ALTERNATING REACTIVITY OF FREE RADICALS COORDINATED TO CHELATED TRANSITION METALS AND TO HEMOPROTEINS*

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Dedicated to Dr R. Zahradník on the occasion of his 60th birthday.

The mean lifetime of free radicals increases by coordination to transition metals of chelates including hemoproteins (hemoglobin, cytochrome *c*, catalase), when the radical generation proceeds in non-polar media in temperature range of physiological ones (290–310 K). In polar media (water, methyl- or ethylalcohol, pyridine), or in the presence of effective ligating agents (e.g. bases of nucleic acids), or at slightly elevated temperatures the intermediately stabilized oxygen centred radicals are liberated from the complex and the original high reactivity of the free radical is renewed. It is assumed that in this way sterically unhindered free radicals derived from chemical carcinogens with alternating reactivity could be transported through the micro-heterogeneous cell matrix.

The living cell as an open system is bound to its environment by the continuous influence of oxygen, metal traces, chelating agents, poisonous chemicals and free radicals generated catalytically in special compartments or randomly under influence of radiation. The operating space of a free radical in a living system depends on its own reactivity, which can be effectively changed after coordination to transition metals[2], and on the actual local reactivity of the biomatrix, where the creation of the radical takes place. According to a long-term study of one-electron transfer reactions between chelated transition metals[3], metalloenzymes[4] and peroxides, a new phenomenon of reactivity alternation during the migration of coordinated radicals on hemoproteins on cell level must be taken into consideration.

The aim of this series of papers is the deeper understanding the role of free and coordinated radicals in the process of transformation of a normal cell, where the radicals operate under enzymatic control, to a cell running out of the control under influence of chemical carcinogens, turning stepwise in a tumorous primitive cell.

Applying ESR spectroscopy we studied the parameters of the one electron transfer from chelated transition metals to hydrogen peroxide and to tert-butyl hydroperoxide

* Part III in the series Reactivity of Free and Coordinated Radicals in Biology and Chemical Carcinogenesis; Part II see ref.[1].

(t-BuOOH) on experimental objects with gradual increase of complexity. Starting from simple models, when Fe, Co or Cr are chelated to acetylacetonate, dithiocarbamate and porphyrine ligands, this paper deals with the problem of radical coordination to components of the oxygen transport mechanism (hemoglobin, erythrocytes, blood), of the respiration chain (cytochrome *c*), and of the enzyme controlling the hydrogen peroxide level (catalase).

EXPERIMENTAL

Materials and methods. Hemoproteins before dissolution in dimethylsulfoxide (DMS), or suspending in benzene, were carefully dried under vacuum at ambient temperature: hemoglobin (Hb) was isolated from bovine blood (Fluka), or from human blood (Lachema, $1{\cdot}4 \cdot 10^{-5}$ mol . l^{-1}). Hemin was prepared from bovine blood (crystalline, Type I, Sigma, m.w. 651·9), cytochrome *c* (Sigma, horse heart, $8 \cdot 10^{-5}$ mol l^{-1}) catalase (beaf liver, Reneal, $4 \cdot 10^{-6}$ mol l^{-1}). Peroxyl radicals were generated after addition of 10-fold molar excess of t-BuOOH (80% Fluka was after drying over silica gel under nitrogen purified by repeated freezing-off crystallization and following high vacuum evaporation at 283 K enriched to 95%) to DMS solution or of 30% H_2O_2, when the electron transfer was studied in water solutions, respectively.

Arterial human blood was obtained from healthy donors and from patients suffering from leukemia (malignant tumor lymphoma). To 2 ml of blood 1 mg of heparin was added to block the clotting. These samples were measured in flat quartz ESR cells or in cylindrical cells, when the liquid phase was eliminated and the plasma-free sediment was isolated and dried under vacuum. t-BuOOH or H_2O_2 were admixed to a suspension or blood coagulum (250 mg) prepared in benzene (0·02 ml of t-BuOOH or 0·05 ml of H_2O_2 to 0·3 ml of blood suspension).

The different methods of preparation of coordinated peroxy radicals in reaction of tris(2,4-pentadionato)iron, $Fe(acac)_3$, $Fe(acac)_2$, $Co(acac)_2$, and of $Cr(acac)_3$ with t-BuOOH or with etheric extract of H_2O_2 were described previously[3,5].

a) Initiation of H-*transfer reactions with coordinated radicals.* Coordinated tert-butylperoxyl radicals were obtained by the reaction at 373 K in vacuum of carefully dried 2% $Co(acac)_2$, $Fe(acac)_2$, $Fe(acac)_3$ or $Cr(acac)_3$ dissolved in water-free benzene, toluene, CCl_4, DMS or in acetone, with a ten-fold molar excess of t-BuOOH, at 293 K. Into an ESR cell filled with 0·2 ml solution of coordinated radicals (c. $1 \cdot 10^{16}$ spins) 0·1 ml of dissolved H-donor (antioxidant) solution in two-molar excess over transition metal chelates was added under a nitrogen blanket and stirred for 1 min by bubbling before ESR measurement.

b) Initiation of H-*transfer reactions with coordinated radicals with exclusion of unreacted* t-BuOOH *and oxygen.* Coordinated tert-butylperoxyl radicals made according to the method *a)* were evaporated under vacuum at 283 K and the powder-like residue was dissolved in benzene, toluene, CCl_4, DMS or acetone. This operation was repeated twice, finally giving a 10^{-4}M solution of coordinated peroxyl radicals. 0·1 ml of a 0·1M benzene solution of H-donor (antioxidant) was then added to 0·3 ml of the initiating system with coordinated radicals.

Apparatus. Free radical signals were measured by Varian E-3 and Bruker SRC-200ESR spectrometers operating in X-band with 100 kHz modulation, equipped with variable temperature accessory.

RESULTS AND DISCUSSION

One Electron Transfer between Transition Metals and Peroxides and Formation of Coordinated Peroxyl Radicals in the Range of Physiological Temperatures

Transition metals (Fe, Co, Cu, Mn, Cr, Ni) bound in metalloenzymes play an essential role in most redox reactions proceeding in biological environment at aerobic conditions. Nevertheless transition metals as free ions or bound to different chelates and also denaturated metalloenzymes can take part in the multistep process of carcinogenesis[5-9]. The same can be stated also about H_2O_2 (refs[10-13]), which is an intermediate product of two-electron reduction of oxygen operating during detoxification of endogenous compounds and biotransformation proceeding in the endoplasmatic reticulum in the presence of peroxidases.

The respiratory burst of $O_2^{\cdot-}$ and H_2O_2 induced by oxidase during phagocytosis[14] accompanied with HO· radical generation can randomly damage also the phospholipides of the cell membrane and DNA (ref.[15]) of components of the activated immunological system. High toxicity[16,17] and tumor promotion ability was proved for t-BuOOH operating in biological systems as well as for H_2O_2 (refs[10,18]). This double function of necessity and toxicity remains valid also for molecular oxygen[16,18,19] which in ground state is a biradical. Increased oxygen pressure[20] can produce free radicals $O_2^{\cdot-}$, HO·, $HO_2^{\bullet}$ in lungs and lungs mitochondria leading to irreparable damages (chromosomal aberrations) and to carcinogenesis.

Besides the oxidative state and high- or low-spin state of the chelated transition metals, a fundamental role in the process of free radical generation and coordination must be ascribed to the actual chelating ligands. This is demonstrated in the reaction of completely ligated iron being in the oxidative state Fe(III) possessing an unpaired electron ($3d^5$, S 1/2) surrounded with six sulfur atoms (dipropyl dithiocarbamate), six or four oxygen atoms (acac) and six or five or four nitrogen atoms (hemoglobin, porphyrine).

Homolytic scission of metal–ligand bond with free ligand radical generation. The ESR signal of the free ligand radical after homolytic scission of the metal–ligand bond, observed at 296 K in benzene solution of 0·05M tris (dipropyl dithiocarbamato) iron (FeL_3) is indicated in Fig. 1. The concentration of the ligand radical L· being in equilibrium with FeL_2 gives a three-line ESR signal (g 2·02) indicating an effective spin delocalization from two sulfur atoms of the ligand to the neighbouring ^{14}N nucleus (I_N 1) with a coupling constant a_N 8 mT, Eq. (*A*).

$$L_2Fe(III)\,L \rightarrow L_2Fe(II) + L\cdot \qquad (A)$$

The simultaneously observed second narrower three-line ESR signal (a_N 1·2 mT)

with the higher g-value 2·04 is ascribed to the paramagnetic ferry state as a product of an internal charge transfer with the delocalization of the transferred electron upon the ligand[21], Eq. (*B*).

$$L_2Fe(II) \rightarrow LFe(III)\,L^{\cdot-} \qquad (B)$$

In the presence of the antioxidant 2,6-di-tert butyl-4-benzylphenol (AOH) as a hydrogen atom donor, the ESR signal of the ligand radical L· disappears and the typical three on three-line signal of the stable phenoxyl radical AO· is formed (g 2·0039). The coupling constants of two protons in *para* and in *meta* position are a_p 1 mT and a_m 0·2 mT (Fig. 1), according to Eq. (*C*).

$$L^{\cdot} + AOH \rightarrow LH + AO^{\cdot} \qquad (C)$$

It is worthy to mention that the dithiocarbamate ligand is an effective deactivator

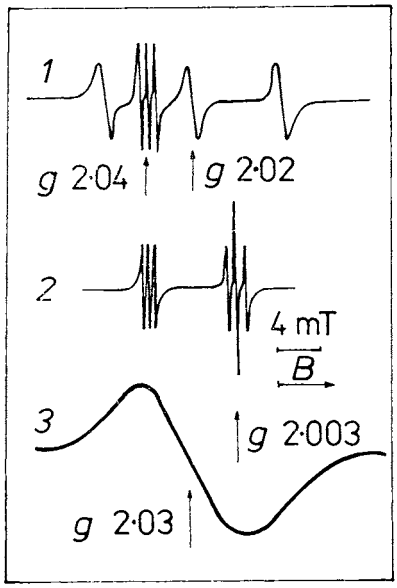


FIG. 1

ESR spectra of tris(dipropyl dithiocarbamato)iron *1* 0·05M benzene solution, *2* after addition of 0·05M-2,6-di-tert-butyl-4-benzylphenol, *3* oxidated with 0·26M-t-BuOOH (*T* 296 K)

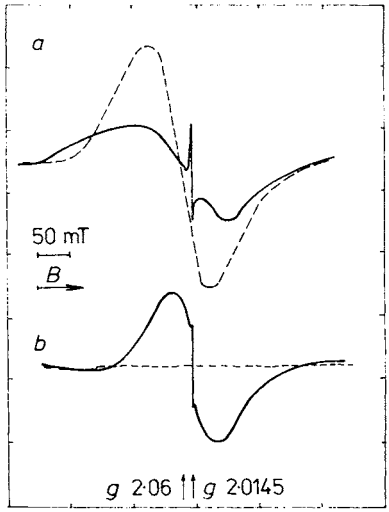


FIG. 2

ESR spectra of *a* 0·05M toluene solution of $Fe(acac)_3$ (dashed line), and after addition 0·1M-t-BuOOH (full line), *b* 0·05M toluene solution of $Fe(acac)_2$ under nitrogen (dashed line), and after addition 0·1M-t-BuOOH (full line)

of the enzyme superoxodismutase (SOD) controlling the level of $O_2^{\cdot-}$ in biosystems[19,22].

Heterolytic scission of metal–ligand bond with ligand elimination. The one-electron transfer to peroxides from totally coordinated Fe(III) can be demonstrated on $Fe(acac)_3$. In toluene solution with tenfold molar excess of t-BuOOH the original broad ESR signal (g 2·06) of the low spin $3d^5$-Fe(III) decreases and is superimposed by the signal at g 2·0147 of coordinated tert-butyl peroxyl radicals ($RO_2^{\cdot}$) (Fig. 2*a*). The reaction is started with a catalytic reduction of $Fe(III)L_3$ and simultaneous ligand elimination, Eq. (*D*).

$$L_2Fe(III)L + \text{t-BuOOH} \rightarrow L_2Fe(II) + RO_2^{\cdot} + HL \qquad (D)$$

This step creates convenient steric conditions for radical coordination during the successive two-electron transfer to t-BuOOH, generating the stable signal (g 2·0147) of coordinated $RO_2^{\cdot}$ radicals on Fe(IV) forming an oxy-ferryl radical complex, Eqs (*E*), (*F*), and (*G*).

$$L_2Fe(II) + \text{t-BuOOH} \longrightarrow L_2Fe(III)OH + RO^{\cdot} \qquad (E)$$

$$L_2Fe(III)OH + [\text{t-BuOOH}]_2 \longrightarrow \left[L_2Fe(IV)\underset{OH}{\overset{R}{O}}-O^{\cdot} \quad {}^{-}OH \right]_{cage} + ROH \qquad (F)$$

$$\left[L_2Fe(IV)\underset{O}{\overset{R}{O}}-O^{\cdot} \right] + H_2O \qquad (G)$$

This mechanism is supported also with the experimental fact that a higher concentration of coordinated peroxyl radicals can be prepared on $Fe(acac)_2$ missing a bidentate ligand after reaction with t-BuOOH (Fig. 2*b*).

The direct electron-transfer to t-BuOOH from Fe(III) surrounded by three bidentate ligands was proved in course of rapid freezing to 152 K of a $2 \cdot 10^{-2}$ M toluene solution of $Fe(acac)_3$ mixed with 5-fold molar excess of t-BuOOH (Fig. 3). The electron transfer starts in the temperature interval, where the frozen solid phase changes to liquid one, near the temperature of 233 K accompanied with an effective increase of free, non coordinated $RO_2^{\cdot}$ radicals (g 2·0090). Reaching a narrow maximum the concentration of free $RO_2^{\cdot}$ rapidly falls down with further temperature increase. At ambient temperature at least 2–5% of all generated peroxyl radicals remain stabilized on the highest oxidation state of Fe(IV) (g 2·0147). The temperature

range, in which the $RO_2^{\bullet}$ is generated, is the same, in which the reversible change of the high spin Fe(III) (S 5/2, g 4·3) to low spin Fe(III) (S 1/2, g 2·0060) is observed.

A similar peroxyl radical coordination (broad ESR signal with g 2·0145) is observed, when the totally coordinated $Cr(acac)_3$ reacts with t-BuOOH excess (Fig. 4). The original high spin Cr(III), $3d^3$ ESR signal (g 3·2) decreases during oxidation and the free radical remains coordinated on the higher oxidation state of Cr(IV) in agreement with the theory of oxidative addition[2]. At the same experimental conditions 10-times higher concentration of coordinated peroxyl radicals can be generated, when $Co(acac)_2$ reacts with t-BuOOH (refs[2-4]).

Electron transfer without ligand elimination. In hemoproteins the reactive center for redox reaction is complexed to porphyrine ligand possessing a strong covalent character with effective spin delocalization on the whole ligand field. The *d*-electrons of the transition metal are according to the competent temperature organized to low or high spin state.

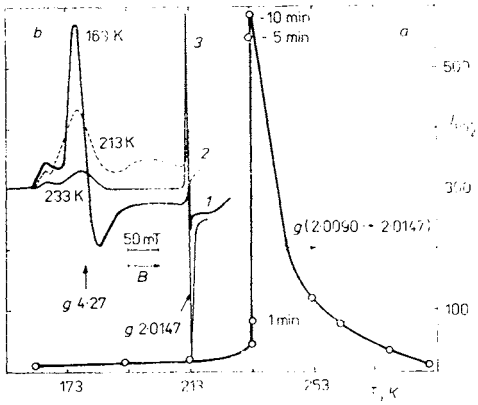


FIG. 3

Dependence of the intensity of ESR signal of free peroxyl radicals (g 2·0090) and to Fe(IV) coordinated radicals (g 2·0147) on time. *a* 0·02M Toluene solution of $Fe(acac)_3$ mixed with 0·16M-t-BuOOH was rapidly frozen to 140 K and then heated up to ambient temperature. *b* ESR signals are registered at stepwise increased temperature *1* 163 K, *2* 213 K, and *3* 233 K

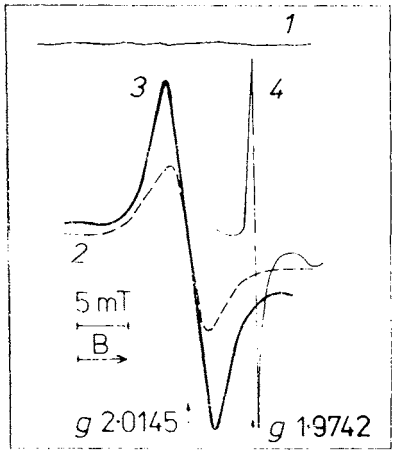


FIG. 4

ESR spectra of $Cr(acac)_3$ at 296 K. *1* 0·05M benzene solution, *2* after reaction with 0·1M-t-BuOOH, *3* after evaporation of the unreacted t-BuOOH, *4* addition of 0·1M-3,5-dimethylphenol

When hemoglobin (Hb) dissolved in DMS is contacted at ambient temperature with t-BuOOH, after rapid two-electron transfer steps the original iron in the oxidation state Fe(II) is transformed to oxyferryl complex [Fe(IV)], on which $RO_2^{\bullet}$ radicals remain stabilized by coordination[4,5].

With time and at slightly elevated temperature the original broad ESR signal of coordinated $RO_2^{\bullet}$ radicals to Hb (g 2·0147) in benzene suspension is transformed to a narrower single line with lower g-value (2·0032) in consequence of an intramolecular electron transfer (Fig. 5). When the electron transfer proceeds between t-BuOOH and hemin, where the iron is complexed in porphyrine without protein substituent, the primarily formed complex of $[Fe(IV)RO_2^{\bullet}]$, (g 2·0147) remains stable without transformation to the singlet line with g 2·0032, as it was observed in the case of

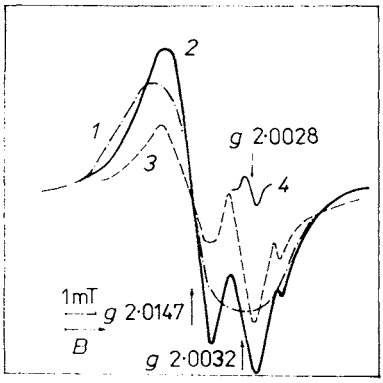


FIG. 5

ESR spectra of 10 mg hemoglobin suspended in 0·3 ml of benzene in presence of 0·05 ml t-BuOOH. *1* Microwave power 30 mW and T 296 K, *2* after 5 min heating at 323 K, *3* decrease of microwave power to 10 mW, *4* weak pitch Varian standard (10^{13} spin/0·1 ml)

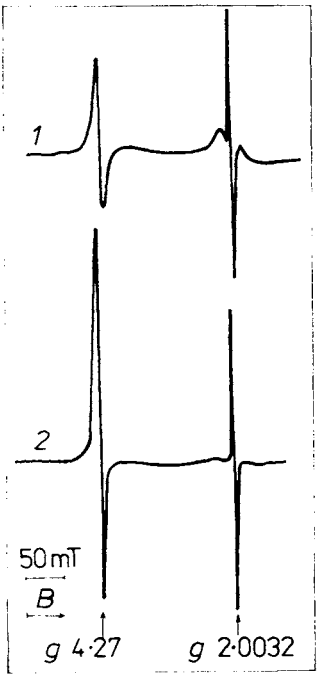
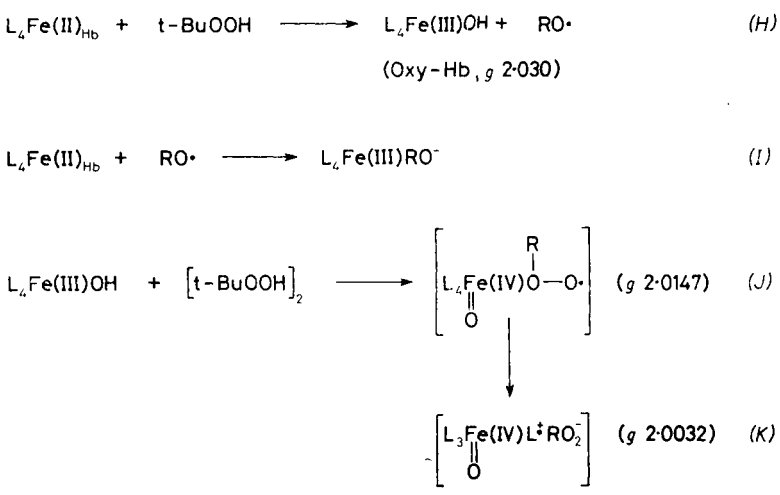


FIG. 6

ESR spectra of *1* 0·3 ml of aqueous solution of hemoglobin (5 mg in 1 ml water) mixed with 0·05 ml of 10% H_2O_2 rapidly frozen to 140 K, *2* cytochrome *c* at the same conditions

oxidation of Hb. It is assumed that the peroxyl radical present in the ligand field of hemoproteins abstracts one electron from a near lying protein side chain having a lone electron pair situated on sulfur component[23].

The singlet signal with g 2·0032 can be generated by treatment of powdered Hb with H_2O_2 (ref.[4]) or in DMS solution, when Hb, cytochrome c or catalase react with t-BuOOH at 296 K. Because this ESR signal does not show hyperfine splitting in consequence of the unpaired electron delocalization to the nuclei of nitrogen atoms of the porphyrine ring, and cannot be changed by H-transfer in the presence of effective antioxidants and can be eliminated only by one-electron reduction, the reaction sequence (Eqs (*H*), (*I*), (*J*), (*K*)) leading to stabilized cation radicals, can be proposed.



The analogous scheme for H_2O_2 reaction with hemoglobin is as follows (Eq. (*L*)).

$$[L_3Fe(IV)LHO^{\bullet}] \rightarrow [L_3Fe(IV)L^{+\bullet}HO^{-}] \qquad (g\ 2{\cdot}0032) \qquad (L)$$

Thus the narrow ESR signal at g 2·0032 observed after heating the powdered Hb in the presence of oxygen can be explained similarly[4] as a paramagnetic radical complex, Eq. (*M*).

$$[L_3Fe(IV)O_2^{-\bullet}L] \rightarrow [L_3Fe(IV)O_2^{2-}L^{+\bullet}] \qquad (M)$$

The direct evidence of one-electron transfer from hemoglobin or from cytochrome c to H_2O_2 in polar solution forming the final ligand $L^{+\bullet}$ free radicals, can be obtained

only applying the technique of rapid freezing and ESR indication at low temperature up to 127 K. In Fig. 6 besides the single ESR line with g 2·0032 is also seen the signal of high-spin iron Fe(III) with g 4·27 of the irreversibly oxidized hemoproteins.

The discussed radical mechanism of peroxide decomposition initiated by hemoglobin remains valid also for erythrocytes isolated from fresh human blood. The complete healthy arterial blood does not show any ESR signal at ambient temperature, but gives an intense signal at g 2·0147 already in the first seconds after contacting with t-BuOOH (Fig. 7).

The primary broad signal (g 2·0147) of the free peroxyl radicals immediately disappears in aqueous media at ambient temperature, followed by simultaneous increase of the ESR signal g 2·0032. The stability of this signal in polar media is comparatively high and can be ascribed to the hemoglobin "ligand" ion-radical $L^{\ddagger}$ generated by internal electron transfer according to Eqs (*L*) and (*M*). Higher production of coordinated peroxyl radicals is observed, when from plasma free dried sediment of the blood composed mainly of erythrocytes and granulocytes is suspended in benzene and then mixed with t-BuOOH (Fig. 8). In non-polar media the coordinated peroxyl radicals express a prolonged mean lifetime, but are still highly

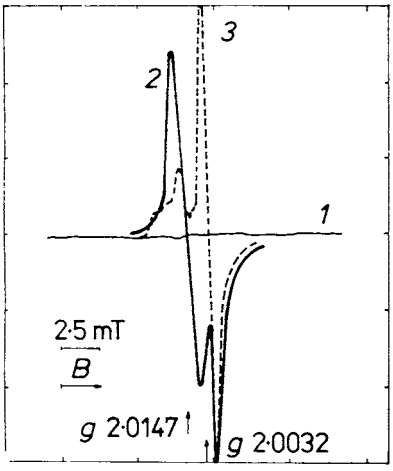


FIG. 7

ESR spectra of *1* 0·3 ml arterial human blood in flat ESR cell at ambient temperature, *2* added 0·05 ml of t-BuOOH and registration in the course of the first three seconds, *3* registration after one min

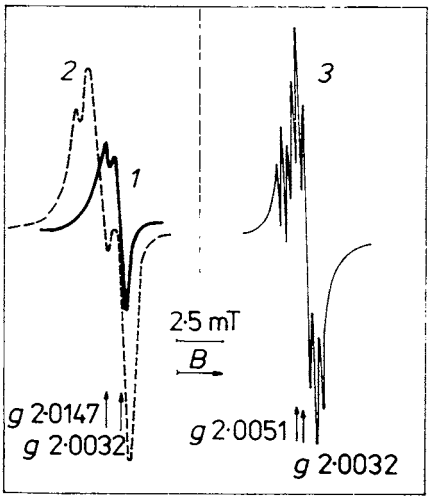


FIG. 8

ESR spectra of *1* 0·3 ml benzene suspension of 150 mg vacuum dried sediment from leukemic blood free of plasma (lymphoma), *2* to previous sample added 0·04 ml of t-BuOOH, *3* to previous sample added 10 mg of 2,6-di-tert-butyl-4-benzylphenol

reactive to hydrogen atom abstraction, for instance from sterically hindered phenolic antioxidants. So when 3,5-di-tert-butyl-4-benzylphenol is admixed as the H-donor, the original ESR signal with g 2·0147 disappears and the new three on three-line signal (g 2·0051) of the phenoxyl radical remains superimposed upon the original signal of the cation-radical $L^{+\cdot}$ (g 2·0032), non-reactive for the H-transfer (Fig. 8, curve 3). In some leukemic blood samples the signal with g 2·0032 can be observed already before contacting the probe with t-BuOOH. Typical for leukemic blood samples (malignant tumor lymphoma) is a 20–60-times higher increase of generated peroxyl radicals in the presence of t-BuOOH, in comparison with the arterial blood samples of healthy donors.

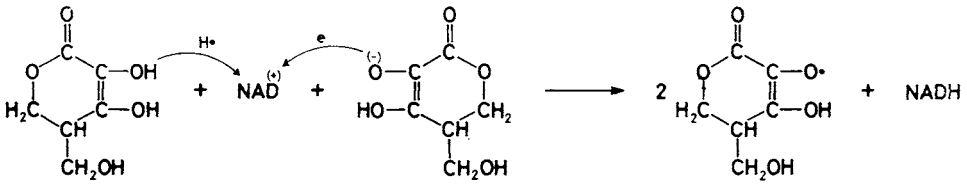
In leukemic blood also an intense ESR signal of low spin Fe(III) (g 2·03) can be observed. It might be supposed that this signal results from irreversible oxidation of Fe(II) of hemoproteins to Fe(III) of hemichromes. We have also proved that denaturated hemoproteins easily transfer one electron to t-BuOOH and H_2O_2 leading to scission of the peroxide bond.

H-*Transfer Cascades Initiated by Coordinated Peroxyl Radicals*

The precondition of the stability of peroxyl radicals complexed on hemoproteins at physiological temperature is the exclusion of stronger coordinating agents as are water, methanol, ethanol, diethylether, pyridine, tetrahydrofuran. These conditions are created in the non-polar media of cell membranes interconnected with hydrophobic channels, where coordinated peroxyl radicals can survive as long as they do not come in contact with effective H-donors owing active OH, SH, SeH or NH_2 groups. Effective H-donors are the biological antioxidants (α-tocopherol, ascorbic acid, glutathione), or some effective procarcinogens and their metabolites (β-naphtylamine, methylaminoazobenzene, benzidine, hydroxylated forms of benzo[*a*]-pyrene[1,24], natural organic compounds with unhindered OH group, e.g. capsaicine, patulin, kojic acid, catechins of quebracho).

We have documented that in multicomponent system of different H-donors the intramolecular H-transfer proceeds as long as the more stable radical form is established[3,25]. Such an H-transfer cascade started by coordinated $RO_2^{\cdot}$ radicals on hemoglobin in DMS solution is demonstrated in Fig. 9, or by radicals coordinated on cytochrome *c* in the Fig. 10. The primary [Fe(IV)$RO_2^{\cdot}$] radical abstracts a hydrogen atom from a sterically hindered phenolic antioxidant 2,6-di-tert-butyl-4-benzylphenol and the original broad ESR signal (g 2·0147) disappears and a new signal composed of three on three-lines of the competent phenoxyl radical is generated (g 2·0051, a_H^p 0·87 mT, a_H^m 0·16 mT). This radical is inactivated in the presence of ascorbic acid in DMS forming the typical narrow doublet line with g 2·0060 of dehydroascorbyl (a_H 0·25 mT). The radical of ascorbic acid can be deactivated in the presence of glutathione and glutathione reductase.

Finally in this H-transfer cascade the intensity of the signal belonging to the dehydroascorbyl radicals is decreased or fully eliminated by glutathione in the presence of glutathione reductase or increased almost twice, when the redox-coenzyme NADH/NAD^+ is added to the DMS solution (Scheme 1).



SCHEME 1

This H-transfer cascade is a part of a protective mechanism in biological systems, when high reactive radicals are randomly formed. On the cell level a great importance must be ascribed to α-tocopherol localized in non-polar media of biomembranes. We have experimentally proved[4] that α-tocopherol effectively deactivates the free and coordinated peroxyl radicals forming characteristic seven-line ground splitting (two *ortho* CH_3 groups) of ESR signal of phenoxyl radicals (*g* 2·0058). But these radicals are effectively reduced by ascorbic acid forming instantly the typical narrow ascorbic acid radical doublet ESR signal (*g* 2·0060). We have also proved that $RO_2^•$

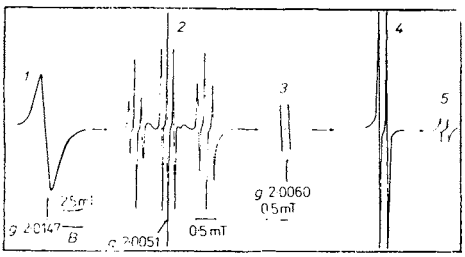


FIG. 9

ESR signal transformations in the course of an H-transfer cascade started by *1* coordinated: peroxyl radicals Fe(IV)$RO_2^•$ on hemoglobin, when to 0·1% solution in DMS 0·2% of t-BuOOH was added. Successively admixed: crystalline *2* 2,6-di-tert-butyl-4--benzylphenol (0·1%), *3* ascorbic acid (0·1%), *4* NADPH (0·01%), *5* last sample after 12 hours at 296 K

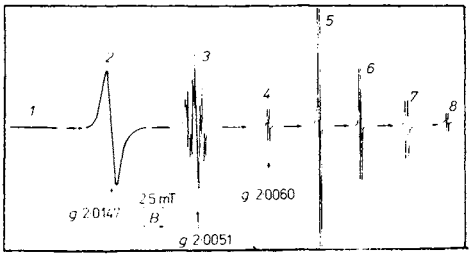


FIG. 10

ESR signal transformations by an H-transfer cascade *1* 0·2% solution of cytochrome *c* in DMS, *2* added 0·2% t-BuOOH. Successively admixed: crystalline *3* 2,6-di-tert-butyl--4-benzylphenol (0·1%), *4* ascorbic acid (0·1%), *5* NADPH (0·01%), *6* reduced glutathione (0·01%), *7* cystein (0·01%), *8* last sample after 12 hours at 296 K

radicals coordinated on hemoproteins are immediately scavanged by vitamin A and cystein possessing the effective H-donor SH group. The expressive synergetic cooperative antioxidant function of a multicomponent system of antioxidants with different redox potential based on α-tocopherol, ascorbic acid and glutathione, was stressed also by other techniques[16,18,26–28].

Aromatic amines and aminoazocompounds, many of them known as carcinogens, are also activated to their radical forms after an H-transfer step from the amine group to coordinated $RO_2^{\bullet}$ radicals on metalloenzymes. In the reaction cage are intermediate radical complexes formed [FeṄAr] with high thermal stability represented by an asymmetric 3-line ESR signal[29] (Fig. 11), which cannot be instantly

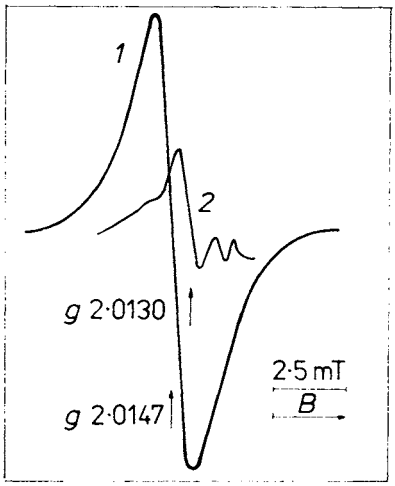


FIG. 11

ESR spectra of peroxyl radicals coordinated on hemoglobin *1* after reaction of 0·05 ml t-BuOOH with 0·3 ml of 0·1% Hb in DMS–acetone 1 : 1, *2* signals observed when successively admixed crystalline 4-dimethylaminoazobenzene, or 4-methylaminoazobenzene, or β-naphtylamine

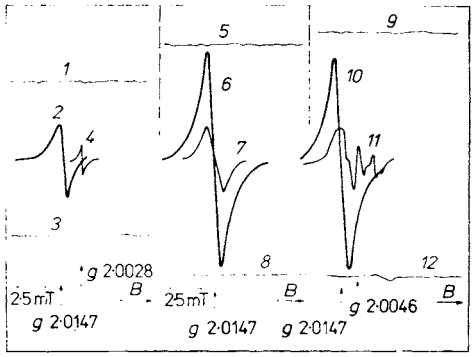


FIG. 12

ESR signals at 296 K of *1* 0·1% of t-BuOOH in DMS, *2* added 5 mg of crystalline hemoglobin, *3* to previous sample added 5 mg of crystalline guanine hydrochloride, *4* weak pitch Varian standard (10^{13} spin/0·1 ml), *5* 0·1% solution of hemoglobin in DMS deaerated with nitrogen, *6* adding of 0·05 ml t-BuOOH to 0·3 ml solution of *5*, *7* to previous sample added 5 mg of crystalline DNA, *8* to previous sample added 5 mg of guanine hydrochloride, *9* 0·1% solution of hemoglobin in DMS, *10* to 0·3 ml of previous sample added 0·05 ml of t-BuOOH, *11* to previous sample added 0·1% of 4-methylaminoazobenzene, *12* to previous sample added 5 mg of guanine hydrochloride

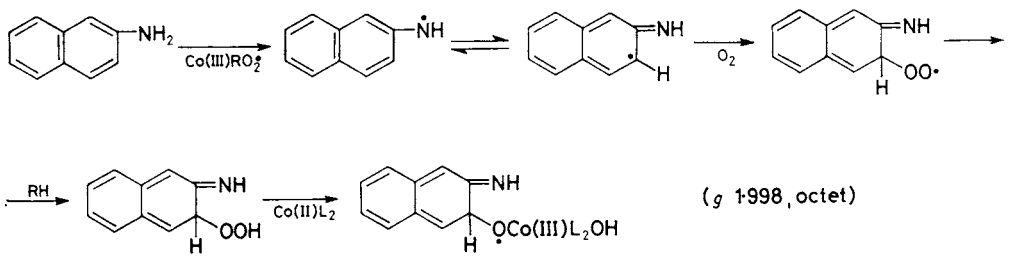
deactivated by an H-transfer step from biological antioxidants, but these complexes are destroyed in the presence of strong coordinating ligands as nucleic acids are, or instantly with the guanine hydrochloride (Fig. 12). Reactive radicals pushed out from the coordination field of transition metals react instantly with H-donor targets, aliphatic double bonds, or they disappear by recombination.

Long-Term Stability of Phenoxyl Radical σ-Complexes Generated from Unhindered Phenols, Carcinogens and Co-Carcinogens

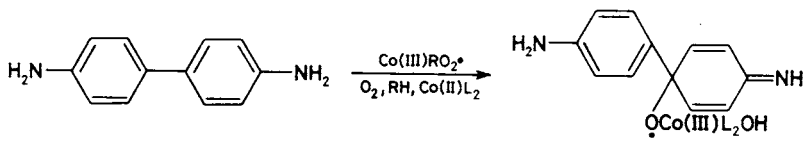
For the direct indication of spin delocalization from a σ-coordinated free radicals up to the transition metal very advantageous are chelated complexes of cobalt, because the cobalt nucleus possesses a discrete magnetic moment (I 7/2). So sterically unhindered phenoxyl radicals can after H-exchange remain stabilized in the coordination field of the cobalt creating ESR signals with basic splitting to eight equal intense lines[3].

In the Fig. 13 the ESR signal with comparatively low g-value (1·998) generated by reaction of peroxyl radicals coordinated on Co(III) chelated by acac with the unhindered 3,5-dimethyl phenol consists of eighteen lines as a result of interaction of an unpaired electron with three protons of one methyl substituent (a_{met} 0·40 to 0·45 mT) and with one cobalt nucleus (a_{Co} 1 mT). This type of σ-complexes could be generated from a great number of unhindered phenols[30,31], but cannot be deactivated in non-polar media by an H-transfer from biological antioxidants in contrast to stable free phenoxyl radicals generated from sterically hindered phenols. On the other hand σ-coordinated radicals are instantly decomposed by a ligand exchange, when they are in contact with DNA or bases of nucleic acids as guanine is (Fig. 13, curve 3). They can also be destroyed by action of strongly coordinating solvents (e.g. methanol, water), namely at elevated temperature.

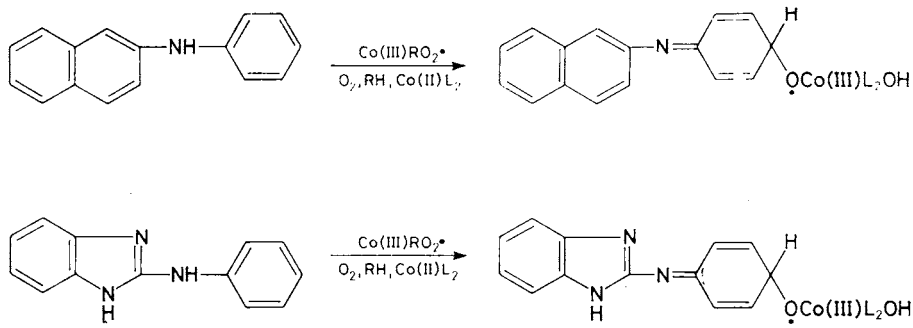
This complexation ability of radicals prepared from aromatic amines (as pro-carcinogens) to cobalt was also demonstrated. The asymmetric three-line ESR signals generated from the carcinogenic β-naphtylamine and from the non carcinogenic α-naphtylamine after reacting with coordinated $RO_2^{\bullet}$ radicals are seen in Fig. 14. The concentration of the primary nitrogen radical complex of the α-naphtylamine decreases with the time in contrast to the radicals generated from the β-naphtylamine, ESR signal of which is stepwise converted to a typical eight-line signal of σ-coordinated naphtoxyl radical (Scheme 2). Very similar octet ESR signal was observed also in the case of carcinogenic benzidine and methylaminoazobenzene (Scheme 3) or of phenyl-β-naphtylamine and phenylaminobenzimidazole (Scheme 4), in reaction with coordinated $RO_2^{\bullet}$ radicals in the presence of oxygen dissolved in polar media. In this way coordinated radicals show great stability and so they can operate in a larger space of non-polar environment, but when they are decomplexed coming in contact with methanol, ethanol or bases of nucleic acids, the original high reactivity is recovered.



SCHEME 2



SCHEME 3



SCHEME 4

The effect of rapid pushing out a coordinated radical from the ligand field of transition metal by water molecules instantly after the electron transfer, when the peroxidic bond of H_2O_2 is homolytically broken to HO· and HO^- fragments, can be minimized by using "dried" diethylether extract of H_2O_2, and by carrying out the redox steps in non-polar solvents (benzene, tetrachloromethane, toluene).

The precondition for obtaining an ESR signal with fifteen lines of the same intensity (a_{Co} 1·2 mT, g 2·0392) in reaction of $Co(acac)_2$ with H_2O_2 ether extract, is the presence of dissolved oxygen in benzene, toluene or tetrachloromethane solution already before mixing the two reactants (Fig. 15, curve *1*). The following bubbling of the solution with inert gases (N_2 or Ar) at 296 K caused an immediate spectral

changes depending on the solvent used. In CCl_4 the fifteen-line signal disappears, but in the benzene or toluene it is transformed to a signal split into eight equal intense lines of σ-complexed radical interacting only with one cobalt nucleus (g 1·998, Fig. 15, curve 5).

An interaction between the formed HO· radical and the molecular oxygen complexed between two cobalt atoms of the dimer $[Co(acac)_2]_2$ must be taken into consideration. The fifteen equi-intense line ESR signal with the high g(2·0392) could be interpreted formally as the signal of coordinated $HO_3^{\bullet}$ radical (Scheme 5).

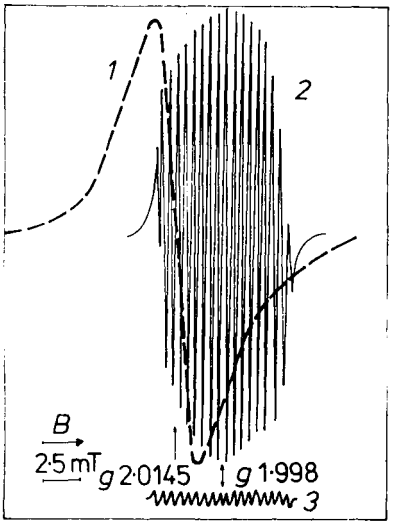


FIG. 13

ESR signal of coordinated $Co(III)RO_2^{\bullet}$ peroxyl radicals prepared at 296 K, *1* by reaction of 2% benzene solution of $Co(acac)_2$ with 10-fold molar excess of t-BuOOH, *2* to previous sample added 5 mg of crystalline 3,5-dimethylphenol, *3* to previous sample added 5 mg of guanine hydrochloride

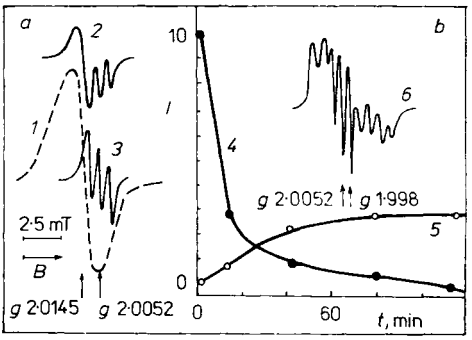


FIG. 14

ESR signals generated from α- and β-naphtylamine in benzene solutions (*a*) and their change with time at 296 K (*b*). *1* Coordinated $Co(III)RO_2^{\bullet}$ peroxyl radicals prepared by oxidation of $Co(acac)_2$ with t-BuOOH, *2* to previous sample added crystalline β-naphtylamine in three-fold molar excess to $Co(acac)_2$ or *3* α-naphtylamine in the same concentration, *4* dependence of the intensity of ESR signal *I* (in arbitrary units of the signal height) on time of radicals generated from α-naphtylamine, *5* from β-naphtylamine, *6* superimposed ESR signal of the free nitroxyl radicals of β-naphtylamine (triplet) and of σ-coordinated naphtoxyl radicals (octet)

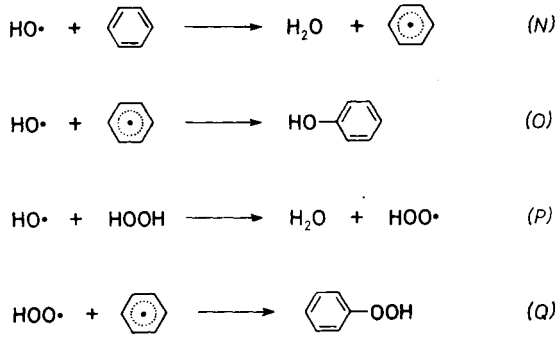
$$[Co(II)L_2]_2 + O_2 \rightleftharpoons [L_2Co(II)O_2Co(II)L_2]$$

$$[L_2Co(II)O_2Co(II)L_2] + 2H_2O_2 \longrightarrow \left[\begin{matrix} L_2 & & H & & L_2 \\ & Co(III)\dot{O}-\dot{O}Co(III) & & & \\ HO & & & & OH \end{matrix}\right] + HO\cdot$$

(g 2·039)

SCHEME 5

The ternary complex can be destroyed by eliminating the oxygen excess from the solution. Simultaneously the high reactivity of the liberated HO· radical is restored. Hydroxyl radicals can oxidize benzene to phenol (Eqs (*N*), (*O*)), while the formed HOO· radicals to benzenehydroperoxide (Eqs (*P*), (*Q*)).



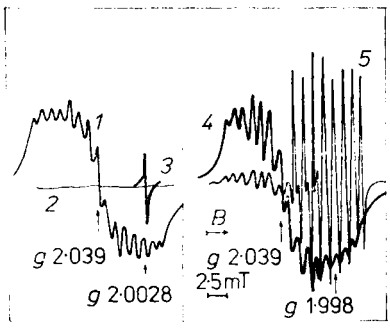
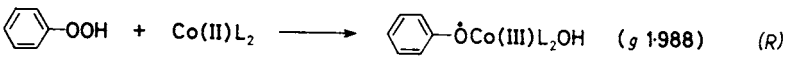


FIG. 15

ESR spectrum of *1* HO· radical bond to ·O—O· complexed between two cobalt nuclei prepared in 0·3 ml 1·5% tetrachloromethane solution of $Co(acac)_2$ saturated with oxygen reacting with 0·1 ml of ethylether extract of H_2O_2, *2* previous sample deoxygenated by nitrogen, *3* Varian weak pitch standard (g 2·0028), *4* HO· radical bond to ·O—O· complexed between two cobalt nuclei prepared in benzene, *5* transformation of the fifteen-line signal of the previous sample to a signal with eight-line basic splitting after deoxygenation with nitrogen at 296 K

H-transfer from phenol to HO· or HOO·, as well as electron transfer from $Co(acac)_2$ to benzenehydroperoxide, generates reactive unhindered phenoxy radicals, which can be stabilized only after σ-coordination (Eq. (*R*)).



(*R*)

The tumour promoter properties of peroxyl or hydroxyl radicals generated from t-BuOOH (ref.[32]) or from H_2O_2 (refs[10,18]), respectively were proved in vivo. This effect is more pronounced in the presence of transition metals as Fe, Cr, Co (refs[5–9]).

Transition metals, besides catalysis of the homolytic scission of peroxides, enable the simultaneous prolongation of the mean lifetime of generated radicals from miliseconds and minutes to hours and days by coordination. Increased mean lifetime leads to increased operating radius of radicals and to their higher steady state concentration. The consequence is the increase of the probability that coordinated radicals will meet biological targets before being deactivated through H-transfer from antioxidants. The original reactivity of radicals is renewed after their decomplexation from the carriers according to the actual polarity and coordination capacity of the biological environment. The proposed mechanism contributes to the explanation of the great complexicity, variability and multifunctional character of chemical carcinogens operating on bases of free radials.

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