cervical dislocation, and washed microsomes (microsomal fractions) prepared as described previously (Slater & Sawyer, 1971). The microsome pellet was resuspended before use in 0.15 M-KCl such that 1 ml of suspension was equivalent to 1 g wet weight of liver. Incubations consisted of 0.3 ml microsomal suspension, 2.2 ml of NADPH-generating system (Slater & Sawyer, 1971) or in some cases 500 μM -NADPH, 0.5 ml of an aqueous spin-trap solution, and 0.1 ml of the metal-ion complex in water. Anaerobic incubations (10–30 min at 37 °C in the dark) were terminated by the addition of 1.5 ml of ice-cold toluene and the spin adducts extracted as described above.

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; PBN, α -phenyl-*N*-butylnitron; MDN, methyl-*N*-durylnitron; DETAPAC, diethylenetriaminepenta-acetic acid; O^* , triplet state.

The photolytic experiments used to check the radical assignments were carried out using focused, but unfiltered, output of a Heraeus 200 W mercury/xenon arc incident through a 50% transmission grating on an e.s.r. sample tube, inserted into the cavity of the spectrometer, containing the substrate, spin trap and, when used, benzophenone, all dissolved in toluene (Davies & Slater, 1987).

All aqueous solutions were made up in deoxygenated (20 min bubbling with O₂-free N₂) doubly distilled water, with the pH adjusted with either HCl or NaOH to pH 7.4, except where stated otherwise. Chemicals were obtained from Fluka AG Chemische Fabrik, Buchs, Switzerland (cumene hydroperoxide), Sigma Chemical Co., Poole, Dorset, U.K. (oleic acid, linoleic acid, linolenic acid and arachidonic acid, lipoxigenase (type 1), glucose 6-phosphate), Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. (NADPH, ADP, glucose-6-phosphate dehydrogenase), Aldrich Chemical Co. Ltd. (DMPO, triethylsilane, allyl bromide, allyl iodide) and BDH Chemicals Ltd., Dagenham, Essex, U.K. (all other chemicals) and used as supplied, with the exception of the fatty acids, which were peroxidized before use by exposure to air for 72 h at room temperature (O'Brien, 1969), and DMPO, which was purified as described previously (Beuttner & Oberley, 1978). No experimental differences were observed between using partially and highly purified (by the method of O'Brien, 1969) fatty acid hydroperoxide samples. Desferrioxamine was a gift from Ciba-Geigy Ltd., Horsham, Sussex, U.K.

E.s.r. spectra were run on a Bruker ER200D X-band spectrometer using 100 kHz modulation and a Bruker ER411VT variable-temperature unit. Hyperfine coupling constants (± 0.005 mT) were measured directly from the field scan, using the 1 mT marker signals from a Bruker ER0354M 'gaussmeter' for calibration. Spectral analyses were confirmed by simulation as described previously (Davies & Slater, 1986).

RESULTS AND DISCUSSION

(1) Cumene hydroperoxide

Reaction of cumene hydroperoxide (70 μ M) with aquated Fe²⁺ (25 μ M) in the presence of the spin trap DMPO (20 mM) led to the detection of two signals, the intensity of which varied linearly with the concentration of the added Fe²⁺ over the range 5–200 μ M (results not shown). Omission of the substrate led to the detection of a weak signal produced from reaction of the trap with Fe²⁺ (Finkelstein *et al.*, 1979), whereas omission of the metal ion was associated with an absence of any signals.

The first of these species, which was present only in low concentration, is assigned on the basis of its coupling constants to a carbon-centred radical, and is consistent with the trapping of methyl radicals formed by β -scission (reaction 2) of an initially formed tertiary alkoxyl radical (Gray & Williams, 1959; Janzen & Liu, 1973). The second species has markedly different parameters, with a small β -hydrogen splitting and a further (γ -hydrogen) splitting (Table 1), characterizing the added radical as having a heteroatom centre (Janzen & Liu, 1973); the similarity of this spectrum to that observed on photolysis of dicumyl peroxide and cumene hydroperoxide (Davies & Slater, 1986) suggests that this species is the cumyloxyl [$\text{PhC}(\text{CH}_3)_2\text{O}^\cdot$] radical adduct.

These assignments are supported by observations on the effects of altering the hydroperoxide concentration. At very low concentrations of cumene hydroperoxide both signals are observed, but, as the hydroperoxide concentration is increased, the signal assigned to the methyl-radical adduct decreases more rapidly in concentration (as measured by signal height) than does the cumyloxyl radical adduct. At very high concentrations a further signal is also observed, which is assigned to the cumene-peroxyl-radical adduct on the basis of the similarity of this signal to that observed in photolytic experiments (Davies & Slater, 1986). The observation of these radicals and their concentration-dependence is in accord with previous work (Gilbert *et al.*, 1981; Bors *et al.*, 1984) on the breakdown of *t*-butyl hydroperoxide, with the initial reaction being the production of alkoxyl radicals by one-electron reduction of the hydroperoxide by Fe²⁺ (reaction 1 below). This species then either fragments (reaction 2) to give the acetophenone and methyl radical (which is subsequently trapped), adds on to the spin traps or reacts with more hydroperoxide by hydrogen abstraction to give the peroxyl radical (reaction 3), which then adds on to the spin trap. At low hydroperoxide concentrations the first two processes occur, whereas at high concentrations all three reactions compete:

The loss of both the CH₃[·] and PhC(CH₃)₂O[·] adduct signals at high hydroperoxide concentrations could be due to: process (i), greater competition between reaction with more hydroperoxide (reaction 3) and fragmentation of the alkoxyl radical (reaction 2); process (ii), increased hydrogen abstraction from the hydroperoxide by methyl radicals, yielding methane and the peroxyl radical (reaction 4); and process (iii), oxidation of the methyl radical by RO₂H, a reaction which yields more RO[·] (reaction 5). If only the first process was occurring, then the adduct signals would be lost at a similar rate; the greater rate of loss of the methyl radical adduct therefore suggests that either (ii) or (iii) is also important. Hydrogen abstraction from RO₂H by CH₃[·] would be expected to be slower than with the cumyloxyl radical, owing to the nucleophilic nature of the former radical species (Veltwisch & Asmus, 1982) as opposed to the electrophilic character of the latter (Howard & Ingold, 1963; Gilbert *et al.*, 1981; Neta *et al.*, 1984), and would therefore produce a more rapid loss of the PhC(CH₃)₂O[·] adduct signal. Oxidation of carbon-centred radicals by H₂O₂ and other powerful oxidants is known to occur (with *k* values of 10³–10⁵ mol⁻¹·dm³·s⁻¹; Gilbert *et al.*, 1974; Davies *et al.*, 1984); oxidation of CH₃[·] by cumene hydroperoxide may therefore occur at a significant rate, whereas oxidation of alkoxyl radicals would not be expected to be rapid. The most likely cause of the observed concentration-dependencies is a combination of process (i) (increased competition between fragmenta-

Table 1. Hyperfine coupling constants of radicals detected during the breakdown of hydroperoxides in the presence of DMPO

See the Materials and methods section for experimental details. Coupling-constant values are ± 0.005 mT with toluene as solvent.

Substrate	Radical added to trap	Hyperfine coupling Constants (mT)	
		a_N	a_H
Cumene hydroperoxide	$\text{CH}_3\cdot$	1.430	2.050
	$\text{PhC}(\text{CH}_3)_2\text{O}\cdot$	1.308	0.888, 0.168
	$\text{PhC}(\text{CH}_3)_2\text{O}_2\cdot$	1.392	1.120
Peroxidized oleic acid*	$\text{RO}\cdot$	1.284	0.648, 0.168
Peroxidized linoleic acid,*	$\text{RO}\cdot$	1.330	0.600, 0.216
peroxidized linolenic acid* or peroxidized arachidonic acid*	$\text{RC}(\text{O})\cdot^\dagger$	1.384	1.672

* Further weak signals from additional radicals also detected; see the text.

† Tentative assignment; see the text.

Fig. 1. E.s.r. spectrum of toluene extracts from reaction of Fe^{2+} with a suspension of peroxidized oleic acid in the presence of DMPO

Final reaction concentrations were: Fe^{2+} , 25 μM ; peroxidized oleic acid, 10 $\mu\text{l/ml}$; DMPO, 20 mM; see the Materials and methods section for details. Lines marked 'O' are assigned to a carbon-centred radical adduct; the stick diagram (bottom) shows lines assigned to the alkoxyl-radical adduct. E.s.r. spectrometer settings: gain 2×10^6 ; modulation amplitude, 0.1 mT; time constant, 1.0 s; scan time, 1000 s; field, 338.5 mT; scan, 6 mT; power, 13 dB; frequency, 9.55 GHz; temperature, 213 K; marker (broken vertical) lines are at 1.0 mT intervals.

tion and reaction with RO_2H) that will lead to a decrease in the intensity of both radical adducts, and the oxidation reaction (iii), which will result in a more rapid loss of the methyl-radical adduct.

(2) Fatty acid hydroperoxides

In some cases the anaerobic reaction of peroxidized fatty acid (10 $\mu\text{l/ml}$) suspensions with aquated Fe^{2+} in the presence of DMPO in the dark led to markedly different behaviour from that observed with cumene hydroperoxide. Peroxidized oleic acid gave two detectable radical adducts (Fig. 1 and Table 1). The main species present at all concentrations studied is assigned, on the basis of its hyperfine splitting constants (Davies & Slater, 1986), to an alkoxyl radical adduct. This

suggests that the dominant reaction is one-electron reduction of the hydroperoxide by Fe^{2+} (reaction 6). The second signal, which is only visible as low-intensity peaks in the wings of the spectra, has an overall spectral width of 4.96 mT. The exact identity of this adduct cannot be determined definitively, but some general conclusions can be obtained from the overall width of the spectra. Thus, as virtually all nitroxide adduct signals obtained with this spin trap in organic solvents have a nitrogen hyperfine splitting of approx. 1.3 mT, this would necessitate a β -hydrogen splitting of the order of 2.3 mT, which is consistent with the trapping of an α -hydroxyalkyl radical (Janzen & Liu, 1973). The only exceptions where the nitrogen hyperfine splitting constant is markedly different from 1.3 mT are oxidation and reduction

products of the spin trap; these species are well characterized, and can be eliminated as the source of this signal.

Unlike the cumene hydroperoxide system, no evidence was obtained for the trapping of peroxy radicals, even though such species have been previously identified (Davies & Slater, 1986). This may be due to the limited concentration range available for study or a lower rate of reaction of the alkoxyl radicals with more hydroperoxide. Either possibility would result in the hydrogen abstraction to produce peroxy radicals being uncompetitive.

On changing the substrate to any of the polyunsaturated fatty acids (linoleic acid, linolenic acid and arachidonic acid), markedly different behaviour was observed. Thus peroxidized linoleic acid gave two detectable signals. The first of these, which was present at low concentrations, is assigned to the alkoxyl radical adduct (Davies & Slater, 1986; Fig. 2a and Table 1). The second, stronger, signal has the following hyperfine splitting constants: a_N 1.384 mT; a_H 1.672 mT. These values are *not* consistent with this species being either an alkyl or peroxy radical adduct (Davies & Slater, 1987). Similar behaviour was observed with peroxidized linolenic and arachidonic acids with weak signals from the alkoxyl radical adduct and strong signals from a further unknown radical, with parameters very similar to those observed with linoleic acid (see Table 1) being obtained. The origin of these unidentified signals will be discussed below. No evidence for any other radical adducts was obtained.

Effect of varying the oxidation state of the metal ion and the chelating ligand

Only very weak signals were observed when aqueous suspensions of Fe^{3+} were used instead of Fe^{2+} , and no evidence was obtained for the production of peroxy radical adducts. This is in agreement with earlier workers (Hiatt *et al.*, 1968), who found no appreciable breakdown of hydroperoxides by Fe^{3+} , but is at variance with several biochemical studies where Fe^{3+} has been suggested to catalyse lipid peroxidation (for a review, see Halliwell & Gutteridge, 1984), though it is possible that, in these studies, the initiating species is Fe^{2+} produced via reduction by, for example, the NADPH-flavoprotein, ascorbate or superoxide (Wills, 1966; Svingen *et al.*, 1979; Aust & Svingen, 1982).

It has been previously shown that chelating ligands can play an important role in determining the thermodynamic favourability and rate of reaction of a metal ion with a hydroperoxide (Kochi, 1973). Consequently several commonly used complexing agents (ADP, EDTA, DETAPAC and desferrioxamine) were investigated in a standard Fe^{2+} (20 μM)/cumene hydroperoxide (0.5 mM)/DMPO (10 mM) reaction system. Only desferrioxamine, which resulted in complete loss of all signals at concentrations greater than 30 μM , had any significant effect; inclusion of ADP (60 μM), EDTA (20 μM), or DETAPAC (20 μM) had no significant effect on alkoxyl radical production, as judged by the peak height of the spin-adduct signals when compared with controls, where the aquated Fe^{2+} complex was used. The inclusion of any of these complexing ligands had no effect on systems where Fe^{3+} was used, i.e. no signals were observed (cf. a report that Fe^{2+} catalysed the decomposition of linoleic acid hydroperoxide approx. 10-fold faster than Fe^{3+} ;

O'Brien, 1969). The lack of inhibition of alkoxyl radical production by ADP, EDTA, and DETAPAC is at variance with several previous studies where addition of these agents to systems undergoing iron-stimulated lipid peroxidation produced an inhibitory effect (Gutteridge *et al.*, 1979; Tien *et al.*, 1981; Halliwell & Gutteridge, 1984). This effect may be due to either the complexing ligand decreasing the *rate* of reaction of the Fe^{2+} with the hydroperoxides or, if the iron is added as Fe^{3+} , by reducing the *rate* of production of Fe^{2+} from the Fe^{3+} complex by reduction (Gardner & Jursinic, 1981) via the effect of the ligand(s) on the stability of the two oxidation states (cf. evidence that Fe^{3+} -DETAPAC complexes are reduced by $\text{O}_2^{\bullet -}$ more slowly than Fe^{3+} -EDTA complexes; Butler & Halliwell, 1982). The present results suggest that the latter process is the most important.

Reaction of lipid hydroperoxides with lipoxygenase

The anaerobic reaction of lipoxygenase with polyunsaturated-fatty-acid hydroperoxides has been shown to produce carbon-centred radicals (Albano *et al.* 1982; Klein *et al.*, 1984), though it is unlikely that these are produced *directly* without the mediation of either alkoxyl or peroxy radicals.

Anaerobic incubation of *polyunsaturated*-fatty-acid hydroperoxides with soybean lipoxygenase (3 mg/ml final concn.) in the presence of DMPO gave signals identical with those observed in the corresponding Fe^{2+} -catalysed-reaction experiments (Fig. 2b), with a weak signal from the alkoxyl radical adduct (suggesting that one-electron reductive cleavage of the hydroperoxide is occurring) and a stronger signal from the second unidentified species being detected; omission of the lipoxygenase led to the loss of all signals.

Microsomal experiments

Similar experiments using rat liver microsomal fractions and an NADPH-generating system were carried out to determine whether analogous radicals are produced in peroxidizing membranes. Incubation of microsomal fractions with either an NADPH-generating system or NADPH itself (500 μM) and DMPO (20 mM) led to the detection of a weak signal (or possibly several weak signals with similar parameters) whose hyperfine coupling constants are similar to those assigned to the alkoxyl radical. Addition of Fe^{2+} (50 μM) to the system increased the intensity of this signal by 210% and produced two further signals, though the lipid-alkoxyl radical signal remains the dominant feature (see Fig. 3). The other species are only observable as lines in the wings of the spectra, with overall spectral widths approx. 4.7 and 5.5 mT respectively. The first of these, assuming a nitrogen hyperfine splitting constant of approx. 1.3 mT (Janzen & Liu, 1973), has a hydrogen splitting of approx. 2.1 mT, which identifies it as a carbon-centred radical adduct. The second species, on the basis of its large spectral width, is assigned to the reduced form of the spin trap (I) which has *two* hydrogen atoms in the β -position (Janzen & Liu, 1973).

Fig. 2. E.s.r. spectra of toluene extracts from reaction of Fe^{2+} (a) or lipoxygenase (b) with a suspension of peroxidized linoleic acid in the presence of DMPO

(a) Final concentrations: Fe^{2+} , 25 μM ; peroxidized linoleic acid, 10 $\mu\text{l/ml}$; DMPO, 20 mM. (b) As (a), except lipoxygenase was used (at 510000 units/ml) instead of Fe^{2+} . Lines marked '○' are tentatively assigned to an acyl-radical adduct; other weak lines are assigned to the alkoxyl-radical adduct. Spectrum (c) was produced by photolysis of benzophenone (25 mM), hex-2-enal (10 mM) and DMPO (25 mM) anaerobically in toluene. Spectrum (d) was obtained in the same way as spectrum (c), except that the aldehyde was omitted; these lines are assigned to the benzyl-radical adduct produced by reaction of the benzophenone triplet with the solvent (see the text). E.s.r. spectrometer settings: (a) and (b), as Fig. 1; (c), as Fig. 1, except: gain, 1×10^6 ; modulation amplitude, 0.0125 mT; (d), as Fig. 1, except: gain, 1.25×10^6 ; modulation amplitude, 0.05 mT.

Fig. 3. E.s.r. spectrum of toluene extract obtained from incubation (10 min at 37 °C) of microsomes with an NADPH-generating system in the presence of Fe^{2+} (50 μM) and the spin trap DMPO (20 mM) (see the Materials and methods section for details)

Lines marked '○' are assigned to the reduced form of the spin trap, DMPO-H; lines marked 'X' are believed to be the outermost lines of a carbon-centred radical adduct. The stick diagram (bottom) shows analysis of signal assigned to an alkoxyl-radical adduct. E.s.r. spectrometer settings: as Fig. 1, except: gain, 4×10^6 ; modulation amplitude, 0.2 mT; time constant, 2.0 s; scan time, 2000 s.

Possible identities of the unassigned radicals detected in the breakdown of polyunsaturated-lipid hydroperoxides

The unidentified signal(s) observed during the reaction of Fe^{2+} and lipoxygenase with peroxidized linoleic acid, linolenic acid and arachidonic acid has unusual hyperfine coupling constants (a_N 1.384 mT; a_H 1.672 mT). The few radical adducts to DMPO known to have splittings of this magnitude are carbon-centred radicals with highly-electron-withdrawing groups attached; the acetyl [$\text{CH}_3\text{C}(\text{O})^\cdot$]-, benzoyl [$\text{PhC}(\text{O})^\cdot$]- and trifluoromethyl ($^\cdot\text{CF}_3$)-radical adducts have β -hydrogen splittings of 1.787, 1.557, and 1.554 mT respectively in benzene (Janzen & Liu, 1973). The observed radicals may also fall into this category, and consequently further experiments were carried to attempt to identify the radical(s) giving rise to these signals. Acyl radicals which have been previously suggested to arise in peroxidation processes (Aoshima *et al.*, 1986) were generated, as described previously (Janzen & Liu, 1973), by photolysis of an appropriate aldehyde (10 mM), DMPO (25 mM), and benzophenone (25 mM) in toluene, using the benzophenone triplet state as the hydrogen-abstracting species (reaction 7 below). In the absence of aldehyde, strong signals attributable to the benzyl ($\text{PhCH}_2^\cdot$)-radical adduct were observed (Fig. 2d) (from hydrogen abstraction from the solvent; Janzen & Liu, 1973); addition of an aldehyde led to a significant decrease in the intensity of this species and the observation of further signals, which are assigned to the acyl-radical adduct (see Fig. 2c and Table 2). In this manner the coupling constants for the acyl-radical adducts from ethanal, 5-hydroxypentanal, *trans*-pent-2-enal, *trans*-hex-2-enal, and *trans*-2-methylpent-2-enal were determined; those produced from the long-chain aldehydes are similar to those observed in breakdown of the polyunsaturated-fatty-acid hydroperoxides, suggesting that these signals *may* be due to acyl adducts:

The mediation of allyl radicals in lipid-peroxidation processes has been postulated on numerous occasions

Table 2. Hyperfine coupling constants of radicals detected during the photolysis of benzophenone and aldehydes in the presence of DMPO in toluene

See the Materials and methods section for experimental details. For each radical added to the trap, an additional signal with a_N 1.471, a_H 2.150 mT due to the benzyl ($\text{PhCH}_2^\cdot$)-radical adduct (which arises from hydrogen abstraction from the solvent) was also detected. Coupling-constants are ± 0.005 mT.

Substrate	Radical added to trap	Hyperfine coupling constants (mT)*	
		a_N	a_H
Ethanal	$\text{CH}_3\text{C}(\text{O})^\cdot$	1.430	1.833
5-Hydroxypentanal	$\text{HO}[\text{CH}_2]_4\text{C}(\text{O})^\cdot$	1.376	1.704
Pent-2-enal	$\text{CH}_3\text{CH}_2\text{CH}=\text{CHC}(\text{O})^\cdot$	1.430	1.700
Hex-2-enal	$\text{CH}_3[\text{CH}_2]_2\text{CH}=\text{CHC}(\text{O})^\cdot$	1.430	1.700
2-Methylpent-2-enal	$\text{CH}_3\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)\text{C}(\text{O})^\cdot$	1.442	1.608

(Tappel *et al.*, 1952). In an attempt to eliminate this type of radical as the cause of the unassigned signals, photolytic experiments were carried out in which either allyl iodide ($\text{CH}_2=\text{CH}-\text{CH}_2\text{I}$, 50 mM) was irradiated directly in the presence of DMPO (20 mM) in toluene (a process which will yield the corresponding allyl radical in high yield, owing to the low carbon-iodine bond strength in this molecule; Vedeneyev *et al.*, 1966), or triethylsilyl radicals, $\text{Et}_3\text{Si}^\cdot$, were generated (from reaction of *t*-butoxy radicals, $^\cdot\text{BuO}$, with the silane) in the presence of allyl bromide (50 mM) and the spin trap (20 mM) in toluene (a known method of generating radicals by abstraction of $\text{Br}^\cdot$ by $\text{Et}_3\text{Si}^\cdot$; Kawamura *et al.*, 1972). In neither case were any signals observed that could be assigned to a trapped allyl radical. This presumably reflects either a slow rate of addition of the resonance-stabilized allyl radical to the spin trap or a very rapid rate of loss of the spin adduct once formed. A similar state of affairs would be expected with allyl radicals generated from the more sterically hindered lipid molecules, so the possibility that the unidentified species are allyl radical adducts appears unlikely.

The results obtained in the present study are consistent with the initial formation of alkoxyl radicals formed by reductive cleavage of the hydroperoxide by Fe^{2+} . These radicals once formed undergo a number of competing reactions, including reaction with more hydroperoxide (to give peroxy radicals; reaction 3), 1,2-hydrogen shifts (which will give α -hydroxyalkyl radicals), C—H hydrogen abstraction (to give carbon-centred radicals), β -scission (for the tertiary alkoxyl radicals, yielding alkyl radicals) and ring closure (to form epoxy species), and these processes appear to be the sources of the observed radicals (Ingold, 1961; Pryor, 1973), with the exception of the tentatively assigned acyl radicals produced from the polyunsaturated-fatty-acid hydroperoxides. The fact that no evidence was obtained for the *direct* production of peroxy radicals (though such species have been successfully identified with the same spin trap in the same solvent; Davies & Slater, 1987), suggests that processes such as reaction (8), which have been postulated as sources of these radicals, either do not occur, or occur at a lower rate compared with the rate of reductive formation of alkoxyl radicals. The only peroxy-radical adduct observed in the present study (from cumene hydroperoxide) has been shown, by its dependence on the concentration of the hydroperoxide, to arise via hydrogen abstraction by alkoxyl radicals. Furthermore, the detection of this adduct appears to eliminate the possibility that it is the *stability* of the peroxy-radical adducts, rather than their rate of production, which is the reason for not observing such species.

The mechanism of formation of the acyl radicals, if these have been correctly identified, cannot be determined from the available data, though it is interesting to note that aldehydes (which may arise from hydrogen abstraction from further lipid molecules by these radicals) are a well characterized *product* of lipid peroxidation. However, whether the observed radicals are arising *directly* from breakdown of the hydroperoxides or from reactions of radicals with the products of peroxidation (e.g. aldehydes) to give these species, is unknown. An example of this latter type of process is

reaction (9), which has been proposed as a peroxidation pathway by Porter (1984). This process would yield alkyl radicals (e.g. the n-pentyl radical) and an aldehyde in close proximity; a hydrogen-abstraction reaction (reaction 10) could then give an acyl radical and pentane that is a known product of peroxidation (Slater, 1972).

It is interesting to note that an unidentified species has also been observed (Smith *et al.*, 1981) in the reaction of arachidonic acid with ram seminal-vesicle microsomes (a source of prostaglandin H synthase) and, although direct comparison of the hyperfine coupling constants is not possible due to the different solvents used, if allowance is made for this solvent effect, then the signal is remarkably similar to the acyl-radical-adduct signal observed in the present study, suggesting that this signal may be attributable to a similar species arising from the known peroxidase activity of this enzyme (Marnett, 1981).

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