

Interaction of *tert*-butyl Hydroperoxide with Hypochlorous Acid. A Spin Trapping and Chemiluminescence Study

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The formation of radical species during the reaction of *tert*-butyl hydroperoxide and hypochlorous acid has been investigated by spin trapping and chemiluminescence. A superposition of two signals appeared incubating *tert*-butyl hydroperoxide with hypochlorous acid in the presence of the spin trap α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitron (POBN). The first signal ($a_N = 1.537$ mT, $a_H^\beta = 0.148$ mT) was an oxidation product of POBN caused by the action of hypochlorous acid. The second spin adduct ($a_N = 1.484$ mT, $a_H^\beta = 0.233$ mT) was derived from a radical species that was formed in the result of reaction of *tert*-butyl hydroperoxide with hypochlorous acid. Similarly, a superposition of two signals was also obtained using the spin trap *N*-*tert*-butyl- α -phenylnitron (PBN). *tert*-Butyl hydroperoxide was also treated with Fe^{2+} or Ce^{4+} in the presence of POBN. Using Fe^{2+} a spin adduct with $a_N = 1.633$ mT and $a_H^\beta = 0.276$ mT was observed. The major spin adduct formed with Ce^{4+} was characterised by $a_N = 1.480$ mT and $a_H^\beta = 0.233$ mT. The reaction of *tert*-butyl hydroperoxide with hypochlorous acid was accompanied by a light emission, that time profile and intensity were identical to those emission using Ce^{4+} . The addition of Fe^{2+} to *tert*-butyl hydroperoxide yielded a much smaller chemiluminescence. Thus, *tert*-butyl hydroperoxide yielded in its reaction with hypochlorous acid or Ce^{4+} the same spin adduct and the same luminescence profile. Because Ce^{4+} is known to oxidise organic hydroperoxides to peroxy radical species, it can be concluded that a similar reaction takes place in the case of hypochlorous acid.

Keywords: *tert*-Butyl hydroperoxide; Hypochlorous acid; Free radicals; Spin trapping; Chemiluminescence

Abbreviations: PBN, *N*-*tert*-butyl- α -phenylnitron; POBN, α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitron; ^oPBN, oxidation product of the spin trap PBN

INTRODUCTION

Hypochlorous acid is a strong oxidising and chlorinating species produced by stimulated polymorphonuclear leukocytes in a myeloperoxidase-catalysed reaction.^[1,2] It is an important constituent of the antimicrobial defence system in human organism. In inflammatory loci where a massive accumulation of polymorphonuclear leukocytes occurs, reactions of neutrophil-derived HOCl can also be directed against the host tissue.

Hypochlorous acid has manifold effects on biological targets such as the oxidation of functional sites of proteins, especially of sulfhydryl and thioether groups,^[3–5] the formation of chloramines,^[6,7] enzyme inactivation^[8] and also the initiation of lipid peroxidation. An accumulation of lipid peroxidation products in liposomes and lipoproteins upon the action of hypochlorous acid has been detected by several groups.^[9–14]

Organic hydroperoxides such as *tert*-butyl hydroperoxide,^[15] cumene hydroperoxide,^[15] or linoleic acid hydroperoxide^[16] have been found to promote the lipid peroxidation induced by hypochlorous acid when they are incorporated into unsaturated phosphatidylcholine liposomes. This increase in lipid peroxidation products can be inhibited by scavengers of free radicals such as butylated hydroxytoluene. Hypochlorous acid reacts with organic hydroperoxides in a totally other way than with hydrogen peroxide. Singlet oxygen, observed as

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the main product in the latter case,^[17,18] has been failed to detect in the reaction with *tert*-butyl hydroperoxide.^[19] A product analysis by ¹H-NMR spectroscopy yielded di-*tert*-butyl peroxide, *tert*-butanol, *tert*-butyl hypochlorite, acetone and acetate as products of the reaction between hypochlorous acid and *tert*-butyl hydroperoxide.^[20] Taking together, these experimental data support the existence of a free radical mechanism of unknown nature in the reaction between both partners.

The present paper is addressed to clarify the nature of the radical species formed upon the reaction of *tert*-butyl hydroperoxide with hypochlorous acid. This hydroperoxide was chosen as model substance for lipid hydroperoxides in biological systems. Spin trapping and chemiluminescence measurements have been used to detect and identify radicals formed upon the reaction of hypochlorous acid with *tert*-butyl hydroperoxide.

MATERIALS AND METHODS

Chemicals

The spin traps α -(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitron (POBN) and *N*-*tert*-butyl- α -phenylnitron (PBN) were obtained from Sigma (Deisenhofen, Germany). *tert*-Butyl hydroperoxide (70% solution in H₂O), FeCl₂ and Ce(NH₄)₂(NO₃)₆ were also purchased from Sigma (Deisenhofen, Germany). Sodium hypochlorite and all other chemicals were from Sigma (Deisenhofen, Germany).

A stock solution of NaOCl was kept in the dark at 4°C. Its concentration was determined at pH 12 using $\epsilon_{290} = 350 \text{ M}^{-1} \text{ cm}^{-1}$.^[21] It was diluted with 0.14 M NaCl, 10 mM phosphate immediately prior to use and adjusted to pH 7.4.

ESR Measurements

Spin trapping assay has been used to evaluate short living radical species formed in the result of the reaction between *tert*-butyl hydroperoxide and hypochlorous acid. ESR spectra were acquired at room temperature using a computerised radio-spectrometer Varian E-4 (Palo Alto, CA, USA). Freshly prepared incubation mixture containing the spin trap was placed into a flat quartz aqueous sample cell and then put into the cavity of ESR spectrometer. ESR spectra registration parameters were: field strength 330 mT, scan width 4.2–5.2 mT, microwave power 10 mW, modulation amplitude 0.05 mT, and time constant 0.03 s. Hyperfine splitting constants on nitrogen and hydrogen nuclei (a_N and a_H^β , respectively), as well as the line width (ΔH_{pp}) have been used to characterise the spin adducts.

Simulation of ESR spectra has been performed using a computer programme developed by Duling (Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC).^[22]

Chemiluminescence Measurements

Chemiluminescence measurements were performed on a LKB-1251 chemiluminometer (LKB-Pharmacia, Sweden) using the whole spectral range of the photomultiplier. No selection of smaller spectral regions by filters has been provided. To induce the reaction, sodium hypochlorite or aqueous solutions of Fe²⁺ or Ce⁴⁺, respectively, were injected into the measuring cuvette containing *tert*-butyl hydroperoxide.

RESULTS

In order to investigate details of the reaction between organic hydroperoxides and hypochlorous acid, *tert*-butyl hydroperoxide has been used as model substance for lipid hydroperoxides. This substance does not contain any other components (double bonds, amino groups etc.) except peroxide group that can react with hypochlorite. Thus, the hydroperoxide group in the *tert*-butyl hydroperoxide molecule should be the only target for HOCl.

It was found that hypochlorous acid reacts with the spin trap POBN under formation of a spin adduct characterised by $a_N = 1.537 \text{ mT}$, $a_H^\beta = 0.148 \text{ mT}$, and $\Delta H_{pp} = 0.042 \text{ mT}$ (Fig. 1). This signal has hyperfine splitting constant values close to the O-centred radical^[23,24] *tert*-Butyl hydroperoxide alone did not yield any ESR signal with POBN. However, the incubation of *tert*-butyl hydroperoxide with hypochlorous acid in the presence of POBN resulted in a complex signal, which can be evaluated as a superposition of two spectra (Fig. 2A). The impact of the individual components of this signal depended on the ratio between *tert*-butyl hydroperoxide and hypochlorous acid concentrations. One of the signal was identical to that presented in Fig. 1 and resulted from the interaction of the spin trap

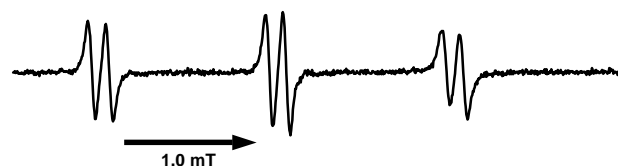


FIGURE 1 EPR signal of the spin adduct produced upon the reaction of hypochlorous acid (1 mM) with the spin trap POBN (20 mM). The reaction was performed in 50 mM phosphate buffer, pH 7.4, at room temperature. EPR spectrum was obtained three minutes after incubation. The spectrum has the following parameters: $a_N = 1.537 \text{ mT}$, $a_H^\beta = 0.148 \text{ mT}$, and $\Delta H_{pp} = 0.042 \text{ mT}$.

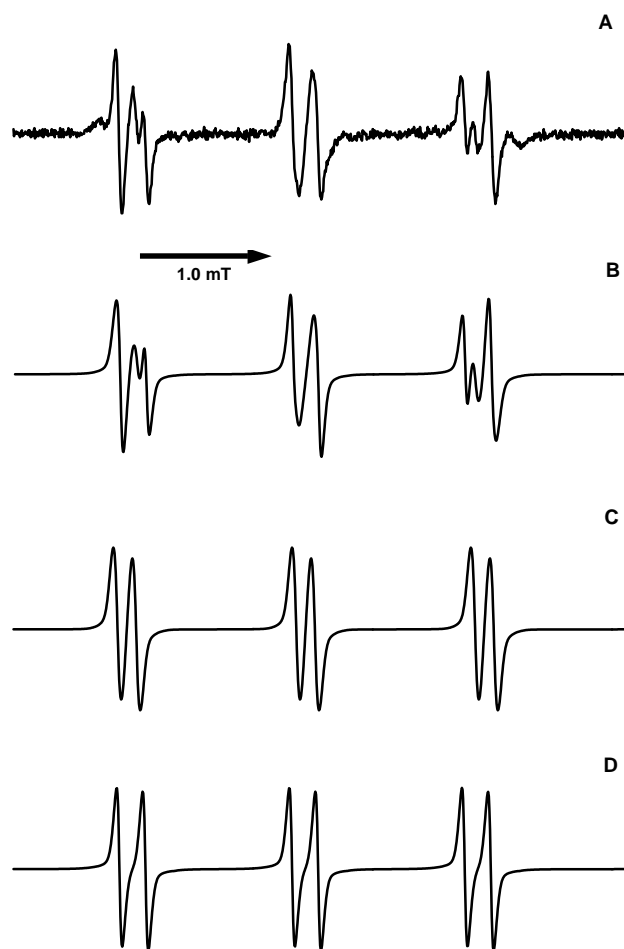


FIGURE 2 EPR signal of spin adducts formed after incubation of hypochlorous acid (1 mM) and *tert*-butyl hydroperoxide (5 mM) in the presence of the spin trap POBN (20 mM) (spectrum A). All other experimental conditions are as in Fig. 1. Simulation of this complex signal (spectrum B) as well as simulation of its both components (spectra C and D) are also given. The signal characteristics of single components are $a_N = 1.537$ mT, $a_H^\beta = 0.148$ mT, and $\Delta H_{pp} = 0.042$ mT (spectrum C) and $a_N = 1.484$ mT, $a_H^\beta = 0.233$ mT, and $\Delta H_{pp} = 0.063$ mT (spectrum B). The superimposed spectrum in B is composed by 40% of the signal presented in C and 60% of the signal shown in D.

with hypochlorous acid. Both individual components of the combined signal in Fig. 2A can be found by computer simulation (Fig. 2B–D). The second spin adduct was characterised by $a_N = 1.484$ mT, $a_H^\beta = 0.233$ mT, and $\Delta H_{pp} = 0.063$ mT. Thus, two spin adducts of POBN were formed incubating *tert*-butyl hydroperoxide together with hypochlorous acid. One adduct is derived from the reaction of POBN with hypochlorous acid, the other—from the interaction of POBN with a radical formed upon the reaction of *tert*-butyl hydroperoxide with hypochlorous acid.

In a similar way, the incubation of the spin trap PBN with *tert*-butyl hydroperoxide and HOCl yielded also the superposition of two different signals (data not shown). Spectral characteristics of the spin adduct of HOCl on PBN were $a_N = 1.558$ mT, $a_H^\beta = 0.190$ mT,

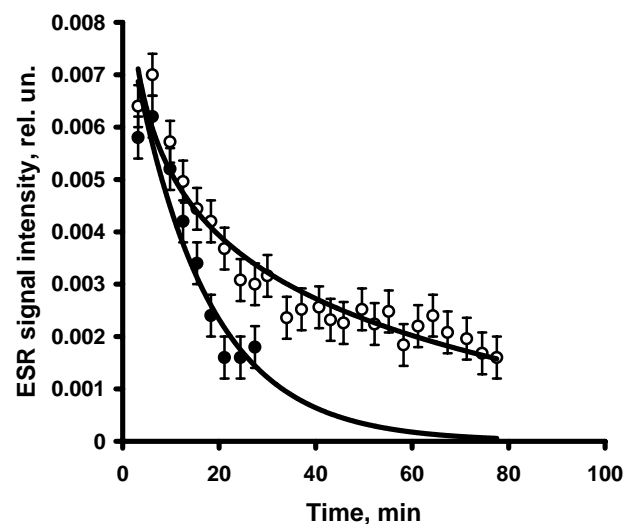
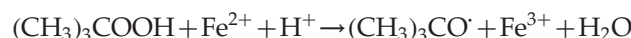


FIGURE 3 Kinetics of EPR signal intensity of spin adducts of POBN (○) or PBN (●) that resulted from a radical that is formed upon the reaction of *tert*-butyl hydroperoxide and hypochlorous acid. All other experimental conditions are as in Fig. 2. All data are the means and standard deviations of three independent experimental series.

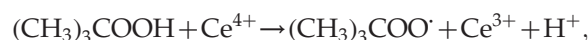
and $\Delta H_{pp} = 0.074$ mT. This signal could be assigned to an oxidation product 'OPBN of the spin trap PBN.^[25] The spectrum of this spin adduct derived from the reaction of *tert*-butyl hydroperoxide with hypochlorous acid was characterised by $a_N = 1.547$ mT, $a_H^\beta = 0.360$ mT, and $\Delta H_{pp} = 0.063$ mT.

Spin adducts of POBN and PBN with the radicals, derived from the *tert*-butyl hydroperoxide/HOCl interaction, loose gradually their intensity with increasing incubation time (Fig. 3). The half life in the case of POBN was 20 min. The spin adduct of PBN decayed with a half life of 12 min. Because there was only a decrease in signal intensity, but no changes in parameters of hyperfine splitting of these signals, any transformation of these spin adducts into other spin adducts can be excluded. Consequently, all ESR spectra using the *tert*-butyl hydroperoxide/HOCl system were monitored 2–3 min after the addition of the incubation mixture to avoid a decrease in signal intensity.

In order to better understand the nature of the radical species that is formed during the reaction between *tert*-butyl hydroperoxide and HOCl and led subsequently to a corresponding spin adduct of POBN, two other model systems have been applied. *tert*-Butyl hydroperoxide can be either reduced to an alkoxy radical by Fe^{2+} ^[26] or oxidised to a peroxy radical by Ce^{4+} ^[27] according to the following reactions:



or



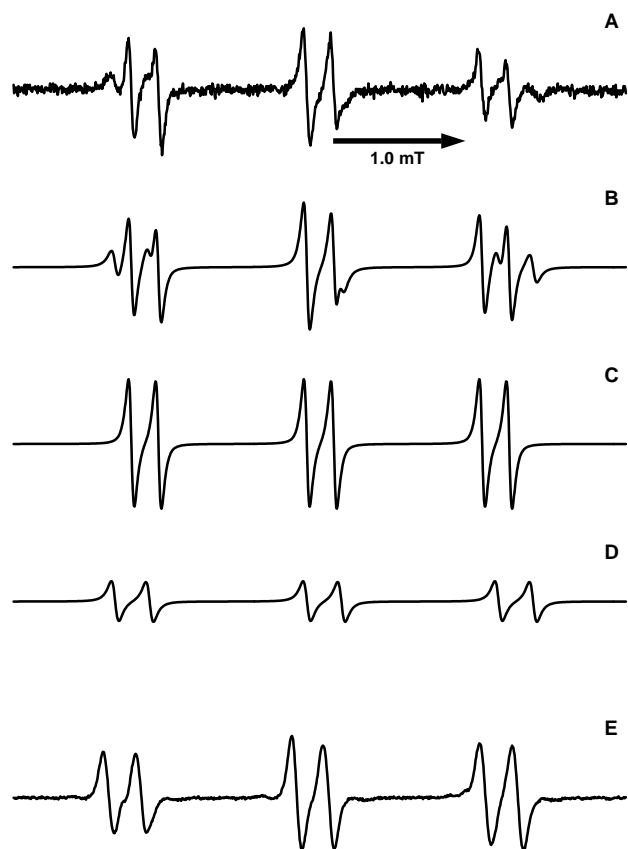


FIGURE 4 EPR spectra of spin adducts of POBN and radicals generated from *tert*-butyl hydroperoxide (20 mM) and Ce^{4+} (5 mM) (spectrum A) as well as from *tert*-butyl hydroperoxide (15 mM) and Fe^{2+} (3 mM) (spectrum E). The reaction was performed in 50 mM phosphate buffer, pH 7.4, at room temperature. EPR spectrum was obtained 3 min after incubation. Spectrum E was characterised by $a_{\text{N}} = 1.633$ mT, $a_{\text{H}}^{\beta} = 0.276$ mT, and $\Delta\text{Hpp} = 0.085$ mT. A simulation of spectrum A is given in B. The both components of this simulated spectrum are shown in C ($a_{\text{N}} = 1.480$ mT, $a_{\text{H}}^{\beta} = 0.233$ mT, and $\Delta\text{Hpp} = 0.055$ mT) and D ($a_{\text{N}} = 1.627$ mT, $a_{\text{H}}^{\beta} = 0.293$ mT, and $\Delta\text{Hpp} = 0.085$ mT).

respectively. Figure 4 presents experimental ESR spectra obtained from POBN incubated with *tert*-butyl hydroperoxide in the presence of either Fe^{2+} (spectrum E) or Ce^{4+} (spectrum A). In case of Fe^{2+} , a single ESR spectrum was obtained with the following spectral parameters: $a_{\text{N}} = 1.633$ mT, $a_{\text{H}}^{\beta} = 0.276$ mT, and $\Delta\text{Hpp} = 0.085$ mT that can be identified as a *tert*-butoxyl radical spin adduct.

The ESR spectrum derived from the reaction mediated by Ce^{4+} (spectrum A) was composed of two components as clearly seen by simulation of this spectrum (spectrum B). Individual spectra of these two components were simulated in C and D. The more intense signal (spectrum C) was characterised by $a_{\text{N}} = 1.480$ mT, $a_{\text{H}}^{\beta} = 0.233$ mT, and $\Delta\text{Hpp} = 0.055$ mT. These spectral data are nearly identical to those obtained in the result of the reaction of *tert*-butyl hydroperoxide with hypochlorous acid. The minor component (spectrum D) had the following parameters: $a_{\text{N}} = 1.627$ mT, $a_{\text{H}}^{\beta} = 0.293$ mT, and

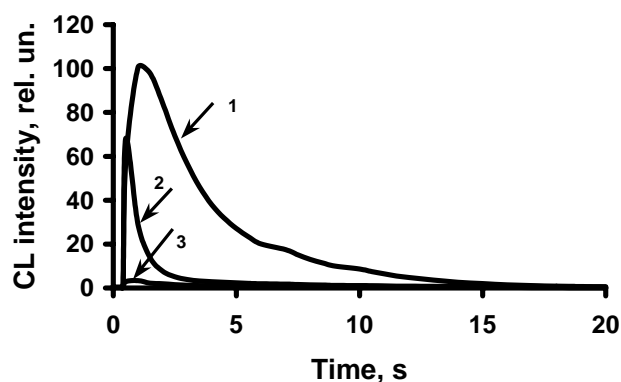


FIGURE 5 Representative examples of chemiluminescence kinetics of the reaction of *tert*-butyl hydroperoxide (22 mM) with 4.4 mM NaOCl (trace 1), 4.4 mM Ce^{4+} (trace 2) or 4.4 mM Fe^{2+} (trace 3). The reaction was performed in 50 mM phosphate buffer, pH 7.4, at room temperature. Each experiment has been repeated five times.

$\Delta\text{Hpp} = 0.085$ mT. It corresponds to a *tert*-butoxyl radical spin adduct because of the similarity of spectral data obtained with Fe^{2+} (spectrum E).

Thus, the spin adduct of POBN derived from the reaction of *tert*-butyl hydroperoxide with hypochlorous acid has the same spectral characteristics as the major spin adduct detected with *tert*-butyl hydroperoxide and Ce^{4+} and these parameters differed from those of *tert*-butoxyl radical spin adduct, produced in the reaction with Fe^{2+} . We can conclude that the *tert*-butyl peroxy radical is primarily formed as the result of the reaction of *tert*-butyl hydroperoxide and hypochlorous acid. This conclusion was supported by further ESR and chemiluminescence investigations.

Ethanol is known to yield an α -hydroxyethyl radical upon abstraction of a hydrogen atom by strong oxidants such as alkoxy radicals.^[28,29] However, the presence of 1.7 M ethanol did not change the spectral characteristics of spin adducts obtained after incubation of *tert*-butyl hydroperoxide with either Ce^{4+} or HOCl (data not shown). The *tert*-butyl peroxy radical appeared to be too weak to abstract a hydrogen atom from ethanol.

It is well known that recombination of peroxy radicals can produce chemiluminescence.^[30] We have tested the reaction of *tert*-butyl hydroperoxide with HOCl and have found that it is accompanied by an emission of light.^[15] This chemiluminescence response has been compared with the light emission observed in the result of reaction of *tert*-butyl hydroperoxide with either Fe^{2+} or Ce^{4+} (Fig. 5). Ce^{4+} yielded a comparable luminescence response as HOCl, while only a small light burst was observed in the case of Fe^{2+} . This chemiluminescence arises apparently from the recombination of *tert*-butyl peroxy radicals.^[30] Singlet oxygen has been excluded previously as a source of light emission in the reaction of *tert*-butyl hydroperoxide and

HOCl ,^[20] because this light emission was not observed in the red region of the spectra, known for the dimol emission of $^1\text{O}_2$,^[17] was not enhanced replacing H_2O by D_2O , and did not show a square dependence on reactant concentration. Moreover, it was impossible to detect an emission of singlet oxygen at 1268 nm in the reaction of *tert*-butyl hydroperoxide and HOCl .^[19]

DISCUSSION

The reaction between *tert*-butyl hydroperoxide and hypochlorous acid yields a radical species that has been identified as *tert*-butyl peroxy radical. A major spin adduct with the same spectral parameters was found incubating *tert*-butyl hydroperoxide with solutions of Ce^{4+} . These ions are known to oxidise organic hydroperoxides to the corresponding peroxy radicals as it was shown by continuous flow ESR experiments.^[27] The direct formation of an alkoxy radical in the reaction between *tert*-butyl hydroperoxide and hypochlorous acid can be excluded because this radical species exhibit other spectral characteristics as evidenced by experiments with Fe^{2+} . *tert*-Butyl alkoxy radical was detected as a minor product in the reaction of *tert*-butyl hydroperoxide with Ce^{4+} . Most probably, this alkoxy species was derived from the recombination of two *tert*-butyl peroxy radicals.^[31]

Hypochlorous acid is a strong two-electron oxidant, but a less efficient one-electron oxidant. The standard reduction potential of the redox couple HOCl/Cl^- , H_2O is 1.08 V at pH 7.0.^[32] Lower standard values at pH 7.0 were reported for one electron reduction of HOCl : 0.25 V ($\text{HOCl}/\text{HOCl}^-$), 0.26 V (HOCl/OH , Cl^-), and 0.17 V (HOCl , $\text{H}^+/\text{H}_2\text{O}$, Cl^-).^[32] For oxidation of lipid hydroperoxides, the standard reduction potential of the redox couple ROO/ROOH has been calculated to be 1.0 V at pH 7.0.^[33] In case of methylperoxy radical, a standard reduction potential in the range 1.02–1.11 V at pH 7.0 was experimentally determined for the redox couple ROO/ROOH .^[34] Unfortunately, the standard reduction potential of the couple *tert*-butyl peroxy radical/*tert*-butyl hydroperoxide is unknown. It should be in the same range as for lipid hydroperoxides.

Our results indicate, that *tert*-butyl hydroperoxide is oxidised by hypochlorous acid to the *tert*-butyl peroxy radical. Only the combination of the two-electron reduction of one molecule HOCl with one-electron oxidation of the two hydroperoxide molecules can be thermodynamically favourable. However, details of the mechanism of this reaction are unknown. From thermodynamics, it seems to be unlikely that Cl^- , OH , or HOCl^- that can derive from one-electron reductions of HOCl are formed in the

reaction of *tert*-butyl hydroperoxide with hypochlorous acid.

Although the formation of *tert*-butyl peroxy radical by incubation of *tert*-butyl hydroperoxide with hypochlorous acid has been shown using two different spin traps, a number of problems arose from these measurements. Both spin traps used were also a target for hypochlorous acid. This is not surprising, considering the high reactivity of hypochlorous acid with a wide number of targets.^[3–7] Thus, the addition of hypochlorous acid to *tert*-butyl hydroperoxide led in the presence of spin traps to a superposition of two signals that can be separated from each other.

tert-Butyl hydroperoxide was incubated with either HOCl , Ce^{4+} or Fe^{2+} in our experiments. In addition to spin adducts caused by the reagent HOCl , two different spin adducts of POBN (and also using PBN) have been detected, that are derived from the *tert*-butyl peroxy radical (HOCl , Ce^{4+}) or the *tert*-butyl alkoxy radical (Fe^{2+}). Spin adducts of peroxy radicals are believed to be unstable and converted to adducts of alkoxy radicals.^[35–38] Especially, the spin trap PBN forms a very unstable adduct with the *tert*-butyl peroxy radical that is converted to a *tert*-butoxy adduct of PBN at higher temperatures.^[35,36] It has been reported that at temperatures higher than 250 K only the PBN-alkoxy radical was detected.^[36] These experiments were performed in organic solvents such as toluene, fluorotrichloromethane or carbon tetrachloride. We used an aqueous environment in all experiments and performed all experiments at room temperature. From our experiments, we cannot exclude any fast conversions of spin adducts of the *tert*-butyl peroxy radical before the start of registration. Spin adducts detected were stable enough to monitor their spectral characteristics. They disappeared slowly without the formation of other spin products with a half life of 20 min (POBN) or 12 min (PBN). On the other hand, we can clearly state that any possible conversion from a spin adduct of a *tert*-butyl peroxy radical did not yield an alkoxy radical species in our case. Spin adducts derived from the *tert*-butyl alkoxy radical have other spectral characteristics than those adducts derived from the *tert*-butyl peroxy radical.

Peroxy radicals are important radical intermediates in lipid peroxidation. We demonstrated previously that the peroxidation of lipids induced by hypochlorous acid^[15,16] or the $\text{MPO} + \text{H}_2\text{O}_2 + \text{Cl}^-$ -system^[16] was favoured by *tert*-butyl hydroperoxide,^[15] cumene hydroperoxide^[15] and also hydroperoxides derived from linoleic acid.^[16] The reaction of hypochlorous acid with organic hydroperoxides leads to the formation of peroxy radicals according to our results. These radicals will promote further radical driven reactions in a lipid matrix.

Acknowledgements

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