Fatty Acids Oxidation Studied by Electron Paramagnetic Resonance I. Initiation with Coordinated Peroxy Radicals and Reactions with Spin-Trapper DMPO

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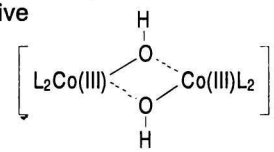
tert-Butyl peroxy radicals ROO$^\bullet$ with prolonged mean lifetime coordinated to Co(III) chelated in acetylacetonate ligand (acac) or to Fe(IV) in porphyrin ligand can effectively abstract hydrogen atom in nonpolar solvents from organic molecules at physiological temperature or form spin-adducts with the spin-trapper 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). The paramagnetic peroxy and spin-adduct nitroxy radicals, when joined through molecular oxygen present in the solution, form reversible diamagnetic complexes.

The experimental conditions controlling the transformation of the primary 12-line EPR signal of ROO$^\bullet$ and HOO$^\bullet$ spin-adducts to the secondary 7-line alkoxy radical signal in benzene solution, in contrast to the 4-line EPR signal of the high-stable HO$^\bullet$ spin-adducts formed in aqueous solutions, are described.

The diamagnetic hydroxy derivative



as the end product of the reaction of $Co(acac)_2$ with *tert*-butyl hydroperoxide resulting after the elimination of the original to Co(III)-coordinated ROO$^\bullet$ radicals, can catalytically generate DMPO spin-adducts of HO$^\bullet$ or HOO$^\bullet$ according to the actual polarity of the used solvent.

All types of studied DMPO spin-adducts (ROO$^\bullet$, HOO$^\bullet$, HO$^\bullet$) effectively abstract hydrogen from sterically hindered phenols as well as from biological antioxidants as α-tocopherol in hydrophobic or ascorbic acid in hydrophilic environment generating simultaneously the high-stable free radicals of vitamin E or C.

The main motivation of this study was to elucidate the principal experimental parameters, which control the reactions leading to the cell membrane peroxidation and to the highly reactive HO$^\bullet$, HOO$^\bullet$, ROO$^\bullet$ radical production in consequence of homolytic scission of the peroxide bond in the presence of effective electron donors (transition metals, semiquinones, asymmetric polyaromates, radiation effects in H_2O) [1, 2].

The prerequisite for correct interpretation of discrete steps of free radicals reactions initiated with defined amount of peroxy radicals stabilized by coordination on chelated transition metals and metalloenzymes, is the detailed knowledge of all experimental factors controlling the process and of all side reactions occurring in competition during the start of the chain oxidation. It concerns the role of oxygen pressure, solvent polarity and coordination ability, hydroperoxide decomposition, isomerization and internal peroxy radical reaction with the own or with the neighbouring molecules possessing a double bond system.

In the last years in many laboratories focussing their research on the radical chemistry in biological systems at physiological conditions, the spin-trapping technique – accumulation of reactive primary radicals with short mean lifetime to secondary nitroxy radicals with prolonged stability, is practised [3—5].

In this paper we intend to describe all reactions which can occur at different conditions when the attack of the biological target is initiated with comparatively high concentration of peroxy radicals coordinated to Co(III) or Fe(IV) in combination with simultaneously or additively applied DMPO as the spin-trapper. A stepwise transformation of the primary spin-adduct EPR signal occurs, when different H-atom donors as chain-breaking antioxidants are present in the system.

The second part of this contribution deals with the process of H-abstraction by free radicals from a methylene group in β-position to one or two conjugated double bonds of unsaturated fatty acids without and in the presence of biological antioxidants.

The experimental method discussed in this paper gives reliable information about the H-transfer cascade antioxidant mechanism, in which the initiating

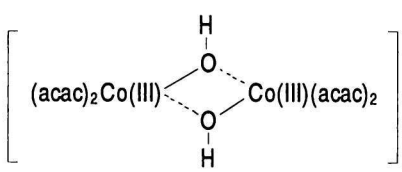
highly reactive radicals as well as their secondary less reactive nitroxy spin-adducts are stepwise transformed in the presence of vitamin E and C as biological antioxidants, to their most stable radicals, which finally are deactivated with glutathione/glutathione-reductase or disappear spontaneously by recombination [6—10].

Applying the described method the rapid freezing technique commonly used to prolong the mean lifetime of the primary radicals generated at physiological conditions, can be omitted.

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.

Coordinated radicals were prepared as described previously [11, 12]. Briefly, cobalt acetylacetonate was dried under vacuum for 20 min at 90—110 °C. The violet powder was dissolved in benzene to the concentration about 5×10^{-2} mol dm^{-3}. 0.3 cm^3 of this solution was in cylindrical EPR-cell mixed with 0.05 cm^3 of TBHP. After staying for approx. 10 min the unreacted surplus of TBHP together with the solvent was completely evaporated under vacuum at ambient temperature. This operation was repeated, the green powder was dissolved in benzene (0.3 cm^3) and evaporated again under vacuum. The concentration of coordinated *tert*-butyl peroxy radicals ROO$^\bullet$ was determined by EPR (broad line with g = 2.0147). After 24 h staying at elevated temperature (30 °C) or instantly by adding of decomplexing polar solvents (water, alcohol, pyridine), the EPR signal of coordinated ROO$^\bullet$ disappears. What remains after evaporation is the so-called hydroxy derivative



which can react with DMPO creating HO$^\bullet$ nitroxy spin-adducts in an aqueous or HOO$^\bullet$ adducts in benzene solution.

The spin-trapper 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was 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.

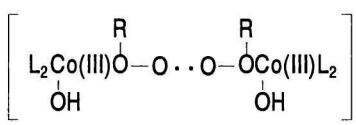
Measurements were carried out on Bruker ESR-SRC 200 spectrometer (Karlsruhe), which operates at X-band with 100 kHz modulation. For the spectra simulations the ASPECT 2000 software (Bruker) was used. Measurements were carried out either in cylindrical EPR-cells for nonpolar solutions or in flat cells for aqueous and biological solutions. For g-values calibration the Varian pitch standard was used (g = 2.0028).

RESULTS AND DISCUSSION

Radicals in Nonpolar Environment

The characteristic EPR signal of coordinated *tert*-butyl peroxy radicals on Co(III) chelated with acetylacetonate ligands or on Fe(IV) chelated in porphyrin ligand of hemin or of metalloenzymes (hemoglobin, cytochromes) [1, 6, 11, 12] is in nonpolar benzene (CCl_4, DMSO, acetone) solution at ambient temperature a broad line with g = 2.0147 (Fig. 1). When the electron transfer from the chelated transition metal, with at least one unpaired d-electron (Co(II), $3d^7$; Fe(III), $3d^5$), proceeds at higher mole surplus of the hydroperoxide (TBHP) (x_r = 1 : 10), a comparatively high concentration of the stabilized *tert*-butyl peroxy radicals can be reached, *ca.* 2×10^{-4} mol dm^{-3} at the temperature of 23 °C.

The reversible *para*-diamagnetic transformation by decreasing the temperature below 5 °C was experimentally proved [6, 12] resulting in consequence of the radical complex dimerization and tetraoxide formation without measurable EPR signal [L≡(acac)]



At ambient temperature, when the homolytic scission of the peroxy bonds in the presence of transi-

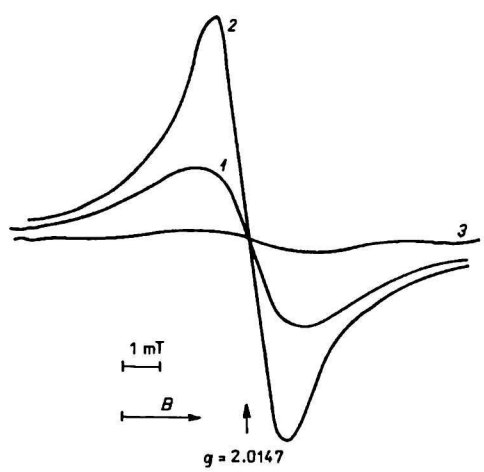
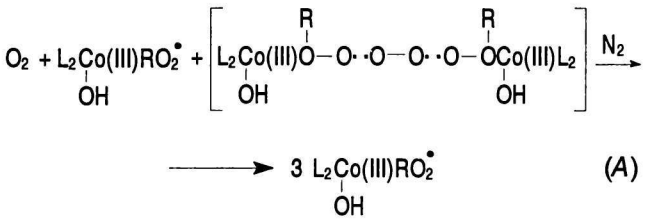


Fig. 1. EPR signal of coordinated *tert*-butyl radicals stabilized on Co(III) prepared in benzene at laboratory temperature and in the presence of air (*1*), bubbled with nitrogen or argon (*2*) and oxygen (*3*). Gain 1.6×10^4, modulation 1.25 mT, power 19.7 mW, θ = 23 °C.

tion metals proceeds in the presence of dissolved oxygen from air (line *1*), the intensity of the EPR signal with $g = 2.0147$ is at least three times lower than in the case, when the system was stripped with N_2 or Ar (line *2*). Oppositely, stripped with oxygen, the signal practically disappears (line *3*). The reversible oxygen effect is not only an EPR-line broadening, but it can be explained by the formation of transient labile diamagnetic associates of coordinated peroxy radicals mediated by the dissolved molecular oxygen possessing two unpaired electrons erasing the EPR signal (reaction *A*)



(*A*)

The high measurable concentration level of stable peroxy radicals cannot be explained as the result of a kinetic effect leading to a high steady-state concentration of peroxy radicals continuously generated in the presence of the steadily decomposing TBHP surplus [6, 12].

The signal intensity of the peroxy radicals namely remains at the same height also after complete elimination of the TBHP surplus by vacuum evaporation and following dissolution of the green diamagnetic powder once again in noncoordinating solvents — benzene, acetone or hexane.

The equilibrium between the paramagnetic coordinated peroxy radicals and their diamagnetic associates can be changed admixing weak organic acids (acetic, palmitic, oleic) into benzene solution.

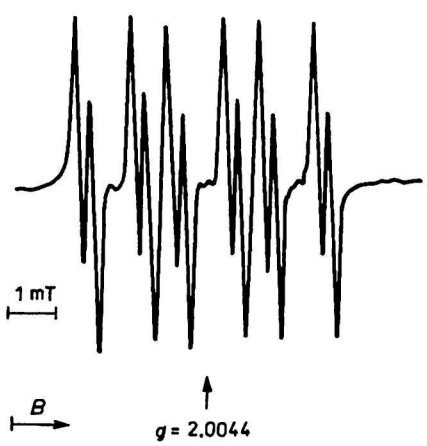
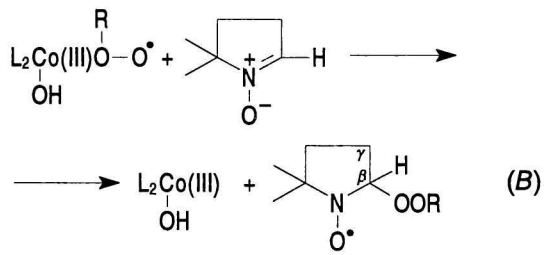


Fig. 2. EPR signal of DMPO spin-adduct of peroxy radicals formed in the reaction of coordinated peroxy radicals on Co(III) or Fe(IV) in nonpolar solvent with the spin-trapper DMPO ($c = 1 \times 10^{-3}$ mol dm^{-3}). Gain 2.5×10^3, modulation 0.05 mT, power 19.7 mW, $\theta = 23$ °C.

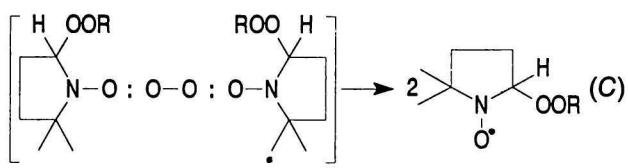
Reactivity of Coordinated Peroxy Radicals with the Spin-Trapper DMPO

Adding stepwise benzene solution of coordinated peroxy radicals (Co(III) or Fe(IV)) free from the unreacted *tert*-butyl hydroperoxide to a benzene solution with mole surplus of the DMPO spin-trapper in the presence of air, the original broad line of the peroxy radicals $g = 2.0147$ is transformed to a 12-line EPR signal (Fig. 2) with lower $g = 2.0044$, typical for the nitroxy spin-adducts with trapped peroxy radicals with characteristic coupling constants: $a_N = 1.30$ mT, $a_H^\beta = 0.81$ mT, $a_H^\gamma = 0.20$ mT (reaction *B*)



(*B*)

Following bubbling of the system with N_2 and simultaneous decomposition of the diamagnetic nitro tetraoxide of the spin-adducts takes place resulting in threefold increase of the intensity of the original 12-line EPR signal of the peroxy spin-adduct compared to the presence of dissolved air in benzene (reaction *C*)



(*C*)

The EPR signal transformation proceeds in principle differently, when the concentration of the spin-trapper is comparatively lower than the concentration of the primary peroxy radicals. In this case stripping with N_2 or following admixing of palmitic, oleic or acetic acid, the original 12-line signal of the DMPO-OOR spin-adduct is immediately transformed to a new type of EPR signal. The signal is composed of 7 lines in a line intensity relation 1 : 2 : 2 : 2 : 2 : 2 : 1 (Fig. 3), and was spectrosimulated with the following coupling constants: $a_N = 0.60$ mT, $a_{2H}^\beta = 0.31$ mT.

This transformation is connected with the change of *g*-value from 2.0044 to 2.0050. The same effect is observed, when to a benzene solution with the spin-trapper drops of coordinated peroxy radicals diluted in benzene are progressively admixed.

The transformation of the 12-line to the 7-line EPR signal during very careful "titration" passes practically through an "equivalent point" (Fig. 4) and is accelerated by the temperature increase. At 10 °C and lower temperature the original ROO$^\bullet$ spin-adduct is stable.

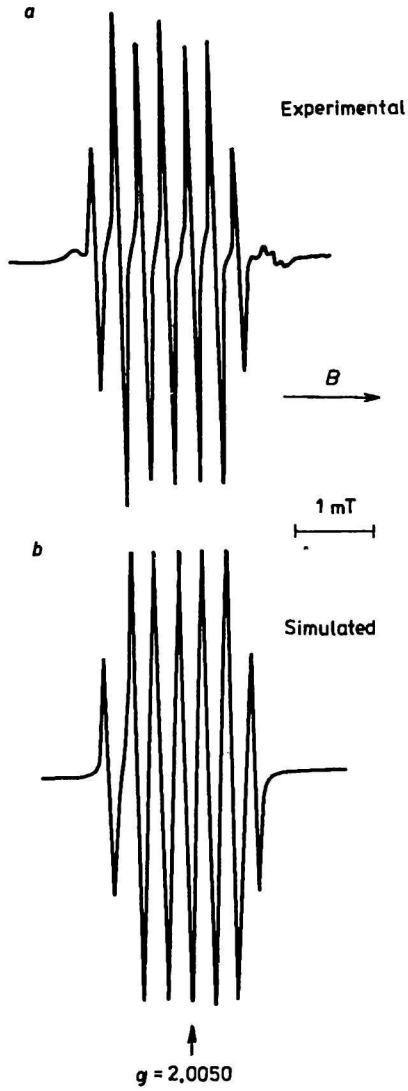


Fig. 3. 7-Line EPR signal produced during the transformation of primary DMPO-OOR spin-adduct (12-line signal) in the consequence of increased concentration of initiating peroxy radicals or adding of oleic or acetic acid (*a*) and simulated experimental signal (*b*). Gain 2,5 × 10^3, modulation 0.05 mT, power 19.7 mW, θ = 23 °C.

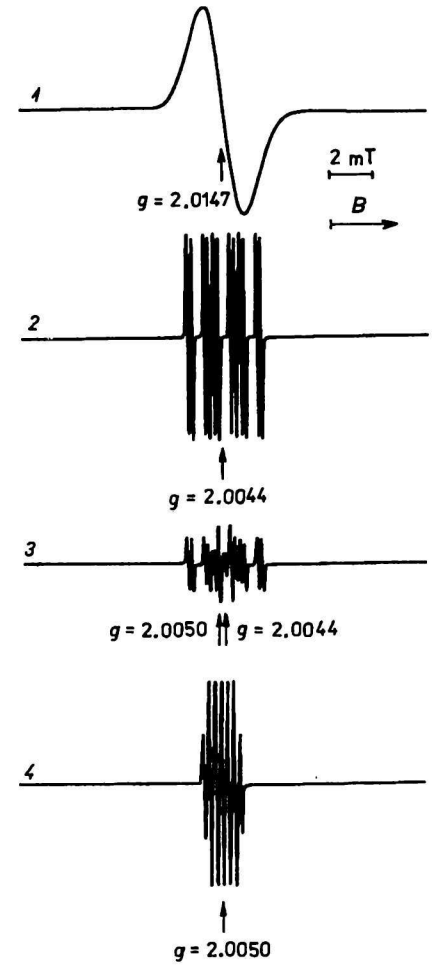
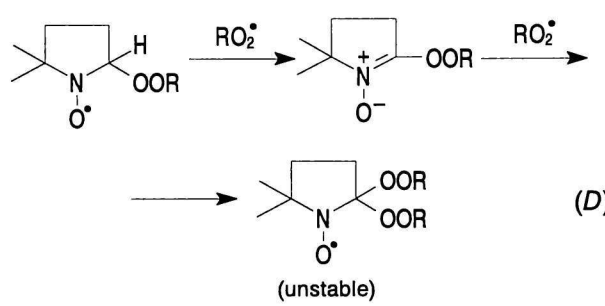


Fig. 4. Transformation of the EPR signal of DMPO spin-adduct (*2*) during stepwise adding of benzene solution of coordinated peroxy radicals (*1*) at laboratory temperature to the 7-line signal (*4*), mixed EPR signal of the original DMPO-OOH spin-adduct and of the forming septet signal near to the equivalent point of the titration (*3*). *1*. Gain 1.6 × 10^4, modulation 1.25 mT; *2*—*4*. Gain 2.5 × 10^3, modulation 0.05 mT, power 19.7 mW, θ = 23 °C.

The stepwise decrease of the 12-line signal intensity practically to zero at ambient temperature is followed by an effective increase of the 7-line signal. The interpretation of this phenomenon is based on acceptance of a secondary radical attack with H-abstraction from the primarily formed DMPO-OOR spin-adduct, with loss of the paramagnetism renewing the double bond and so the secondary spin-trapping capacity is created (reaction *D*).



(*D*)

The 7-line EPR signal is still highly reactive for additional H-atom abstraction from sterically hindered phenolic antioxidants or from the biological antioxidant α-tocopherol (vitamin E) operating in nonpolar media inclusive when it is localized in cell membranes (Fig. 5).

The theoretical interpretation of the 7-line signal is not unambiguous and is based on effective three-fold decrease of the spin density upon the heterocyclic nitrogen atom (from $a_{N_{12}}$ = 1.30 mT to a_{N_7} = 0.60 mT) and its transfer on two equivalent β-protons (a^{β}_{2H} = 0.31 mT).

Simultaneously taking into consideration the effective change also of the *g*-value a shift of the paramagnetic centre must be accepted on the pyrroline heterocyclic ring and an internal rearrangement of

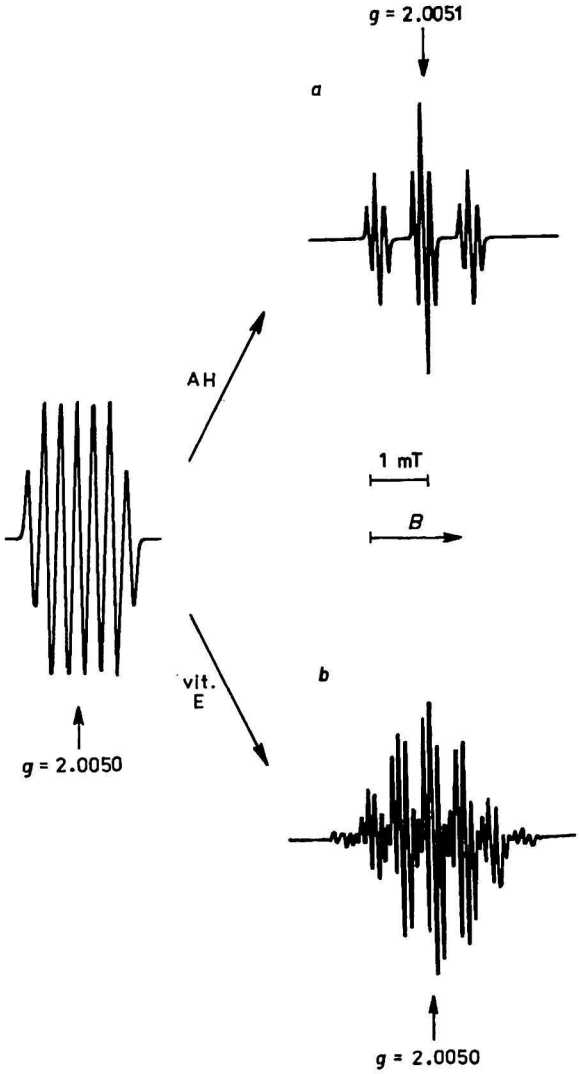


Fig. 5. Transformation of the alkoxy radical with 7-line EPR signal in benzene solution at ambient temperature after adding of phenolic antioxidant (AH, 2,6-di-*tert*-butyl-4-benzylphenol) (*a*) and of vitamin E (α-tocopherol) (*b*). *a*) Gain 10 × 10^5, modulation 0.125 mT; *b*) Gain 6 × 10^5, modulation 0.125 mT, power 19.7 mW, θ = 23 °C.

the spin-trapper after trapping the second peroxy radical is assumed. A similar effect can be achieved also by trapping of HOO$^\bullet$ radicals catalytically formed from the spontaneous decomposition of the hydroxy derivative present in the reaction system. This problem is discussed later in the text. When the spin-trapper is already present during the homolytic scission of ROOH accepting one electron from the chelated Co(II) or Fe(III) combined with ROO$^\bullet$ radical generation "in status nascendi" (reactions *E*)

$$\begin{aligned} \mathrm{ROOH} + \mathrm{L_2Co(II)} &\rightarrow \mathrm{RO^\bullet} + \mathrm{L_2Co(III)OH} \\ \mathrm{RO^\bullet} + \mathrm{ROOH} &\rightarrow \mathrm{RO_2^\bullet} + \mathrm{ROH} \end{aligned} \qquad (E)$$

besides the characteristic 12-line EPR signal of the DMPO-OOR spin-adduct simultaneously the 6-line signal (a_N = 1.25 mT, a_H^β = 1.46 mT) of trapped primary alkoxy radicals DMPO-OR is detectable. Also in this case, when the concentration of the spin-trapper is comparatively low in respect to the rapidly formed initiating ROO$^\bullet$ radicals, in a short time the final dominating 7-line EPR signal is established.

Spin-Trapping in Water and Buffer Medium

By dissolution of the vacuum-dried and from TBHP surplus free coordinated peroxy radicals on Co(III) in aqueous solutions containing the DMPO spin-trapper a comparatively high concentration of trapped HO$^\bullet$ radicals in the form of DMPO-OH spin-adduct can be proved. The characteristic EPR signal is a quartet with 1 : 2 : 2 : 1 line intensity relation resulting from similar coupling constants for the nitrogen atom a_N = 1.47 mT and for the one β-hydrogen a_H = 1.47 mT (Fig. 6).

In the presence of ascorbic acid after a rapid H-transfer to the DMPO-OH spin-adduct the new narrow doublet signal of ascorbyl radical is seen (g = 2.0050, a_H = 0.16 mT). The intensity of the quartet EPR signal runs with increasing pH through a maximum dominating in the range of 7.5—8.0.

The mechanism of immediately generating HO$^\bullet$ radicals in the presence of primary coordinated peroxy radicals was cleared after experiments with the hydroxy derivative free from coordinated ROO$^\bullet$ radicals.

Spin-Adducts Formed in the Presence of the Hydroxy Derivative of Co(III)

In the course of the preparation of coordinated peroxy radicals on Co(III) in the reaction with *tert*-

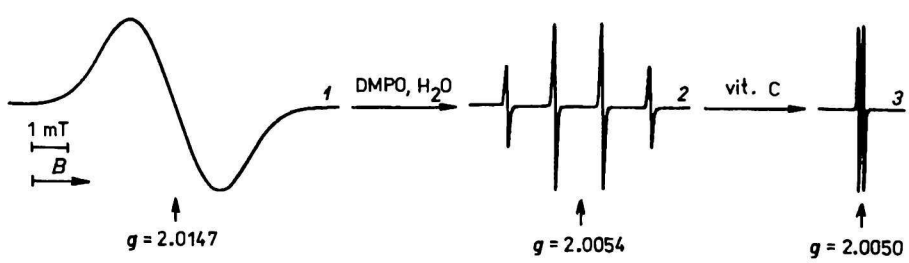
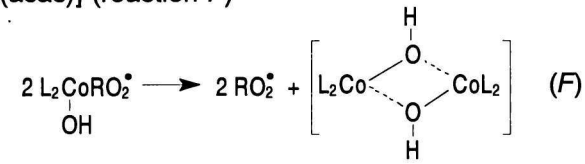


Fig. 6. Generation of HO$^\bullet$ spin-adduct in aqueous solution of DMPO (*2*) after admixing of coordinated peroxy radicals (*1*) and transformation of 4-line EPR signal to a doublet signal when additionally vitamin C is added (*3*). *1*. Gain 1.6 × 10^4, modulation 1.25 mT; *2*. Gain 5 × 10^4, modulation 0.125 mT; *3*. Gain 6.3 × 10^4, modulation 0.25 mT, power 19.7 mW, θ = 23 °C.

butyl hydroperoxide a diamagnetic hydroxy derivative associate after the peroxy radicals elimination from the coordination field is prevailingly formed [L≡(acac)] (reaction *F*)



(*F*)

When an internal electron-transfer takes place connected with homolytic bond scission, HO• radical creation can be accepted as an explanation (reaction *G*)

$$L_2Co(III)OH \rightarrow L_2Co(II) + HO^{\bullet} \qquad (G)$$

This suggestion was proved according to the following experiment summarized in Fig. 7.

At laboratory temperature, in benzene solution free from TBHP, the concentration of peroxy radicals coordinated to Co(III) after 24 h staying at ambient temperature decreases to zero, according to the disappearance of the original intense broad EPR signal of ROO• (curves *1*, *2*). The process can be accelerated by increasing of the temperature to 50 °C. Now, when the solvent is evaporated under vacuum and the dried green diamagnetic powder residue is under N_2 stirring once again, dissolved in water using the spin-trapper DMPO, the characteristic quartet signal of the HO• spin-adduct is registered (curve *3*). The intensity of the signal is of the same height as indicated in the freshly prepared system with the presence of coordinated radicals and is stable with the time (curve *4*). Once again nitroxy spin-adduct abstracts hydrogen from the ascorbic acid and the typical doublet signal of its radical is formed (curve *5*).

In nonpolar benzene in contrast to polar aqueous solution the interaction of the hydroxy derivative (after disappearance of the coordinated ROO• radicals) with the spin-trapper proceeds differently. In DMPO mole surplus the primarily formed 12-line EPR signal (curve *6*) of the spin-adduct differs in the a_H^{γ} coupling constant 0.155 mT from the DMPO-OOR spin-adduct a_H^{γ} = 0.200 mT, but not in a_N = 1.30 mT and a_H^{β} = 0.80 mT, which is typical for the HOO• spin-adduct [4, 13, 14]. The original 12-line EPR signal of DMPO-OOH is spontaneously transformed to the 7-line signal (curves *7*, *8*), which after contact with α-tocopherol is instantly transformed to the more stable phenoxy radical of the vitamin E (curve *9*).

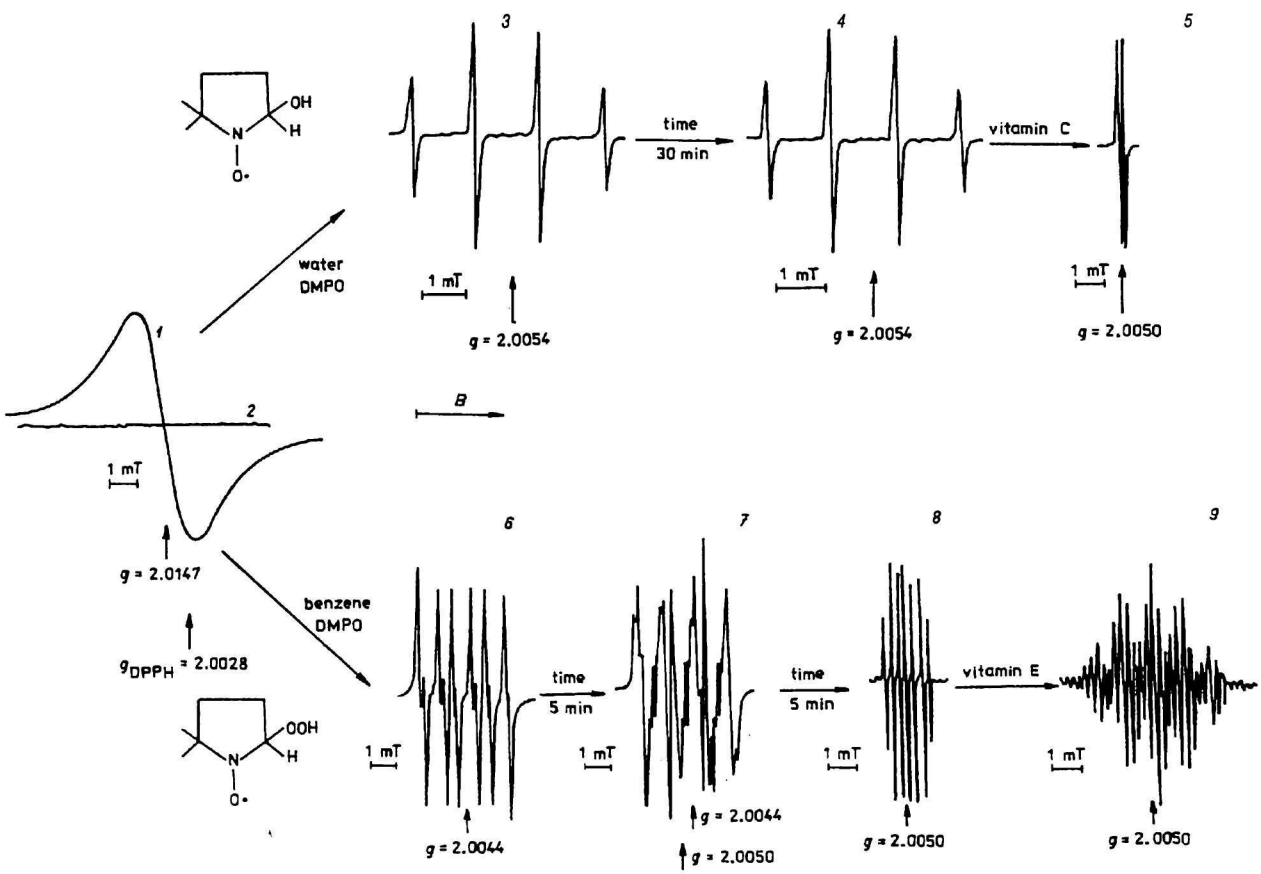


Fig. 7. Radical reactions represented with EPR signals of the hydroxy derivative formed after elimination of coordinated peroxy radicals from Co(III). The original signal of coordinated peroxy radicals (*1*), without EPR signal after elimination of ROO• radicals (30 °C, 24 h) (*2*). Reactions in aqueous solutions with DMPO ($c = 1 \times 10^{-3}$ mol dm^{-3}) stable with time (*3*, *4*), abstracting hydrogen from vitamin C and formation of EPR signal of vitamin C (*5*). Reactions in benzene solution forming DMPO-OOH spin-adduct (*6*), which is unstable with time (*7*) and continuous transformation to the 7-line EPR signal (*8*). H-Transfer from vitamin E to the alkoxy radical and formation of the radical of vitamin E at ambient temperature (*9*). Parameters as in figures above.

The acceleration of the transformation of the 12-line EPR signal of DMPO-OOH to the 7-line signal with addition of some drops of oleic or acetic acid can be explained by scission of the hydroxy derivative complexed through hydrogen bridges in the presence of polar compounds. Spin-adduct formation only from DMPO in the presence of organic acids was not proved. The formed labile monomer (a strong oxidative agent) undergoes an internal redox transfer induced in the presence of DMPO creating the transient $HO^{\bullet}$ radical and Co(acac) (reaction *H*)

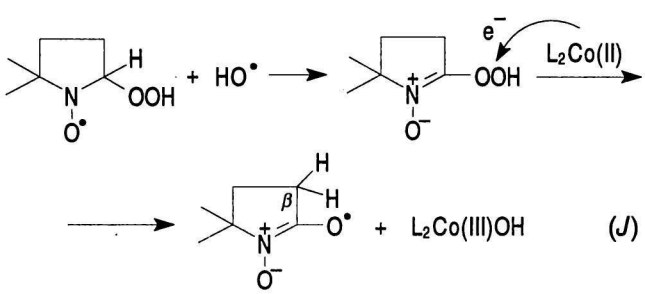
$$L_2Co(III)OH \xrightarrow{DMPO} L_2Co(II) + HO^{\bullet} \qquad (H)$$

Recombination of $HO^{\bullet}$ to H_2O_2 and successive radical transfer (reaction *I*)

$$HOOH + HO^{\bullet} \rightarrow H_2O + HOO^{\bullet} \qquad (I)$$

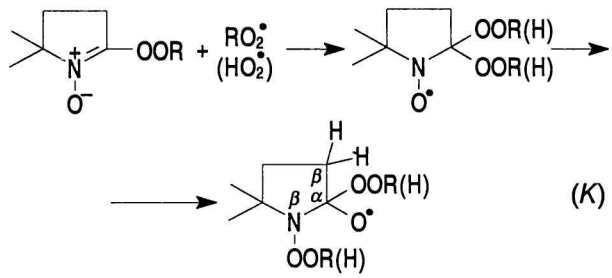
explain the origin of trapped hydroperoxy radicals in the beginning of the reactions.

The spontaneous change of the original 12-line EPR signal of DMPO-OOH to the 7-line EPR signal with the shift of the *g*-value to lower magnetic field ($g = 2.0050$), typical for alkoxy radical and the reactivity of this new formed radical for abstraction of a hydrogen atom from antioxidants can be explained as an intrinsic partial spin-density transfer from the nitrogen in the heterogeneous ring ($a_N = 0.60$ mT) to an alkoxy radical centre and as a spin-delocalization from the new radical centre to the two protons on the ring now in the β-position ($a_H^{\beta} = 0.31$ mT) (reaction *J*)



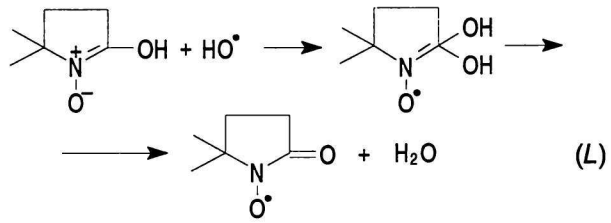
(*J*)

The proposed reaction (*J*) proceeds parallelly to the primary initiation step started with coordinated peroxy radicals, after the accumulation of the hydroxy derivative, and also when compounds which can split the associate to form the free $L_2Co(III)OH$ are present. Another possible explanation of the transformation of the 12-line to the 7-line EPR signal at increased $ROO^{\bullet}$ or $HOO^{\bullet}$ radical concentration comparative to DMPO is an internal decomposition of the destabilized spin-trapper after accepting two $ROO^{\bullet}$ or $HOO^{\bullet}$ radicals. The supposed final stable alkoxy radical, reactive to different H-donors, can manifest the same EPR spectral parameters as was described for the previous interpretation (reaction *K*)



(*K*)

The superposition of the 12-, 7- or 4-line EPR signals of DMPO spin-adducts with a triplet-signal $a_N = 1.20$ mT, $g = 2.0050$ without any interactions of protons can be explained according to the decomposition of the DMPO-$(OH)_2$ spin-adduct (reaction *L*)



(*L*)

All these facts must be taken into account, when coordinated peroxy radicals are used as initiators of radical reactions in biological systems in combination with spin-trappers to avoid false intepretations.

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