Reaction of Hypochlorous Acid with Hydrogen Peroxide and *tert*-Butyl Hydroperoxide. ^{1}H NMR Spectroscopy and Chemiluminescence Analyses

Jürgen Arnhold[a], Oleg M. Panasenko[b], Jürgen Schiller[a], Klaus Arnold[a], Yurij A. Vladimirov[b], Valerij I. Sergienko[b]

[a] Institut für Medizinische Physik und Biophysik, Medizinische Fakultät, Universität Leipzig, Liebigstr. 27, D-04103 Leipzig, Bundesrepublik Deutschland

[b] Research Institute of Physico-Chemical Medicine, M. Pirogovskaya 1a, RUS-119828, Moscow, Russia

Z. Naturforsch. **51c,** 386–394 (1996); received January 26/February 27, 1996

Hypochlorous Acid, Hydrogen Peroxide, *tert*-Butyl Hydroperoxide, Singlet Oxygen, Free Radicals, ^{1}H NMR Spectroscopy, Chemiluminescence

In contrast to the well-known reaction of hypochlorous acid with hydrogen peroxide, no singlet oxygen is formed as the result of reaction between hypochlorous acid and *tert*-butyl hydroperoxide. The reaction with hydrogen peroxide yielded a quadratic dependence of light intensity on reactant concentration, a drastic enhancement of luminescence yield using D_2O as solvent and only an emission of red light, that are typical characteristics of emission resulting from two molecules of delta singlet oxygen. Other chemiluminescence properties were observed using *tert*-butyl hydroperoxide. There was a linear dependence of light intensity on reactant concentration using *tert*-butyl hydroperoxide in excess with a decline of emission at higher concentrations.

^{1}H-NMR spectroscopic analysis revealed di-*tert*-butyl peroxide, *tert*-butanol and also *tert*-butyl hypochlorite, acetone and acetate as products of the reaction between hypochlorous acid and *tert*-butyl hydroperoxide. The formation of di-*tert*-butyl peroxide is only possible assuming a *tert*-butyloxy radical as primary intermediate product of this reaction.

Our results demonstrate that alkoxy radicals derived from organic hydroperoxides can participate in lipid peroxidation induced by hypochlorous acid. On the other hand, singlet oxygen did not influence the yield of peroxidation products. Changing H_2O for D_2O in suspension of egg yolk phosphaditylcholine no differences in accumulation of thiobarbituric acid reactive products were observed.

Introduction

Stimulated neutrophils generate the powerful oxidant hypochlorous acid in a myeloperoxidase-catalysed reaction between hydrogen peroxide and chloride anions (Thomas, 1979; Albrich *et al.*, 1981). Hypochlorous acid contributes to tissue injury in a number of diseases including rheumatoid arthritis (Schiller *et al.*, 1995; 1996), coronary heart disease and others (Fliss, 1988; Klebanoff, 1988; Smith *et al.*, 1989). An involvement of low density lipoproteins modified by hypochlorous acid into foam cell formation during arteriosclerosis is also under discussion now (Hazell *et al.*, 1993; 1994).

Hypochlorous acid has manifold effects on cell constituents. Among these are the oxidation of functional sites of proteins, especially of sulfhydryl and thioether groups (Winterbourn, 1985; Arnhold *et al.*, 1991) the formation of chloramines (Weiss *et al.*, 1982), enzyme inactivation (Wasil *et al.*, 1987; Klebanoff, 1988) and also the initiation of lipid peroxidation. An increase of primary (diene conjugates, hydroperoxides) and secondary (thiobarbituric acid reactive compounds) products of lipid peroxidation in liposomes and lipoproteins upon the action of hypochlorous acid has been detected by several groups (Stelmaszynska *et al.*, 1992; Evgina *et al.*, 1992; Panasenko *et al.*, 1994a,b).

Although hypochlorous acid reacts in lipid systems with different targets only its reaction with organic hydroperoxides previously accumulated by autoxidation or other processes leads to a promotion of new lipid peroxidation (Panasenko *et al.*, 1995). Other possible reactions of hypochlorous acid such as the chlorohydrin formation with olefinic double bonds of phospholipid molecules (Winterbourn *et al.*, 1992; Arnhold *et al.*, 1995) or

Reprint requests to Dr. Jürgen Arnhold.
Fax: 0341-9715709.

0939–5075/96/0500–0386 $ 06.00

the oxidation of molecules with an aldehydic function did not contribute to additional peroxidation reactions (Panasenko *et al.*, 1995). On the other hand, the chlorohydrin formation diminishes the number of double bonds in lipid systems and thus also the amount of available substratum for lipid peroxidation. Consequently, sufficient products of lipid peroxidation are only detectable at relatively low concentrations of hypochlorous acid (Panasenko *et al.*, 1994a).

In the present paper we focused our interest on the reaction mechanism between hypochlorous acid and organic hydroperoxides and the role of reaction products to promote lipid peroxidation. *Tert*-butyl-hydroperoxide and cumene hydroperoxide have been found to promote additional production of thiobarbituric acid (TBA) reactive products if they are incorporated into egg yolk phosphatidylcholine liposomes (Panasenko *et al.*, 1995). This increase in TBA-reactive compounds can be inhibited by the free radical scavenger butylated hydroxytoluene. On the other hand, hydrogen peroxide did not influence the yield of TBA-reactive products (Panasenko *et al.*, 1994a), although hydrogen peroxide is known to yield singlet oxygen in its reaction with hypochlorous acid (Khan *et al.*, 1970; Held *et al.*, 1978). Singlet oxygen itself is assumed to be a potent agent to induce peroxidation reactions (Thomas *et al.*, 1980; Frankel *et al.*, 1982).

The objection of the present paper is to compare the reaction of hypochlorous acid with hydrogen peroxide and with organic hydroperoxides to establish differences between these two reactions and to characterize mechanisms responsible for initiation of lipid peroxidation. Using chemiluminescence detection and ^{1}H-nuclear magnetic resonance spectroscopy results are obtained favouring the appearance of free radicals immediately during the reaction of hypochlorous acid with organic hydroperoxides.

Material and Methods

Chemicals

Tert-butyl hydroperoxide (70% solution in H_2O), cumene hydroperoxide, *tert*-butanol, di-*tert*-butyl peroxide, *tert*-butyl methyl ether, 2-methylpropene, egg yolk phosphatidylcholine, D_2O, $CDCl_3$ and all organic solvents (in UV-spectroscopy-grade) were obtained from Fluka, Switzerland. H_2O_2 was a product from Merck, Germany. Hexamethylethane was purchased from Aldrich, Germany. Sodium hypochlorite was from Sigma, Germany. Luminol was obtained from Boehringer, Mannheim, Germany. 2-Thiobarbituric acid was a product from Serva, Germany. *Tert*-butyl hypochlorite was prepared bubbling chlorous gas through an alkaline solution of *tert*-butanol (Metzger, 1989).

Solutions

A stock solution of NaOCl was kept in the dark at 4 °C. Its concentration was determined at pH 12 using $\varepsilon_{290} = 350\ M^{-1}cm^{-1}$ (Morris, 1966). It was diluted with 0.14 mol/l NaCl, 10 mmol/l phosphate immediately prior to use and adjusted to pH 7.4. In some cases the buffer used for dilution was prepared with D_2O.

A stock solution of H_2O_2 was prepared in phosphate buffer using H_2O or D_2O. Its concentration was determined spectrophotometrically using $\varepsilon_{230} = 74\ M^{-1}cm^{-1}$ (Beers *et al.*, 1952).

Liposome preparation and incubation with hypochlorous acid

1 ml of egg yolk phosphatidylcholine dissolved in chloroform (6 mg/ml) were given in a round-bottom flask and evaporated to dryness using a rotary evaporator. Multilamellar liposomes were prepared by dissolving the lipid film in 3 ml 0.14 mol/l NaCl, 10 mmol/l phosphate (pH 7.4) and vortexing rigorously for 30 seconds.

In order to induce peroxidation aliquots of liposome samples (final concentration 2 mg/ml) were incubated with NaOCl at 37 °C for 40 min. Final concentrations of NaOCl varied from 0.05 to 3 mmol/l. Control measurements revealed that the pH value remains at 7.4 after the addition of NaOCl.

A number of experiments was made using buffer and NaOCl solutions prepared on D_2O.

Malondialdehyde was determined by its reaction with thiobarbituric acid (Uchiyama *et al.*, 1978).

Chemiluminescence

200 µl of NaOCl solution (1–50 mmol/l, final concentration) in phosphate buffer was placed

into plastic vials of the luminometer AutoLumat LB 953 (Laboratorium Prof. Dr. Berthold, Wildbad, Germany). Then 50 µl of H_2O_2 (final concentration $2 \cdot 10^{-1}$ mol/l) or *tert*-butyl hydroperoxide (final concentration $2 \cdot 10^{-1}$ mol/l) was added by means of an injector device. Total light intensities were determined using an integration time of 10 s. In some cases a cut-off filter (λ>600 nm) was placed between the photomultiplier and reaction tube. Only red light photons were counted by this approach.

NMR measurements

Solutions of *tert*-butyl hydroperoxide either prepared in phosphate buffered saline or D_2O were incubated with NaOCl at 25 °C for 15 min. The concentration ratios between *tert*-butyl hydroperoxide and hypochlorous acid are indicated in the Results section. Then 1 ml of the reaction mixture was thoroughly mixed with 1 ml $CDCl_3$. After a short centrifugation to allow phase separation, the water and chloroform phases were analysed by ^{1}H-NMR spectroscopy.

Proton spectra were obtained on a Bruker AMX-NMR spectrometer at 300.13 MHz. The spectra were accumulated 64 times. No line-broadening or Gauß-broadening was used.

Chemical shifts were referenced to sodium 3-(trimethylsilyl)propane-1-sulphonate in aqueous solution or to the $CHCl_3$ resonance in deuterated chloroform.

Results

Chemiluminescence resulting from reaction of hypochlorous acid with hydrogen peroxide

Hypochlorous acid and hydrogen peroxide react in a strong 1:1 (molar ratio) stoichiometry to yield singlet oxygen (Held *et al.*, 1978). Although some details of this reaction are under discussion, it is well known that free radicals are not involved in this process (Held *et al.*, 1978). The formation of singlet oxygen can be followed by appearance of a red chemiluminescence due to dimol emission of excited oxygen molecules (Khan *et al.*, 1970; Kanofsky, 1989). Fig. 1 shows the luminescence yield after the addition of excess of hydrogen peroxide to a solution of hypochlorous acid as a function of reactant concentration. No significant differences were found measuring the total luminescence or those at wavelengths higher than 600 nm using a cut-off filter. In both cases a square dependence of light emission intensity on the reactant concentration is found.

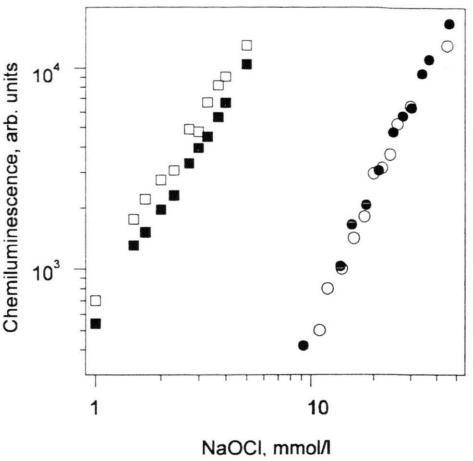

Fig. 1. Chemiluminescence intensities in the result of reaction of different amounts of NaOCl and hydrogen peroxide ($2 \cdot 10^{-1}$ mol/l, final concentration). 50 µl H_2O_2 was injected into 200 µl NaOCl solution made in 0.14 mol/l NaCl, 10 mmol/l phosphate, pH 7.4. Photons were counted over 10 s. A cut-off filter (λ>600 nm) was placed between reaction tube and photomultiplier in some experiments (full symbols). H_2O_2 and NaOCl solution were prepared with H_2O (circles) or D_2O (squares). Means of four measurements are given. S.D. was lower than 6%.

Changing H_2O for D_2O the light emission is drastically enhanced in reaction between hydrogen peroxide and hypochlorous acid. D_2O is known to prolong the lifetime of singlet oxygen from 2–4 µs to 20 µs (Merkel *et al.*, 1972; Rodgers *et al.*, 1980). The same range of intensities of light emission in D_2O is shifted to lower reactant concentrations by about an order of magnitude in comparison to H_2O (Fig. 1). The emission in D_2O consists also completely of red light and has a square dependence on reactant concentration.

The red nature of luminescence, its quadratic dependence on reactant concentrations, and the enhancement of light emission by D_2O are characteristic features of the dimol emission of singlet oxygen.

Chemiluminescence resulting from reaction of hypochlorous acid with organic hydroperoxides

Hydrogen peroxide was then replaced by *tert*-butyl hydroperoxide or cumene hydroperoxide. A chemiluminescence was also measured in the reaction between *tert*-butyl hydroperoxide and hypochlorous acid. Using an excess of *tert*-butyl-hydroperoxide and variable concentrations of NaOCl a linear dependence between light intensity and NaOCl concentration has been found up to approximately 10^{-3} mol/l (Fig. 2). Then the luminescence raises more slowly and decreases at higher concentrations of hypochlorous acid. The application of a cut-off filter ($\lambda > 600$ nm) into the equipment drastically diminishes the light emission. Most of this emission is of non-red nature. Only 1% of total light intensity can come through the cut-off filter. D_2O did not enhance the luminescence yield. A decrease of light emission was found in this case. Similar results were obtained using cumene hydroperoxide (data not shown).

The linear dependence between light intensity and the concentration of NaOCl in its reaction with *tert*-butyl hydroperoxide supports the view that a primary intermediate product of these reactants or one of its subsequent products is formed in an excited state. A direct reaction of this primary intermediate product with the original *tert*-butyl hydroperoxide would explain the deviation of the linear dependence between light intensity and NaOCl concentration using higher amounts of hypochlorous acid in Fig. 2.

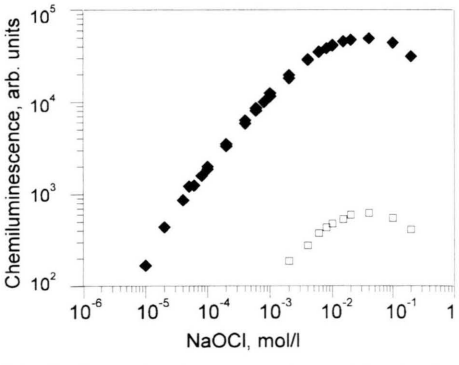

Fig. 2. Chemiluminescence intensities in the result of reaction of different amounts of NaOCl and *tert*-butyl hydroperoxide ($2 \cdot 10^{-1}$ mol/l, final concentration). 50 µl *tert*-butyl hydroperoxide was injected into 200 µl NaOCl solution made in 0.14 mol/l NaCl, 10 mmol/l phosphate, pH 7.4. Photons were counted over 10 s. A cut-off filter (λ>600 nm) was placed between reaction tube and photomultiplier in some experiments (full symbols). Means of four measurements are given. S.D. was lower than 6%.

A likely candidate for this primary intermediate product is the *tert*-butyloxy radical $(CH_3)_3CO^{\cdot}$. It is known to abstract a $H^{\cdot}$ from *tert*-butyl hydroperoxide (Metzger, 1989):

$$(CH_3)_3C{-}O^{\cdot} + (CH_3)_3COOH \rightarrow (CH_3)_3COH + (CH_3)_3COO^{\cdot}.$$

These data show that the light emission in the result of reaction between *tert*-butyl-hydroperoxide and hypochlorous acid is not caused by singlet oxygen. Differences in chemiluminescence mechanisms using hydrogen peroxide or *tert*-butyl hydroperoxide in their reactions with hypochlorous acid confirm previous results (Panasenko *et al.*, 1995) of influence of these hydroperoxides on the product yield in lipid peroxidation induced by hypochlorous acid. Whereas hydrogen peroxide did not cause additional peroxidation of unsaturated phospholipids, *tert*-butyl and also cumene hydroperoxides favour this deterioration process.

Accumulation of TBA-reactive products in D_2O

Singlet oxygen is formed in the result of reaction between hydrogen peroxide and hypochlorous acid. It is known to promote the lipid peroxidation reaction (Thomas *et al.*, 1980; Frankel *et al.*, 1982). These results are in contrast to our previous observation (Panasenko *et al.*, 1994a). A possible reason could be the very short lifetime of singlet oxygen. To increase the lifetime of singlet oxygen egg yolk phosphatidylcholine liposomes were prepared in D_2O. Fig. 3 shows the accumulation of TBA-reactive products in these liposomes in the presence of H_2O or D_2O. Lipid peroxidation was initiated by addition of sodium hypochlorite. However, there were no differences in the yield of peroxidation products using H_2O or D_2O. These experiments confirm our previous data (Panasenko *et al.*, 1994a) obtained by incubation of liposomes with hydrogen peroxide that singlet oxygen does not play any role in initiation of a lipid peroxidation induced by hypochlorous acid.

Therefore, an other mechanism besides the formation of singlet oxygen is responsible for en-

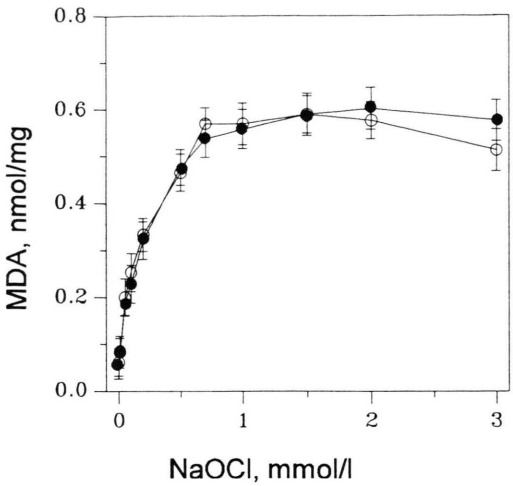

Fig. 3. Concentration of TBA-reactive products (in nmol/mg phospholipids) in phosphatidylcholine liposomes (2 mg/ml) as a function of NaOCl concentration. Liposomes were prepared in 0.14 mol/l NaCl, 10 mmol/l phosphate, pH 7.4 and incubated for 40 min with NaOCl. Puffer and NaOCl were prepared on H_2O or D_2O. All data are given as means and S.D. (n=4).

hanced lipid peroxidation after the reaction of hypochlorous acid with organic hydroperoxides.

^{1}H-NMR analysis of reaction products between tert-butyl hydroperoxide and hypochlorous acid

In order to reveal further details of the reaction mechanism between *tert*-butyl hydroperoxide and hypochlorous acid the products of this reaction were analysed by ^{1}H-NMR spectroscopy. If *tert*-butyl hydroperoxide would react like hydrogen peroxide with hypochlorous acid products with a single *tert*-butyl group should be formed. On the other hand, if free radicals would be formed in the result of reaction between *tert*-butyl hydroperoxide and hypochlorous acid products of recombination of free radicals should appear.

^{1}H-NMR spectra of *tert*-butyl hydroperoxide, *tert*-butanol, *tert*-butyl chloride and di-*tert*-butyl peroxide are shown in Fig. 4. All spectra were recorded using deuterated chloroform as solvent. In this solvent different chemical shifts for the *tert*-butyl group of these substances can be obtained. On the other hand, using D_2O as solvent, all these protons have the same chemical shift at 1.24 ppm (data not shown).

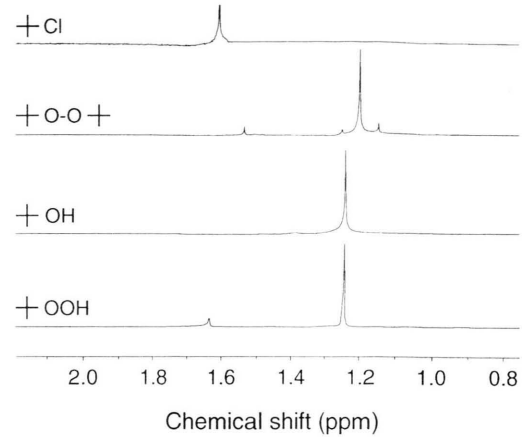

Fig. 4. ^{1}H NMR spectra of *tert*-butyl hydroperoxide, *tert*-butanol, di-*tert*-butyl peroxide and *tert*-butyl chloride in deuterated chloroform.

Tert-butyl hydroperoxide yielded in $CDCl_3$ an intense singlet at 1.245 ppm for all methyl protons and a much less intense singlet at 7.329 ppm for the proton of the hydroperoxide group. Methyl protons of *tert*-butanol and *tert*-butyl chloride gave singlets at 1.252 and 1.60 ppm, respectively. Additionally, a very small resonance appeared at 1.382 for the OH group of *tert*-butanol. Di-*tert*-butyl peroxide was characterized by an intense resonance at 1.193 ppm. A mixture of both *tert*-butyl hydroperoxide and di-*tert*-butyl peroxide yielded two well separated resonances at chemical shifts indicated above (data not shown).

Tert-butyl hydroperoxide dissolved in D_2O or phosphate buffer on the basis of D_2O was treated with different amounts of hypochlorous acid for 15 min. After that deuterated chloroform was added. Both phases were separated and analysed by ^{1}H-NMR spectroscopy. A typical ^{1}H-NMR spectrum of the water phase is given in Fig. 5. There is an intense signal at 1.24 ppm for protons of *tert*-butyl groups. Three small signals appeared at 1.90, 2.20, and 3.47 ppm. By comparing with resonances of known substances and signal enhancement after the addition of these substances the signals were attributed to acetate, acetone and methanol, respectively.

Much more complex are the ^{1}H-NMR spectra of chloroform phase of reaction products between hypochlorous acid and *tert*-butyl hydroperoxide (Fig. 6). The resonance for methyl protons of *tert*-butyl hydroperoxide at 1.245 ppm decreases con-

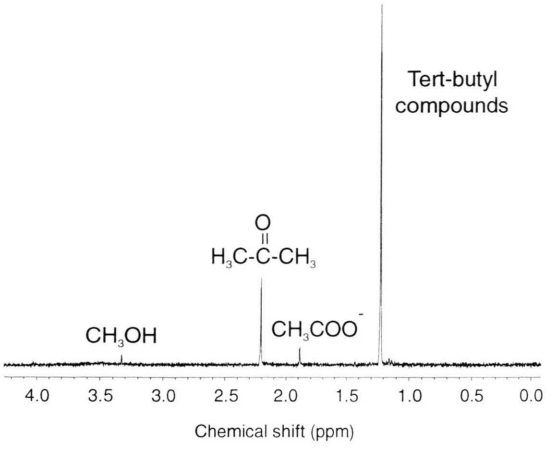

Fig. 5. ^{1}H NMR spectrum in D_2O of reaction products of *tert*-butyl hydroperoxide and NaOCl. *Tert*-butyl hydroperoxide (10 mmol/l) was incubated with NaOCl (10 mmol/l) for 15 minutes. All solutions were diluted with 0,14 mol/l NaCl, 10 mmol/l phosphate, pH 7.4 made on D_2O. After incubation 1 ml of reactants was thoroughly mixed with 1 ml $CDCl_3$.

tinuously with increasing concentrations of hypochlorous acid (data not shown). Surprisingly, marked amounts of *tert*-butyl hydroperoxide were still present using a twofold excess of hypochlorous acid. There are only traces of *tert*-butyl hydroperoxide at a fivefold excess of hypochlorous acid.

Seven new resonances appeared in chloroform phase of samples treated with hypochlorous acid. They are listed in Table I. All signals should arise

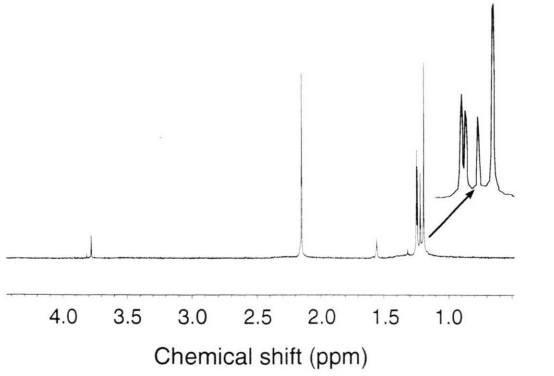

Fig. 6. ^{1}H NMR spectrum in $CDCl_3$ of reaction products of *tert*-butyl hydroperoxide and NaOCl. *Tert*-butyl hydroperoxide (10 mmol/l) was incubated with NaOCl (10 mmol/l) for 15 minutes. All solutions were diluted with 0,14 mol/l NaCl, 10 mmol/l phosphate, pH 7.4 made on D_2O. After incubation 1 ml of reactants was thoroughly mixed with 1 ml $CDCl_3$.

Table I. Chemical shifts and fine structures of ^{1}H NMR signals in the $CDCl_3$ phase of reaction products between *tert*-butyl hydroperoxide and $HOCl/OCl^-$.

Chemical shift	Formula	Fine structure	Intensity
1.193	$(CH_3)_3C-O-O-C(CH_3)_3$	singlet	+++
1.22	$(CH_3)_3COCl$	singlet	++
1.245	$(CH_3)_3COOH$	singlet	
1.252	$(CH_3)_3COH$	singlet	+++
1.32	non identified	singlet	traces
1.54	H_2O	singlet	
1.60	$(CH_3)_3CCl$	singlet	traces
2.15	CH_3COCH_3	singlet	+++
3.47	CH_3OH	singlet	traces
3.78	non identified	singlet	+
3.81	non identified	singlet	traces
7.24	$CHCl_3$	singlet	

from relatively simple molecule groups because they are singlets. Signal identification was performed by the comparison of chemical shifts with resonances of known substances and signal enhancement after the addition of these compounds. New signals at 1.193, 1.22 and 1.253 ppm were attributed to di-*tert*-butyl peroxide, *tert*-butyl hypochlorite and *tert*-butanol, respectively. Whereas only traces of di-*tert*-butyl peroxide were detected using equimolar or lower amounts of hypochlorous acid, the yield of this product raises markedly using an excess of hypochlorous acid. An intense resonance was observed for acetone at 2.15 ppm in $CDCl_3$. The acetone resonance appears already at the lowest concentration of hypochlorous acid and raises continuously at higher $HOCl/OCl^-$ concentrations. Methanol was found in traces at 3.47 ppm. Resonances at 1.32, 3.78, and 3.81 ppm could not be exactly assigned. Signal intensities for *tert*-butyl hydroperoxide, water and chloroform were not indicated in Table I. *Tert*-butyl hydroperoxide is the original compound. Water is contained in traces after phase separation in the $CDCl_3$ layer. Chloroform is also found in low amounts in $CDCl_3$.

Furthermore, the ability of *tert*-butanol, di-*tert*-butyl peroxide and acetone to react with hypochlorous acid has been studied. Whereas *tert*-butanol and di-*tert*-butyl peroxide did not show any reaction with hypochlorous acid under our experimental conditions, acetone yielded acetate and chloroform in a slow reaction with hypochlorous

acid. The latter reaction is known as the so-called haloform reaction:

$$CH_3-CO-CH_3 + 3\ HOCl \rightarrow CH_3COOH + CHCl_3 + 2\ H_2O.$$

^{1}H-NMR spectra of $CDCl_3$ solutions of 2,2′,3,3′-tetramethylbutane $(CH_3)_3CC(CH_3)_3$, *tert*-butyl methyl ether $(CH_3)_3COCH_3$, and 2-methylpropene $(CH_3)_2C=CH_2$ were also recorded. They yielded singlets at 0.85 ppm (2,2′,3,3′-tetramethylbutane), 1.17 and 3.19 ppm (*tert*-butyl methyl ether) or 1.71 and 4.19 ppm (2-methylpropene). Therefore, these compounds could not be identified as reaction products between *tert*-butyl hydroperoxide and hypochlorous acid.

In some cases the reaction between *tert*-butyl hydroperoxide and hypochlorous acid was studied using higher concentrations of chloride or after bubbling argon through all solutions to remove oxygen. Neither of these approaches leads to significant changes in the product composition (data not shown).

Discussion

NMR and chemiluminescence investigations of the reaction between *tert*-butyl hydroperoxide and hypochlorous acid indicate the formation of a free radical intermediate. One the of main reaction products is di-*tert*-butyl peroxide. It arises from the recombination of two *tert*-butyloxy radicals.

$$2\ (CH_3)_3C-O^{\cdot} \rightarrow (CH_3)_3C-O-O-C(CH_3)_3.$$

A scheme of this reaction sequence and some other proposed reactions of *tert*-butyloxy radical is given in Fig. 7. *Tert*-butanol can also arise from $(CH_3)_3C$-$O^{\cdot}$ by abstraction of a proton from the original *tert*-butyl hydroperoxide

$$(CH_3)_3C-O^{\cdot} + (CH_3)_3COOH \rightarrow (CH_3)_3COH + (CH_3)_3COO^{\cdot}.$$

The *tert*-butylperoxy radical is more stable than *tert*-butyloxy radical. The recombination of two *tert*-butylperoxy radicals will also yield di-*tert*-butyl peroxide according to a Russell mechanism (Russell, 1959).

According to the scheme in Fig. 7 *tert*-butyl hydroperoxide is involved into two reactions. First of all it yields the *tert*-butyloxy radical as the result of reaction with hypochlorous acid. Secondly, *tert*-butyl hydroperoxide reacts also with the *tert*-butyloxy radical.

A formation of di-*tert*-butyl peroxide from $(CH_3)_3C^{\cdot}$ and $(CH_3)_3COO^{\cdot}$ seems to be unlikely because the appearance of other recombination products as $(CH_3)_3C$-$C(CH_3)_3$ should be also expected for this reaction sequence. Furthermore, a bubbling of argon through all solutions to inhibit the formation of $(CH_3)_3COO^{\cdot}$ from $(CH_3)_3C^{\cdot}$ and O_2 did not result in a lower yield of di-*tert*-butyl peroxide.

These results demonstrate that $(CH_3)_3CO^{\cdot}$ radicals are formed in the result of reaction between *tert*-butyl hydroperoxide and hypochlorous acid. Such radicals are assumed to be a promoter of free radical lipid peroxidation (Small *et al.*, 1979; Schöneich *et al.*, 1990). Therefore, our previous finding (Panasenko *et al.*, 1995) of an enhancement of the production of TBA-reactive products in liposomes composed of egg yolk phosphatidylcholine treated with hypochlorous acid in the presence of *tert*-butyl or cumene hydroperoxide is caused by the appearance of $RO^{\cdot}$ radicals. Such radicals are also formed in the metal-catalysed decomposition of hydroperoxides (Vladimirov *et al.*, 1972).

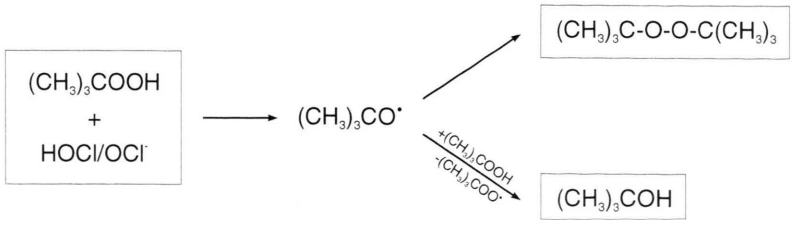

Fig. 7. Formation of di-*tert*-butyl peroxide ($(CH_3)_3C-O-O-C(CH_3)_3$) and *tert*-butanol ($(CH_3)_3COH$) in the result of reaction of *tert*-butyl hydroperoxide ($(CH_3)_3COOH$) with hypochlorous acid. $(CH_3)_3CO^{\cdot}$ and $(CH_3)_3COO^{\cdot}$ indicate *tert*-butyloxy radical and *tert*-butylperoxy radical, respectively.

The exact mechanism of formation of $(CH_3)_3CO^{\cdot}$ from *tert*-butyl hydroperoxide under the influence of hypochlorous acid remains unknown. One possible pathway is the homolytic disruption of $(CH_3)_3COOH$ into $(CH_3)_3CO^{\cdot}$ and $^{\cdot}OH$. On the other hand, the attack of hypochlorous acid on *tert*-butyl hydroperoxide can be accompanied by the formation of $(CH_3)_3COOCl$, whose homolytic disruption will be the source for *tert*-butyloxy radical.

Electron spin resonance experiments to detect free radicals are under progress. They are not included in this paper.

Two other main products of the reaction between *tert*-butyl hydroperoxide and hypochlorous acid are *tert*-butyl hypochlorite and acetone. There are several pathways for their formation. *Tert*-butyl hypochlorite can be formed from a direct reaction between hypochlorous acid and either *tert*-butyl hydroperoxide or *tert*-butanol. A third possibility is the interaction between the *tert*-butyloxy radical and a chlorous atom.

Acetone can also be formed in the result of several reactions. A β-scission of *tert*-butyloxy radicals yields acetone. The decomposition of *tert*-butyl hypochlorite is also accompanied by the formation of acetone. Finally, acetone can result from a direct reaction between *tert*-butyl hydroperoxide and hypochlorous acid.

Acetone itself is known to react with hypochlorous acid to yield acetic acid and chloroform.

These results demonstrate that the reaction mechanism of $HOCl/OCl^-$ with organic hydroperoxides differs considerably in comparison to its reaction with hydrogen peroxide. The reaction with hydrogen peroxide yields singlet oxygen as the main product. Free radicals are not involved in this process (Held *et al.*, 1978). On the other hand, free radicals are formed during reaction of $HOCl/OCl^-$ with *tert*-butyl or cumene hydroperoxides. Singlet oxygen is not generated in this reaction.

These properties are also responsible for the different behaviour of hydrogen peroxide (Panasenko *et al.*, 1994a) or *tert*-butyl hydroperoxide (Panasenko *et al.*, 1995) in peroxidation of unsaturated phosphatidylcholine induced by $HOCl/OCl^-$. Only *tert*-butyl hydroperoxide has been found to promote an additional accumulation of thiobarbituric acid-reactive substances.

Organic hydroperoxides are always present in biological membranes at low concentrations. Free radicals generated as the result of reaction between $HOCl/OCl^-$ and organic hydroperoxides can initiate new oxidation processes.

Acknowledgements

This work was supported by the German Ministry of Research and Technology (Grant 01 ZZ 9103/9-R-6) and by the Deutsche Forschungsgemeinschaft (Grant INK 23/A1–1). One of the authors (O.M. Panasenko) obtained a grant from the Alexander von Humboldt Foundation, Bonn, Germany. A grant of the Graduiertenkolleg (Molecular and cell biology of connective tissue) was provided for J. Schiller by the Deutsche Forschungsgemeinschaft.

Albrich J.M., Mc Carthy M.C. and Hurst J.K. (1981), Biological reactivity of hypochlorous acid: implication for microbicidal mechanism of leukocyte myeloperoxidase. Proc. Natl. Acad. U.S.A. **78,** 210–214.

Arnhold J., Hammerschmidt S. and Arnold K. (1991), Role of functional groups of human plasma in scavenging of NaOCl and neutrophil-derived hypochlorous acid. Biochim. Biophys. Acta **1097,** 145–151.

Arnhold J., Panasenko O.M., Schiller J., Vladimirov Yu.A. and Arnold K. (1995), The action of hypochlorous acid on phosphatidylcholine liposomes in dependence on the content of double bonds. Stoichiometry and NMR analysis. Chem. Phys. Lipids **78,** 55–64.

Beers R.F. and Sizer I.W. (1952), A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195,** 133–140.

Evgina S.A., Panasenko O.M., Sergienko V.I. and Vladimirov Yu.A. (1992), Peroxidation of human plasma lipoproteins induced by hypochlorite-anion. Biol. Membr. (russ.) **9,** 946–953.

Fliss H. (1988), Oxidation of proteins in rat heart and lungs by PMN oxidants. Mol. Cell Biochem. **59,** 177–188.

Frankel E.N., Neff W.E., Selke E. and Weisleder, D. (1982), Photosensitized oxidation of methyl linoleate: secondary and volatile thermal decomposition products. Lipids **17,** 11–18.

Hazell L.J. and Stocker R. (1993), Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. Biochem. J. **290,** 165–172.

Hazell L.J., Van den Berg J.J.M. and Stocker R. (1994), Oxidation of low-density lipoprotein with hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. Biochem J. **302,** 297–304.

Held A.M., Halko D.J. and Hurst J.K. (1978), Mechanisms of chlorine oxidation of hydrogen peroxide. J. Am. Chem. Soc. **100,** 5732–5740.

Kanofsky J.R. (1989), Singlet oxygen production by biological systems. Chem.-Biol. Interact. **70,** 1–28.

Khan A.U. and Kasha M. (1970), Chemiluminescence arising from simultaneous transitions in pairs of singlet oxygen molecules. J. Am. Chem. Soc. **92,** 3293–3300.

Klebanoff, S.J. (1988), Phagocytic cells: Products of oxygen metabolism. In: Inflammation. Basic Principles and Clinical Correlates (Gallin, J.I., Goldstein, I.M., Snyderman, R., Ed.). Raven Press, New York, 391–443.

Merkel P.B., Nilsson R. and Kearns, D.R. (1972), Deuterium effects on singlet oxygen lifetimes in solutions. A new test of singlet oxygen reactions. J. Am. Chem. Soc. **94,** 1030–1031.

Metzger J.O. (1989), Herstellung (Erzeugung) von Radikalen durch homolytische Spaltung von C,H-Bindungen mit Radikalen. In: Houben-Weyl. Methoden der Organischen Chemie. 4. Auflage, Bd. E19a, C-Radikale, Georg-Thieme Verlag Stuttgart, New-York, 60–145.

Morris J.C. (1966), The acid ionization constant of HOCl from 5° to 35°. J. Phys. Chem. **70,** 3798–3805.

Panasenko O.M., Arnhold J., Schiller J., Arnold K. and Sergienko V.I. (1994a), Peroxidation of egg yolk phosphatidylcholine liposomes by hypochlorous acid. Biochim. Biophys. Acta **1215,** 259–266.

Panasenko O.M., Arnhold J., Vladimirov Yu.A., Arnold K. and Sergienko V.I. (1995), Hypochlorous acid-induced peroxidation of egg yolk phosphatidylcholine is mediated by hydroperoxides. Submitted for publication.

Panasenko O.M., Evgina S.A., Aidyraliev R.K., Sergienko V.I. and Vladimirov Yu.A. (1994b), Peroxidation of human blood lipoproteins induced by exogenous hypochlorite or hypochlorite generated in the system of "myeloperoxidase+H_2O_2+Cl^-". Free Rad. Biol. Med. **16,** 143–148.

Rodgers M.A.J. and Snowden P.T. (1980), Lifetime of $O_2(^1\Delta_g)$ in liquid water as determined by time-resolved infrared luminescence measurements. J. Am. Chem. Soc. **15,** 544–548.

Russell G.A. (1959), Deuterium-isotope effects in the autoxidation of aralkyl hydrocarbons. Mechanism of the interaction of peroxy radicals. J. Am. Chem. Soc. **79,** 3871–3877.

Schiller J., Arnhold J. and Arnold K. (1995), NMR studies of the action of hypochlorous acid on native pig articular cartilage. Eur. J. Biochem. **233,** 672–676.

Schiller J., Arnhold J., Sonntag K. and Arnold K. (1996), NMR studies on human, pathologically changed synovial fluids. Role of hypochlorous acid. Magn. Res. Med., in press.

Schöneich C. and Asmus K.-D. (1990), Reaction of thiyl radicals with alcohols, ethers and polyunsaturated fatty acids: a possible role of thiyl free radicals in thiol mutagenesis. Radiat. Environ. Biophys. **29,** 263–271.

Small R.D., Jr., Scaiano J.C. and Patterson L.K. (1979), Radical processes in lipids. A laser photolysis study of t-butoxy radical reactivity toward fatty acids. Biochem. J. **212,** 549–554.

Smith J.K., Grisham M.B., Granger D.N. and Korthuis, R.J. (1989), Free radical defence mechanisms and neutrophil infiltration in postischemic skeletal muscle. Am. J. Physiol. **256,** H789-H793.

Stelmaszynska T., Kukovetz E., Egger G. and Schaur R.J. (1992), Possible involvement of myeloperoxidase in lipid peroxidation. Int. J. Biochem. **24,** 121–128.

Thomas E.L. (1979), Myeloperoxidase, hydrogen peroxide, chloride antimicrobial system: nitrogen-chlorine derivatives of bacterial components in their actions against *Escherichia coli*. Infect. Immun. **25,** 110–116.

Thomas M.J. and Pryor W.A. (1980), Singlet oxygen oxidation of methyl linoleate: isolation and characterization of the $NaBH_4$-reduced products. Lipids **15,** 544–548.

Uchiyama M. and Michara M. (1978), Determination of malondialdehyde precursor in tissues by thiobarbituric test. Anal. Biochem. **86,** 271–278.

Vladimirov Ju.A. and Archakov A.I. (1972), Perekisnoe okislenie lipidov v biologiceskich membranach. Moskva.

Wasil M., Halliwell B., Grootveld M., Moorhouse C.P., Hutchison D.C.S. and Baum, H. (1987), The specificity of thiourea, dimethylthiourea and dimethyl sulphoxide as scavengers of hydroxyl radicals. Their protection of α_1-antiproteinase against inactivation by hypochlorous acid. Biochem. J. **243,** 867–870.

Weiss S.J., Lampert M.B. and Test S.T. (1982), Long-lived oxidants generated by human neutrophils: characterization and bioactivity. Science **222,** 625–628.

Winterbourn C.C. (1985), Comparative reactivities of various compounds with myeloper-oxidase-hydrogen peroxide-chloride, and similarities of the oxidant hypochlorite. Biochim. Biophys. Acta **840,** 204–210.

Winterbourn C.C., Van den Berg J.J.M., Roitman E. and Kuypers F.A. (1992), Chlorohydrin formation from unsaturated fatty acids reacted with hypochlorous acid. Arch. Biochem. Biophys. **296,** 547–555.