Fatty Acids Oxidation Studied by Electron Paramagnetic Resonance II. Detection of Transient Free Radicals of Oleic, Linoleic, and Linolenic Acids

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Coordinated *tert*-butyl peroxy radicals on Co(III) or Fe(IV) abstract from mono- and polyunsaturated fatty acids at ambient temperature in benzene solution hydrogen atom situated at β-position to the double bond. At low concentration of the initiator and in the presence of dissolved oxygen in a narrow temperature range of 10—40 °C a 3-line EPR signal with g = 2.0050 is observed. According to analysis of the reaction mechanisms of oxidation this signal is ascribed to the primary pentadienyl free radical transiently stabilized by complexing with molecular oxygen. The interaction of this radical with the spin-trapper DMPO is discussed.

To the elucidation of the oxidation mechanism of biological membranes, especially of their individual building components — the fatty acids of phospholipids with different number of double bonds (oleic, linoleic, linolenic, and arachidonic acids) is devoted a long-dated research interest [1—6]. While the proof of the end products of the oxidative chain reactions is analytically well elaborated (detection of hydroperoxides, aldehydes, alcohols, and acids) and reliably interpreted [7], the direct detection of transient reactive free radicals (alkyl, allyl, alkoxy, and peroxy) at physiological conditions (θ = 37 °C, hydrophilic-hydrophobic phase boundary) applying EPR is limited [3, 4, 6], in consequence of low steady-state concentrations of radicals with short mean lifetime often under the threshold sensitive level of common spectrometers (*ca.* 10^{-8} mol dm^{-3}).

In a set of papers concerning the radical chemistry in biological systems the high efficiency of peroxy radicals stabilized by coordination on chelated transition metals (Ti(IV), Mn(III), Cr(IV), Fe(IV), Co(III), Ni(0), Cu(III)) [8, 9] or metalloenzymes inclusive hemine, hemoglobin, cytochromes as initiators of oxidative chain reaction was presented [10, 11]. This experimental technique also used in this contribution was discussed in detail in our previous paper [12]. Simultaneously the role of nonpolar medium for simulation of the inner part of the cell membrane was stressed.

As it was shown, this initiation technique can be combined with simultaneous or consecutive applying of spin-trapper DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) transforming the generated short-lived radicals as well as the oxidation-starting radicals to spin-adducts with different stability.

EXPERIMENTAL

All chemicals (Sigma) were of high purity. Dried benzene (pure) was from Lachema, Brno, *tert*-butyl hydroperoxide (TBHP) from the Institute of Macromolecular Chemistry, Brno. Before using TBHP was distilled under vacuum and dried. Oleic, linoleic, and linolenic acids were purified by vacuum molecular distillation and dissolved to the concentration 1 × 10^{-2} mol dm^{-3} of double bonds instantly before use.

The preparation of the coordinated *tert*-butyl peroxy radicals on Co(III) chelated in acetylacetonate and on Fe(IV) chelated in the hemine ligand field [11] and their purification as of the initiator of radical chain oxidation reaction was described in detail in our previous paper [12]. Dissolved in benzene the coordinated ROO$^{\bullet}$ radicals in the concentration range of 10^{-5}—10^{-4} mol dm^{-3} can start H-abstraction when admixed to the dissolved purified unsaturated fatty acids. The concentration of oleic, linoleic, and linolenic acids was 1 × 10^{-2} mol dm^{-3}.

The spin-trapper 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Sigma) was used dissolved either in water or in benzene. Before EPR measurements the solution was bubbled with nitrogen or argon to eliminate the dissolved oxygen and to prevent the direct oxidation of the spin-trapper.

Measurements were carried out on Bruker ESR-SRC 200 spectrometer (Karlsruhe), which operates

at X-band with 100 kHz modulation. For the spectra simulations the ASPECT 2000 software (Bruker) was used. Measurements were carried out in cylindrical EPR-cells for nonpolar solutions. For *g*-values calibration the Varian pitch standard was used (g = 2.0028).

RESULTS AND DISCUSSION

In principle there are two possible ways to abstract hydrogen atom from β-methylene group attached to a double bond of an unsaturated fatty acid with coordinated peroxy radicals on Co(III) or Fe(IV) in nonpolar solvents at physiological temperature:

a) either the dissolved unsaturated acids are stepwise admixed to the radical initiating system,

b) or oppositely, to the solution of fatty acids is gradually added the benzene solution of coordinated peroxy radicals.

In the first case the concentration of the peroxy radicals progressively decreases from the beginning, as the solution of oleic acid, with one double bond, similarly as of linoleic acid with two double bonds, or linolenic acid with three double bonds is admixed (Fig. 1).

The rate of disappearance of the EPR signal intensity during the stepwise adding of oleic acid with two sensitive H-atoms in β-position to the surplus of coordinated ROO• radicals decreases from the beginning of the titration as long as the steady-state concentration of peroxy radicals is established (curve *1a*, *b*). The elimination of the reactive ROO• radicals is compensated with the increase of more stable LOO• radicals possessing the same EPR signal (g = 2.0147).

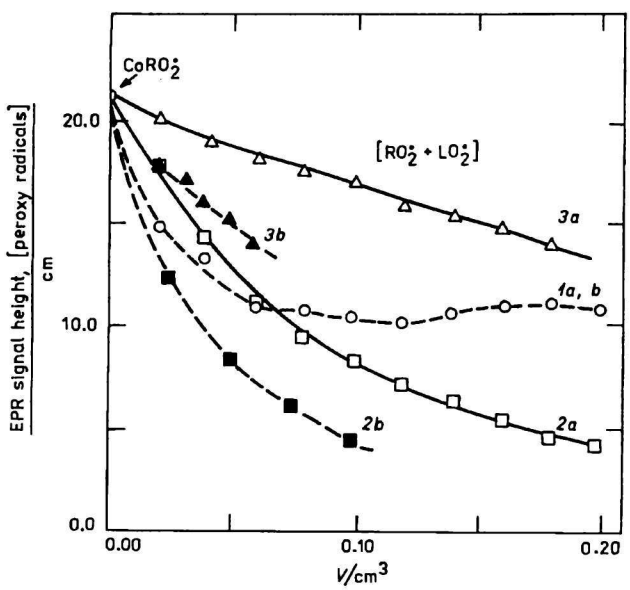


Fig. 1. Decrease of the EPR signal intensity of peroxy radicals during the successive adding of fatty acids (c = 1 × 10^{-2} mol dm^{-3}) to 0.3 cm^3 of Co(III)RO$_2^•$ in benzene (c = 5 × 10^{-4} mol dm^{-3}). *1*. Oleic acid, *2*. linoleic acid, *3*. linolenic acid. *a*) Related to the amounts of substance of acids. *b*) Corrected to the same amount of double bonds.

In Fig. 1 also the reactivity for H-transfer abstraction of the studied fatty acids adjusted to the same concentration (*a*) and to the same amount of double bonds (*b*) against the initiating ROO• is compared.

The more effective decrease of the concentration of initiating ROO•, when linoleic acid is stepwise admixed with six H-atoms in β-position, is simultaneously followed with intramolecular secondary reactions of the primarily formed LOO• with the neighbouring double-bond system leading to cyclization and endoperoxide formation (curve *2a*, *b*).

The linear decrease of ROO• radicals, when linolenic acid with three double bonds and with twelve sensitive H-atoms is progressively added (curve *3*), can be explained in the starting phase of titration with accumulation of comparatively more stable LOO• radicals and then with their continuous decrease through intrinsic cyclization and chain transfer reactions with the colliding fatty acid molecules forming hydroperoxides (reaction *A*)

$$\mathrm{LOO^• + —LH— \rightarrow LOOH + —\dot{L}—} \qquad (A)$$

In the solution oxygen is continuously formed from ROO• recombination (reaction *B*)

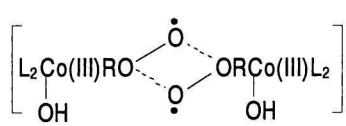
$$\mathrm{2\ RO_2^• \rightarrow ROOR + O_2} \qquad (B)$$

In the absence of oxygen, when the initiation reaction proceeds under nitrogen or argon screen, the dominating reaction is dimerization and recombination (reaction *C*)

$$\mathrm{—\dot{L}— + RO_2^• \rightarrow LOOR} \qquad (C)$$

with the consequence of rapid disappearance of the initiating ROO• radicals.

Without any H-donors the concentration of coordinated ROO• radicals in benzene solution proceeds with increasing temperature through a maximum. From the temperature 0 °C up to *ca*. 25 °C a continuous opening of the tetraoxides



is observed, then from about 27 °C starts the stepwise thermal decomposition of coordinated radicals followed with oxygen formation. Near 47 °C the EPR signal of ROO• radicals disappears during one minute.

In the presence of oleic acid in the temperature interval 27 to 32 °C the decrease of the initiating ROO• radicals is accelerated (Fig. 2). Over the "critical" temperature 35 to 37 °C in benzene solution the decrease of ROO• is overlapped with simultaneous accumulation of more stable oleic acid peroxides LOO• in consequence of oxygen formation from accelerated ROO• recombination with increased temperature.

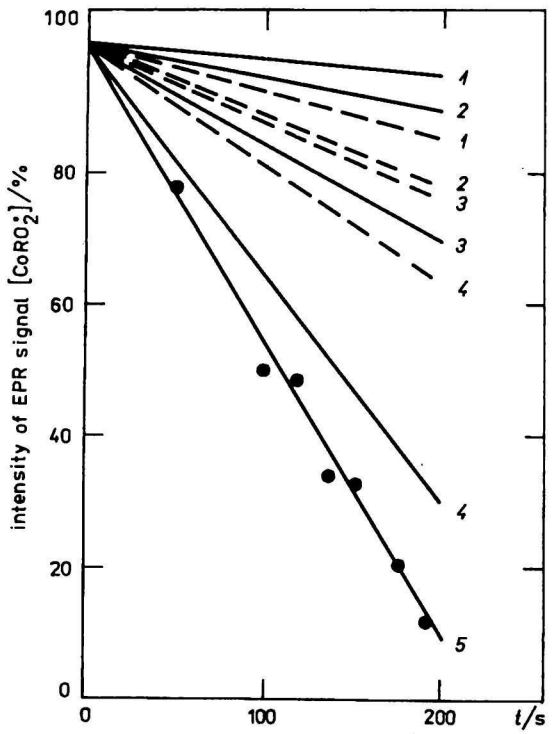


Fig. 2. Decrease of peroxy radicals with temperature (in °C) according to the EPR signal of coordinated peroxy radicals without oleic acid (solid lines) and with oleic acid ($c = 10^{-2}$ mol dm^{-3}) (dashed lines). θ/°C: 1.27, 2.32, 3.37, 4.42, 5.47. The secondarily formed LOO• radicals possess the same EPR signal (g = 2.0147) as the initiating ROO• radicals.

In spite of higher —CH bond dissociation energy for oleic acid (368 kJ mol^{-1}) in comparison with linoleic and linolenic acid (313 kJ mol^{-1}) the sensitivity for H-abstraction through initiating peroxy radicals remains still high at physiological temperature. As lipid component of hydrophobic membranes in the presence of vitamin E the primary allyl radicals of oleic acid are continuously deactivated and so they can be involved also in the H-transfer cascade protecting the cell membrane from random radical attacks (H_2O_2, ROOH, transition metals, metalloenzymes, hemichromes) close with vitamin E regeneration in the presence of ascorbic acid or glutathione (glutathione reductase). Oppositely, in depletion of biological antioxidant and presence of oxygen, the dangerous lipid hydroperoxides are formed — starting the chain oxidative destruction — as the beginning of many degenerative diseases, inclusive aging.

Applying the method *a*), when the initiation of oxidation of the fatty acids proceeds in the permanent surplus of peroxy radicals, with the exception of the immediate decrease of the broad EPR line with g = 2.0147, no other spectral changes are registered. On the other hand, by the opposite way of "titration" using method *b*), when to the surplus of the unsaturated acids with one, two or three double bonds, small doses of coordinated peroxy radicals are stepwise admixed a new EPR signal with lower g = 2.0050 in a very narrow range of experimental parameters can be observed. The precondition is: the initiating peroxy radicals should be admixed at permanent presence of dissolved air in the benzene solution.

The increasing signal of the transient new radical is composed of not completely resolved three lines and was theoretically spectrosimulated as two overlapping doublets of two nonequivalent protons (Fig. 3) with coupling constants a_H^α = 1.35 mT, a_H^β = 0.73 mT, p/p = 0.80 mT.

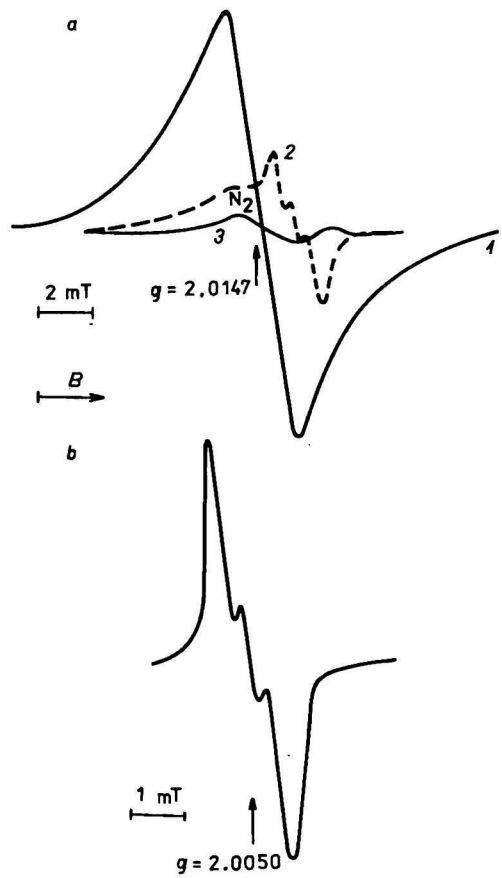


Fig. 3. Experimental EPR signals (*a*) of coordinated peroxy radicals (*1*) used for the initiation of H-abstraction, allyl radicals generated from oleic, linoleic, and linolenic acids in benzene solution ($c = 1 \times 10^{-2}$ mol dm^{-3}) in the presence of dissolved air (*2*) and EPR signal after bubbling with nitrogen or argon (*3*). Simulated EPR signal of the allyl radicals (*b*): *1*. Gain 1.6×10^4, modulation 1.25 mT; *2*, *3*. gain 6.3×10^5, modulation 0.4 mT, power 19.7 mW, θ = 23 °C.

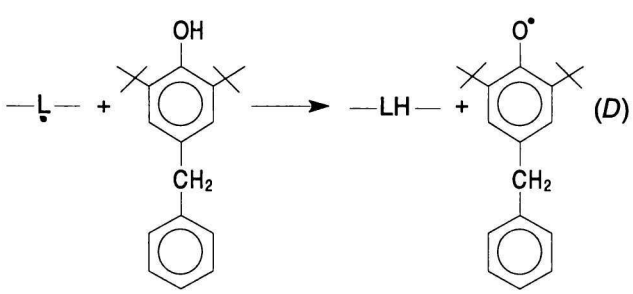
The high peak-to-peak (*p/p*) EPR line broadening in the presence of oxygen makes uncertain the detection of additional hyperfine splittings of one or more protons being in weaker interaction with the unpaired electron of the radical centre.

No difference in signal or *g*-values was detected, when the β-methylene hydrogen was the component of oleic, linoleic, or linolenic acids, or the initiating peroxy radicals were coordinated to Co(III) or Fe(IV).

This new, nonperoxidic signal instantly disappears, when the system is stripped with N_2 or Ar. This fact indicates that the low concentration of the dissolved oxygen in the solution takes part in the steady-state equilibrium, when the rate of generated radicals is equal with the rate of their transformation or recombination. In consequence of oxygen elimination with the bubbling nitrogen, the equilibrium is destroyed and the new simultaneously generated $ROO^{\bullet}$ radicals during the decomposition of the diamagnetic radical associate, stabilized by oxygen — $[L_2Co(III)RO_2\cdot\cdot O_2\cdot\cdot O_2RCo(III)L_2]$ — recombine immediately with the formed reactive alkyl resp. allyl radicals.

The EPR signal of the transient primary radicals measurable only in the presence of low oxygen concentration in benzene solution free from any other H-donors irreversibly disappears when the ambient temperature is increased over 40 °C. This fact is an indication that for chemical binding of oxygen to the generated radical a comparatively low activation energy is required.

The transiently formed radicals from the unsaturated fatty acids after H-abstraction through the peroxy radicals are still high-reactive for additional H-abstraction from an admixed sterically hindered phenolic antioxidant as 2,6-di-*tert*-butyl-4-benzylphenol (reaction *D*).



The result is a new triplet-triplet EPR signal in consequence of two protons of the methylene bridge, a_{CH_2} = 0.87 mT and two *meta*-protons, a_H^m = 0.16 mT (Fig. 4) interacting with the unpaired electron.

Similarly, in the presence of the highly effective biological antioxidant operating in the cell membrane — vitamin E — the original triplet signal is transformed to the signal of α-tocopherol free radical (Fig. 4).

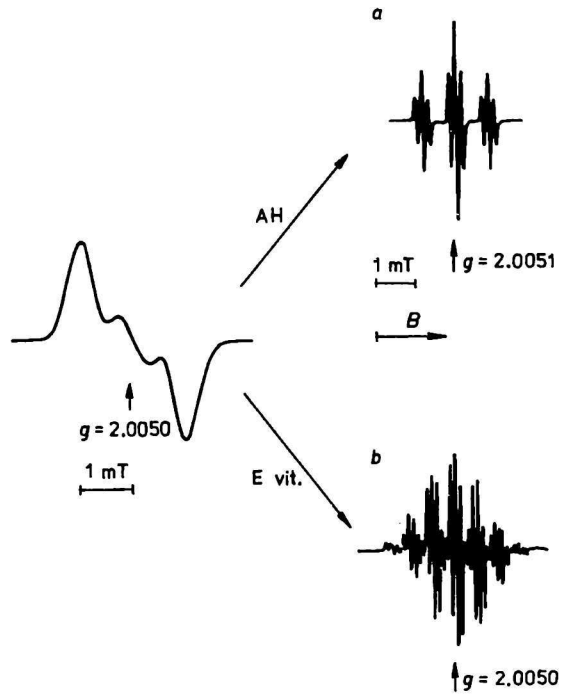
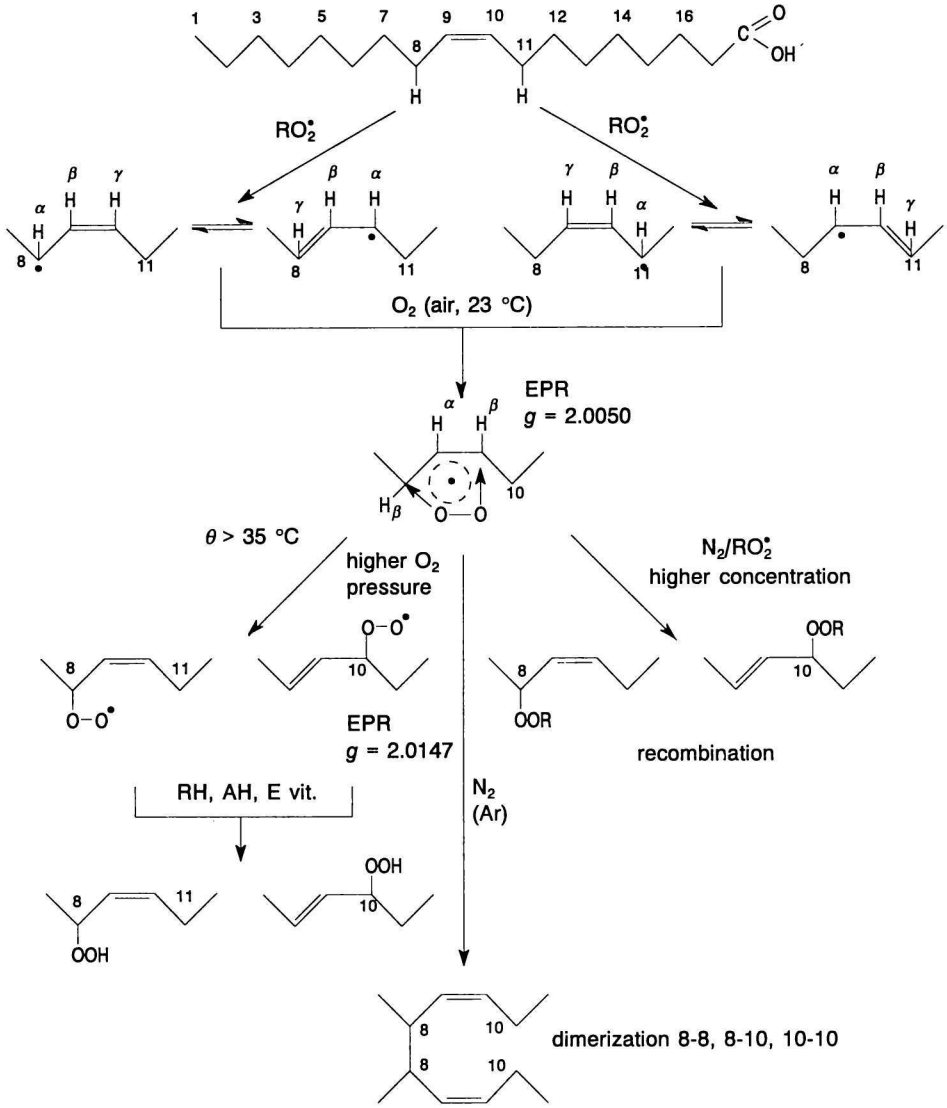


Fig. 4. Transformation of the transient allyl radical in the presence of phenolic antioxidant (AH, 2,6-di-*tert*-butyl-4-benzylphenol) (*a*) and natural antioxidant (α-tocopherol) (*b*) at laboratory temperature. *a*) Gain 10 × 10^5, modulation 0.125 mT; *b*) gain 6 × 10^5, modulation 0.01 mT, power 19.7 mW, θ = 23 °C.

Consequently in the presence of vitamin E, the transient allyl radicals of the unsaturated fatty acids will not be generated, because in the hydrophobic medium vitamin E will scavenge the initiating peroxy radicals. Vitamin E can be regenerated in the presence of vitamin C as the ultimate H-donor, preferentially in increased polar water medium.

The structure of the primarily formed "pentadienyl" radical detectable according to its EPR signal only in the presence of low concentration of oxygen dissolved in benzene with poor hydrogen-donating capacity and in the limited temperature range between 10—40 °C and generated immediately after abstraction of one hydrogen from the methylene group in β-position to one, two or three double bonds is in practice the same. The explanation is demonstrated on oleic acid (c = 1 × 10^{-2} mol dm^{-3}) after abstraction of one hydrogen at low concentration of coordinated peroxy radicals (*ca.* 5 × 10^{-6} mol dm^{-3}) from the methylene group in position 8 or 11 (Scheme 1).

The existence of the intermediate oxygen complex with primary allyl radical was supported by the experimental fact that the observed EPR signal cannot be saturated by increasing of the microwave radiation (up to 45 mW) and also was assumed by *Chan et al.* [13], studying the β-scission of oxygen combined with the release of O_2 and by *Porter* and



Scheme 1

Wujek [14] discussing the alkylic hydroperoxide rearrangement by β-scission.

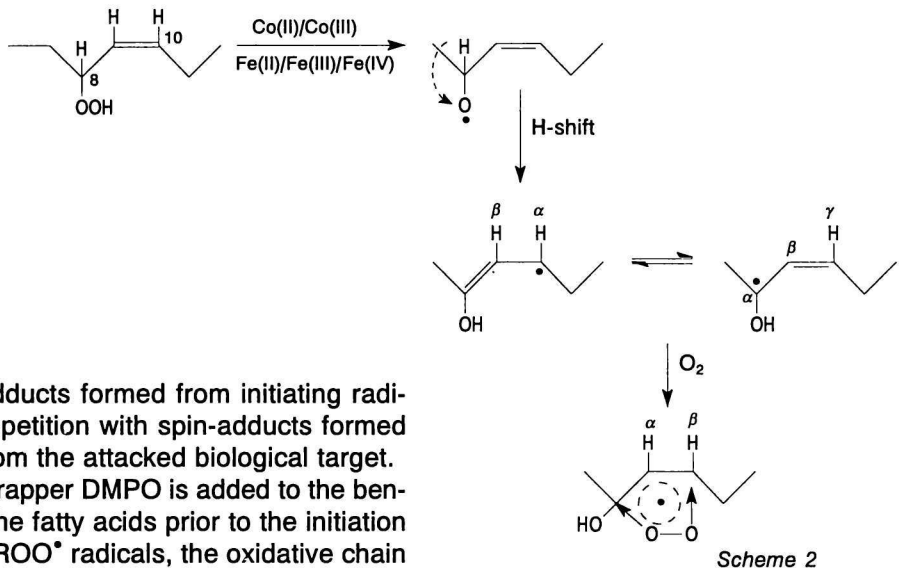
The consecutive reactions of the formed fatty acid peroxy radicals are discussed [6] and the different competitive pathways for oleic, linoleic, and linolenic acids are stressed: double bond isomerization of the *cis,trans* diene to *trans,trans* cyclization by intramolecular rearrangement which requires an inner peroxyl with three or more double bonds. Since peroxyl radicals of oleic acid cannot cyclize during autooxidation, only a competition between H-transfer and β-scission can occur [15, 16].

Intramolecular addition of peroxyl radicals to the β,γ double bond leads to both cyclic peroxides and tricycloendoperoxides with the structures similar to natural prostaglandins [17].

A different theoretical explanation of stable allyl radicals with measurable concentration by EPR without applying the spin-trapper technique at ambient temperature and presence of oxygen can be proposed on the assumption of homolytic scission of hydroperoxide in the presence of an electron donor (*i.e.* Co(II), $3d^9$; Fe(II), $3d^4$; Fe(III), $3d^5$) and followed H-shift during a steady-state autooxidation process (Scheme 2).

Epoxyallylic radicals were proposed [18—20] for intermolecular rearrangement of alkoxyl radicals by addition to the α,β double bond (Scheme 3).

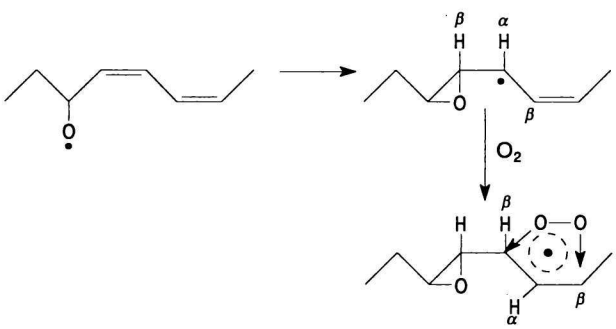
Carbon-centred free radical intermediates in the hematin and ram seminal vesicle catalyzed decomposition of fatty acid hydroperoxides were spin-trapped and characterized by EPR [4]. The authors have identified two carbon-centred radicals in the presence of oxygen using spin-trapper (*t*-nitrosobutane) but not spin-adducts of lipid peroxides LOO$^\bullet$. This observation is in agreement with our results. Applying spin-trappers in biological systems, the fundamental problem is still the distinction be-



Scheme 2

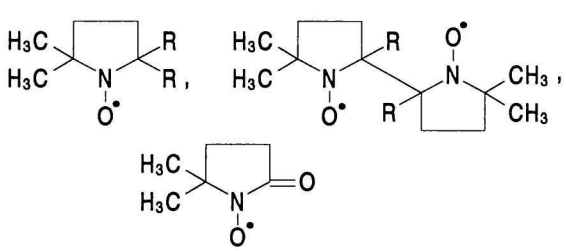
tween the spin-adducts formed from initiating radicals being in competition with spin-adducts formed simultaneously from the attacked biological target.

When the spin-trapper DMPO is added to the benzene solution of the fatty acids prior to the initiation with coordinated ROO• radicals, the oxidative chain reaction is prevailingly blocked and the dominant EPR signal is composed of 12 lines of the DMPO-OOR spin-adduct. But adding stepwise DMPO benzene solution to pentadienyl radicals already gener-

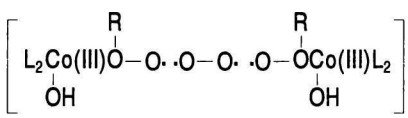


Scheme 3

ated in the presence of oxygen dissolved in benzene an intense 3-line EPR signal is generated (a_N = 1.25 mT). Spin-adduct without any protons interacting with the nitroxy radical centre is obviously formed. The possible structures of the new DMPO spin-adduct resulting from the reaction of the allyl radical R• complexed to activated oxygen are shown in the following formulas



After stripping the system with nitrogen or argon, the 3-line EPR signal remains unchanged, but an intense increase of the ROO• spin-adduct characterized by its 12-line EPR signal is generated — mainly from decomposing diamagnetic oxygen complex followed with the releasing of the coordinated ROO• radicals present in the solution [12] [L≡acac]



CONCLUSION

A requisite for the direct proof of pentadienyl-type free radical formed after peroxy radical attack of fatty acids at ambient temperature in nonpolar solvents is the transient stabilization of it by complexing with molecular oxygen. At low concentration of the initiating ROO• the observed unresolved 3-line EPR signal with g = 2.0050 instantly disappears, when the equilibrium between the labile oxygen radical complex and the dissolved oxygen molecules is changed after stripping the solvent with gaseous nitrogen or argon. The uncomplexed free pentadienyl-type radicals disappear by dimerization. On the other hand, at increased temperature (θ > 35 °C) and higher dissolved molecular oxygen concentration, the pentadienyl oxygen complex accepting the required activation energy is transformed to peroxy radicals of the competent fatty acids LOO• giving the same EPR signal and g-value as the initiating ROO• radicals (g = 2.0147).

Both the peroxy radical types abstract hydrogen atom from α-tocopherol or ascorbic acid forming the more stable free radicals of the competent E or C vitamin. At physiological temperature an intermolecular H-transfer starts the chain reaction with hydroperoxy groups (LOOH) accumulation. At higher concentration of the initiating ROO• and exclusion of

oxygen the primarily formed pentadienyl-type radicals instantly recombine with the surplus of ROO$^\bullet$ forming the diamagnetic alkylperoxy groups (LOOR).

Hydroperoxy groups are very sensitive to follow homolytic decomposition forming alkoxy radicals after accepting one electron in the presence of electron donors as chelated transition metals, semiquinones or asymmetric polyaromates.

This basic mechanism describing well the radical reactions for the oleic acid possessing only one double bond (a frequent component of phospholipidic biological membranes) is modified, when two double bonds are present (linoleic acid) in consequence of simultaneous or successive radical reactions. For instance, in consequence of internal H-shift HO—, epoxy or aldehydic groups can result. When three double bonds are present (linolenic acid) with the greatest probability for the free electron delocalization, the highest stability of the generated peroxy radicals LOO$^\bullet$ at ambient temperature is observed.

The unambiguous interpretation of the 3-line EPR signal of the spin-adduct of the pentadienyl—oxygen complex with the DMPO spin-trapper is not possible due to the presence of oxygen in the system. When during the radical attack of fatty acids the spin-trapper DMPO together with an H-donor (*e.g.* antioxidants, vitamins E or C) is present, in the overlap EPR spectra the lines of the more stable antioxidant radicals dominate.

During the process of peroxidation of the unsaturated membrane components, the membranes are damaged, holes and random hydrophilic channels are formed with the increase of uncontrolled transport of xenobiotics, co- and final carcinogens into cell and finally to the nucleus. Stabilized peroxy radicals on metalloenzymes in the nonpolar hydrophobic intrinsic membrane environment can be instantly deliberated from the transition metal after contact with stronger coordinating agents, like water, alcohol, ether, pyridine, basis of nucleic acids [8, 9]. Under continuous free radical attack the defense capacity of the cell plasma is decreasing by successive depletion of the biological antioxidants, localized in the hydrophobic cell membrane, as vitamin E, glutathione, glutathione reductase or diffuse spread in the hydrophilic cell inside, as vitamin C, catalase, peroxidase, superoxide dismutase (SOD), and superoxide reductase.

In this way the gradual transformation of enzymatically controlled radical reactions to random uncontrolled radical reactions triggers the chain of reactions with the final result of aging, in consequence of damaging organelles, mainly mitochondria and the immune system.

Leakage in mitochondrial respiration, bound to oxidation phosphorylation, is the beginning of uncontrolled electron transfer to oxygen dissolved in membranes starting the formation of superoxide $O_2^{\bullet-}$ transformed in the presence of SOD to H_2O_2, which is under control of catalase. TBHP, H_2O_2, and LOOH are carcinogens and accepting one electron from transition metals, semiquinones or superoxide can randomly form the high-reactive HO$^\bullet$ and HOO$^\bullet$ radicals eliminating the biological antioxidants and in this way stimulating the beginning of uncontrolled gene transcription [10, 11]. LOO$^\bullet$ radicals can additively transform different H-donor carcinogens (*e.g.* hydroxybenzo(*a*)pyrene, dimethylazobenzene, methylaminoazobenzene, benzidine, β-naphthylamine) to their free radicals with prolonged mean lifetime and greater operation space in cells and finally take part in activation of oncogenes coded in nucleus [9]. These events introduce finally the loss of cell contact inhibition blocking the cell differentiation, which is the beginning of uncontrolled tumour growth [10, 11, 21—23].

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