# REACTIVITY OF PEROXY RADICALS COORDINATED TO VITAMIN $B_{12}$ STUDIED BY ELECTRON SPIN RESONANCE SPECTROSCOPY

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One-electron transfer from chelated Co(II)($3d^7$) of vitamin $B_{12}$ (cyanocobalamine) to *tert*-butylhydroperoxide forms at ambient temperature in non-polar solvents peroxy radicals stabilized by π-coordination to Co(III)($3d^6$) of the oxidized vitamin. In the absence of oxygen, the peroxy radicals manifest themselves as singlet ESR lines at $g$ = 2.0174. After addition of sterically hindered phenols, apart from the decreased original singlet line, a new signal of stable phenoxy radicals, formed by H-abstraction from the phenols, is observed. Phenoxy radicals generated from unhindered phenols remain stabilized by σ-coordination to the cobalt(III) centre, giving rise to an ESR signal split into eight lines due to interaction of the unpaired electron with the magnetic moment of the $^{59}$Co nucleus ($I$ = 7/2) of vitamin $B_{12}$. Large molecules with unhindered OH groups, such as testosterone or cholesterol cannot be coordinated after the primary radical attack and the generated radicals disappear by recombination. By this technique, the sensitivity of biological targets to primary peroxy radical attack as well as the efficiency of different antioxidants (vitamin E, Stobadine) can be tested.

**Keywords**: Vitamin $B_{12}$; Peroxy radicals; Antioxidants; H-transfer reactions; Vitamin E; ESR spectroscopy; Cobalt; Electron transfer.

Relatively high concentrations of stable *tert*-butylperoxy radicals (2 × $10^{15}$ spin/0.2 ml) were prepared at ambient temperature using different transition metal chelates such as Co(III) (acetylacetonate)[1], Fe(IV) (metalloenzymes – hematin, hemoglobin, cytochrome c, catalase)[2], and Cu(II) (superoxy dismutase (SOD), cytochrome oxidase (COX) of mitochondria)[3], as proved by ESR.

In the case of vitamin $B_{12}$ with Co(II) coordinated to four nitrogen atoms of the porphyrin ring, fundamental papers have been published[4,5] about the ability to form with oxygen paramagnetic $Co^{III}O_2^{\bullet-}$ complexes stabilized on single crystals at 0 °C. These complexes exhibit typical octet ESR signals characteristic of interaction of the unpaired electron of the superoxy

radical with the magnetic moment of the $^{59}Co$ nucleus. In general, the high activity of Co(II) in formation of oxo complexes after transfer of one electron to molecular oxygen was also proved for the atomic oxygen generated in the cold microwave plasma in the presence of crystalline $CoCl_2$ [6].

In this paper it is shown that the typical π-coordinated peroxy radicals $(OH)B_{12}(ROO^{\bullet})$ can be stabilized by vitamin $B_{12}$ (cyanocobalamine) with Co(III) centres in non-polar solvents (benzene, $CCl_4$, hexane) at room temperature, and then used to study different H-transfer reactions in the presence of organic H-donors (mainly with OH, NH, and SH substituents) or spin-traps.

## EXPERIMENTAL

For the preparation of π-peroxy or σ-phenoxy radicals coordinated to vitamin $B_{12}$ commercial 0.22-g tablets containing 100 μg of vitamin $B_{12}$ (Nature's Bounty Inc., U.S.A.), were used. The materials employed for tablet production (e.g., microcrystalline cellulose, mannitol) have no effect on the extraction of vitamin $B_{12}$ into non-polar solvents (benzene, $CCl_4$, hexane). For ESR measurements, two tablets were treated with dry benzene (2 ml) at about 50 °C under inert atmosphere ($N_2$, Ar). Next, the clear solution containing ca. 0.015% of vitamin $B_{12}$ (0.3 ml) was transferred into a closed quartz cylindrical cell ($d$ = 3 mm), followed by addition of *t*-BuOOH (Fluka, 92%) (0.05–0.08 ml) dried over $P_2O_5$ and vacuum-distilled at room temperature.

The highest concentration of peroxy radicals coordinated to vitamin $B_{12}$ ($2 \times 10^{15}$ spin/0.3 ml) was reached in 10 min at about 23 °C. After this period, the solution was shortly bubbled with $N_2$ or Ar before the ESR measurement.

To study the H-abstraction reaction in the presence of different organic substances such as hindered phenols (vitamin E, 4-phenyl-2,6-di-*tert*-butylphenol), compounds with unhindered (4-*tert*-butylphenol, cholesterol, testosterone) and a nitrogen-based antioxidant with NH groups pyridoindole structure (Stobadine), the mentioned compounds in crystalline or liquid forms were gradually added into the ESR cell with $B_{12}$-coordinated peroxy radicals and shortly mixed by bubbling with argon before starting the kinetic measurement.

An X-band Varian E-3 ESR spectrometer with 100 kHz modulation was used. The ESR spectra were simulated using Bruker ER/SRC-200 E spectral computer.

## RESULTS AND DISCUSSION

The reactions occurring during the reaction of vitamin $B_{12}$ with an excess of *tert*-butylhydroperoxide (as $(t\text{-BuOOH})_2$) at room temperature are as follows.

$$B_{12}Co^{II} + ROOH \rightarrow B_{12}Co^{III} + OH^- + RO^{\bullet} \qquad (1)$$

$$RO^{\bullet} + ROOH \rightarrow ROO^{\bullet} + ROH \qquad (2)$$

$$B_{12}Co^{II} + (ROOH)_2 \rightarrow (OH)B_{12}Co^{III}(ROO^{\bullet}) + ROH \qquad (3)$$

R = *t*-Bu

The ESR signal of the π-coordinated peroxy radicals is a singlet line with a typical shift of 2 mT to lower magnetic field ($g$ = 2.0174) with regard to the position of free electron resonance (pitch standard $g$ = 2.0028). Bubbling oxygen through the solution causes broadening of the ESR line and ultimately its disappearance due to the formation of a labile dioxygen complex.

$$(OH)B_{12}Co^{III}(ROO\uparrow\downarrow O–O\uparrow\downarrow OOR)Co^{III}B_{12}(OH)$$

Removal of the dissolved oxygen by bubbling with argon or nitrogen resulted in decomposition of the diamagnetic complex and the ESR signal gained its original intensity.

It has been proved that peroxy radicals coordinated to vitamin $B_{12}$ at ambient temperature readily abstract hydrogen from the OH group of the dissolved phenols. An example of producing stable radicals from the efficient antioxidant vitamin E is showen in Fig. 1. The original ESR signal, the broad line (*1*) ($\Delta H$ = 20 mT) at $g$ = 2.0147, representing concentration of $2 \times 10^{15}$ spin/0.3 ml, is instantly transformed after adding traces of vitamin E (0.01 ml) to the typical septet signal of the generated stable tocopheroxy radical (with two methyl groups in ortho positions; $a_H$ = 0.65 mT) at $g$ = 2.0050.

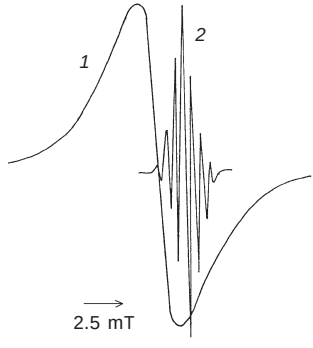


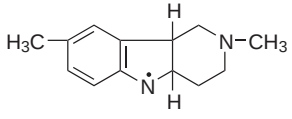

FIG. 1

ESR signal of *t*-BuOO$^{\bullet}$ radicals π-coordinated to vitamin $B_{12}$ (*1*), equivalent to $5 \times 10^{14}$ spin/0.3 ml. Benzene solution (with acetone traces) of ca. $3 \times 10^{-5}$ M $B_{12}$ (23 °C, Ar). Typical septet ESR signal of α-Toc-O$^{\bullet}$ radicals at lower resolution (*2*), generated after adding a small drop of vitamin E

The concentration of vitamin $B_{12}$ was ca. $3 \times 10^{-5}$ mol dm$^{-3}$ and the concentration of the coordinated *t*-BuOO$^{\bullet}$ radicals ca. $3 \times 10^{-6}$ mol dm$^{-3}$. This means that 7–10% of vitamin $B_{12}$ remains in the $(OH)B_{12}(ROO^{\bullet})$ form.

During the formation of the $B_{12}$-coordinated peroxy radicals, the Co(II) centre becomes oxidized to Co(III) by the one-electron transfer to $(t\text{-BuOOH})_2$ and binds concomitantly also the simultaneously formed $HO^-$ group (Eq. (*3*)).

The steady concentration of $(OH)B_{12}(ROO^{\bullet})$ along with time at room temperature is shown in Fig. 2 (curve *1*). After adding the antiallergic antioxidant Stobadine, the concentration of the peroxy radicals coordinated to vitamin $B_{12}$ rapidly decreases (curve *2*). Simultaneously, a new ESR signal rises at $g = 2.0064$ due to the new stable radicals of Stobadine (curve *3*). The signal shows a large splitting due to interaction with nitrogen (1:1:1, $a_N$ = 0.9 mT), each line being further split to triplet (1:2:1, $a_H$ = 0.3 mT) due to two magnetically equivalent protons (see below):



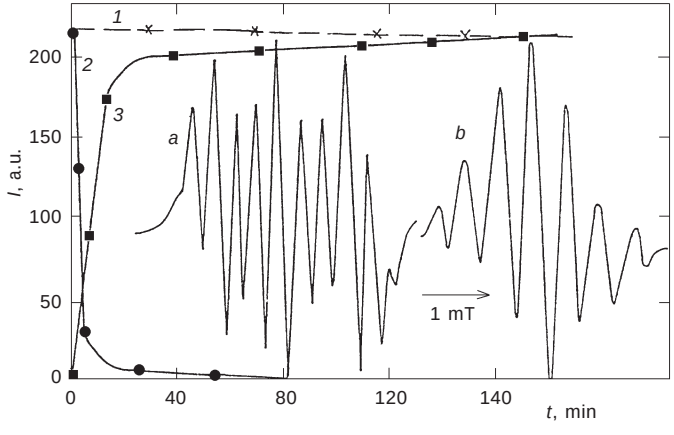




FIG. 2

Time dependence of the ESR signal of peroxy radicals π-coordinate to vitamin $B_{12}$, after the reaction of $B_{12}$ with $(t\text{-BuOOH})_2$ in benzene solution, 23 °C, under Ar atmosphere (*1*). Decay of the original ESR signal of *t*-BuOO$^{\bullet}$ after adding crystalline Stobadine (*2*) and the simultaneous appearance of the ESR signal of the generated free Stobadine radicals (*3*). Transformation of the ESR signal of Stobadine radicals (*a*) to the septet signal of α-tocopheroxy radicals after the addition of vitamin E (*b*)

In the presence of oxygen, this signal can be converted to that of nitroxy radicals, according to Staško et al.[7] The Stobadine radical (Fig. 2, spectrum *a*) is less stable than the free radical of vitamin E and, after adding α-tocopherol, its ESR signal is instantaneously transformed to the characteristic septet of α-tocopheroxy radicals (Fig. 2 spectrum *b*).

In general, it was proved that all types of reactions so far recorded with peroxy radicals coordinated to Co(II) organic chelates[1] can also be realized with vitamin $B_{12}$-stabilized *t*-BuOO$^{\bullet}$ radicals. For instance, their reactions with sterically hindered phenols lead to free radicals stable at ambient temperature in an oxygen-free non-polar solvent. For the free radical derived from 4-phenyl-2,6-di-*tert*-butylphenol (Chart 1), the basis doublet signal due to $H_{(p)}$ ($a_H$ = 0.48 mT) shows additional splitting by two $H_{(o)}$ nuclei ($a_H$ = 0.18 mT); $g$ = 2.0053 (Fig. 3, spectrum *a*).

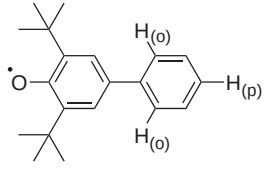



CHART 1

In the case of unhindered phenols, such as in 4-*tert*-butylphenol, the ESR signal proves the interaction of the generated phenoxy radical with the $^{59}$Co nucleus ($I$ = 7/2), resulting in the basis octet signal ($a_{Co}$ = 1 mT), each line being further split into doublet by interaction with the hydrogen in one ortho position ($a_H$ = 0.4 mT); the $g$-value lies typically bellow the free-electron value ($g$ = 1.9990) (Fig. 3, spectrum *b*). However, in the case of large biologically important compounds containing unhindered aromatic OH groups, such as testosterone or cholesterol, only decrease of the original signal of peroxy radicals is observed after the attack of (OH)$B_{12}$(ROO$^{\bullet}$). This result excludes the formation of the Co complex with σ-coordinated phenoxy radicals.

Figure 4 shows the steady concentration of coordinated peroxy radicals (OH)$B_{12}$(ROO$^{\bullet}$) (curve *1*) and its decrease in time (as determined from the decaying ESR signal) after the addition of testosterone (curve *2*) and cholesterol (curve *3*) to the solution. Under the same conditions, addition of acetylsalicylic acid with the hindered OH group, no decrease in the original ESR signal intensity of the (OH)$B_{12}$(ROO$^{\bullet}$) radical is observed (curve *5*), in contrast to the addition of salicylic acid with unhindered OH group (curve *4*).

Data in the literature show that phenoxy radicals π-coordinated to vitamin $B_{12}$ are able to attack also neurotransmitters (e.g., adrenaline, dopamine)[8], and can also act as peroxy radical donors for spin trapping of β-carotene[9], activate oncogenes[10], deactivate mitochondria[3], attack phos-

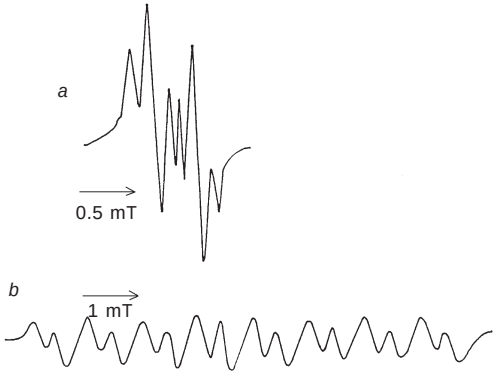



FIG. 3
ESR signal of free phenoxy radicals generated from 4-phenyl-2,6-di-*tert*-butylphenol after attack of the *t*-BuOO$^{\bullet}$ radicals on vitamin $B_{12}$ (*a*). Octet ESR signal of σ-coordinated phenoxy radicals on $B_{12}Co^{III}$ observed in the case of unhindered 4-*tert*-butylphenol (*b*)

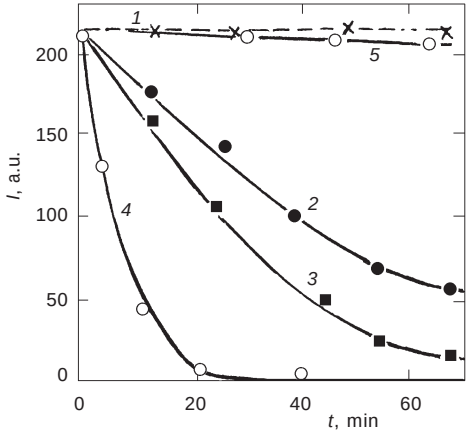



FIG. 4
Time dependence of the ESR signal of π-coordinated (OH)$B_{12}$(ROO$^{\bullet}$) radicals in benzene solution at 23 °C (*1*) and after the addition of acetylsalicylic acid (*5*). Decrease in the concentration of (OH)$B_{12}$(ROO$^{\bullet}$) radicals in presence of testosterone (*2*), cholesterol (*3*) and salicylic acid (*4*)

pholipidic membranes[11], leading in this way to the decompartmentization of cells and locally also to acceleration of cell death apoptosis.

The local oxygen status (e.g., pressure), namely the presence of hydrogen peroxide ($2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$) in a biological environment, can initiate one-electron transfer also from $B_{12}Co^{II}$ and the formation of highly reactive $HO^{\bullet}$ radicals. In a hydrophilic environment, the following secondary reaction of these radicals proceeds with such a high speed that the relevant ESR signal detection can only be realized by the rapid freezing of the system deep below 0 °C. Therefore, to keep the functional structure of vitamin $B_{12}$ unchanged, the local presence of endogenous and exogenous antioxidants is demanded, in particular, as we have shown, the constant presence of α-tocopherol, and in a hydrophilic bioenvironment, vitamin C.

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