Biochemistry (Moscow), Vol. 67, No. 8, 2002, pp. 880-888. Translated from Biokhimiya, Vol. 67, No. 8, 2002, pp. 1061-1070.

Peroxyl Radical Is Produced upon the Interaction of Hypochlorite with *tert*-Butyl Hydroperoxide

O. M. Panasenko[1]*, A. N. Osipov[2], A. V. Chekanov[1], J. Arnhold[3], and V. I. Sergienko[1]

[1]Research Institute of Physico-Chemical Medicine, ul. Malaya Pirogovskaya 1a, Moscow, 119828 Russia;
fax: (095) 246-4490; E-mail: oleg-panasenko@newmail.ru
[2]Russian State Medical University, ul. Ostrovityanova 1, Moscow, 117513 Russia; E-mail: an-osipov@mail.ru
[3]Institute of Medical Physics and Biophysics, University of Leipzig, Liebigstrasse 27, Leipzig, 04103, Germany;
fax: (49-341) 971-5709; E-mail: arnj@server3.medizin.uni-leipzig.de

Received May 4, 2001
Revision received June 9, 2001

Abstract—As we reported previously, hypochlorite interacting with organic hydroperoxides causes their decomposition ((1995) *Biochemistry* (Moscow), **60**, 1079-1086). This interaction was supposed to be a free-radical process and serve as a source of free radicals initiating lipid peroxidation (LP). The present study is the first attempt to detect and identify free radicals produced in the reaction of hypochlorite with *tert*-butyl hydroperoxide, $(CH_3)_3COOH$, which we have used as an example of organic hydroperoxides. We have used a direct method for free radical detection, EPR of spin trapping, and the following spin traps: N-*tert*-butyl-α-phenylnitrone (PBN) and α-(4-pyridyl-1-oxyl)-N-*tert*-butylnitrone (4-POBN). When hypochlorite was added to $(CH_3)_3COOH$ in the presence of a spin trap, an EPR spectrum appeared representing a superposition of two signals. One of them belonged to a spin adduct formed as a result of direct interaction of hypochlorite with the spin trap (hyperfine splitting constants were: $a_H^\beta = 0.148$ mT; $a_N = 1.537$ mT; and $\Delta H_{PP} = 0.042$ mT for 4-POBN and $a_H^\beta = 0.190$ mT; $a_N = 1.558$ mT; and $\Delta H_{PP} = 0.074$ mT for PBN). The other signal was produced by hypochlorite interactions with $(CH_3)_3COOH$ itself (hyperfine splitting constants were: $a_H^\beta = 0.233$ mT; $a_N = 1.484$ mT; $\Delta H_{PP} = 0.063$ mT and $a_H^\beta = 0.360$ mT; $a_N = 1.547$ mT; $\Delta H_{PP} = 0.063$ mT for 4-POBN and PBN, respectively). Comparison of spectral characteristics of this spin adduct with those of *tert*-butoxyl or *tert*-butyl peroxyl radicals produced in known reactions of $(CH_3)_3COOH$ with Fe^{2+} and Ce^{4+}, respectively, showed that the radical $(CH_3)_3COO^\bullet$ is produced from the interaction of hypochlorite with $(CH_3)_3COOH$. Like Ce^{4+} but not Fe^{2+}, hypochlorite addition to $(CH_3)_3COOH$ was accompanied by a bright flash of chemiluminescence characteristic of the reactions in which peroxyl radicals are produced. Thus, all these results suggest peroxyl radical production in the reaction of hypochlorite with hydroperoxide. This reaction is one of the most possible ways for the initiation of free-radical LP that occurs *in vivo*, when hypochlorite interacts with unsaturated lipids comprising natural protein—lipid complexes, such as lipoproteins and biological membranes.

Key words: hypochlorite, hydroperoxide, *tert*-butyl hydroperoxide, free radicals, peroxyl radical, lipid peroxidation, spin traps

The process of lipid peroxidation (LP) plays an important role in the progression of many disorders. Along with transition metal ions, active oxygen forms, such as $^\bullet O_2^-$, H_2O_2, $^\bullet OH$, and HOCl, produced by stimulated neutrophils or monocyte-macrophages initiate LP *in vivo* [1]. As recently reported, both superoxide anion-radical $^\bullet O_2^-$ and hydrogen peroxide H_2O_2 are themselves weak lipid oxidizers [1]. The studies [2-4] suggested that highly reactive hydroxyl radical $^\bullet OH$ produced from the interaction of these compounds with transition metal ions is far from playing the dominant role in oxidative modification of protein—lipid complexes by phagocytes. Along with this, hypochlorous acid (HOCl) or its ionized form, hypochlorite (OCl^-), which is produced in a reaction catalyzed by myeloperoxidase, an enzyme present in neutrophils and monocytes, was recently reported to be a strong oxidizer that can initiate free-radical LP of blood lipoproteins and other protein—lipid complexes [5-11]. Considering that the p*K* of HOCl is near 7.5 [12] and under physiological conditions about a half of the acid is present in molecular form and the other half in the anionic form, the mixture $HOCl/OCl^-$ present in the medium under study is termed below as hypochlorite.

Abbreviations: LP) lipid peroxidation; PBN) N-*tert*-butyl-α-phenylnitrone; 4-POBN) α-(4-pyridyl-1-oxyl)-N-*tert*-butylnitrone; EPR) electron paramagnetic resonance; $(CH_3)_3COOH$) *tert*-butyl hydroperoxide.

* To whom correspondence should be addressed.

A reaction of hypochlorite with organic hydroperoxides that are always present in unsaturated lipids is a candidate to the role of the initiation stage of hypochlorite-induced LP. Indeed, the body of data we obtained previously suggests the formation of free-radical intermediates in the course of this reaction:

1) hypochlorite actually interacts with hydroperoxide group, but not with other peroxides, with stoichiometric ratio more than 1 [11, 13];

2) this reaction is accompanied by chemiluminescence [11, 14, 15] that is characteristic for free-radical reactions [16];

3) singlet oxygen is not produced in this reaction [14, 17], unlike the reaction of hypochlorite with hydrogen peroxide [18]:

$$HOCl + H_2O_2 \rightarrow {}^1O_2 + Cl^- + H^+ + H_2O;$$

4) in the presence of hydroperoxides, an additional accumulation of LP products occurs in phospholipid liposomes incubated with hypochlorite or with the system myeloperoxidase + H_2O_2 + Cl^- catalyzing hypochlorite formation [11, 15, 17, 19];

5) accumulation of LP products is inhibited by the known free-radical quencher, 2,6-di-*tert*-butyl-*p*-cresol (ionol) [15, 17, 19];

6) the main molecular product of the reaction of hypochlorite with *tert*-butyl hydroperoxide is di-*tert*-butyl peroxide, whose production is explainable only from the hypothesis of free radical formation in the course of this reaction [11, 14, 17].

All these data give evidence, although indirectly, for the reaction of hypochlorite with the hydroperoxyl group involved in free-radical intermediate formation. However, there were have been no reports on the direct registration of free radicals that might be produced in the course of the given reaction.

In connection with this, the present study using the method of direct free radical detection, EPR spin trapping, is the first attempt to determine whether free radicals are produced in the reaction of hypochlorite with organic hydroperoxide and what is the chemical nature of the free-radical intermediates formed.

MATERIALS AND METHODS

Chemicals. Spin traps: N-*tert*-butyl-α-phenylnitrone (PBN) and α-(4-pyridyl-1-oxyl)-N-*tert*-butylnitrone (4-POBN) and also NaOCl were purchased from Sigma (USA); other chemicals: NaH_2PO_4, $FeCl_2 \cdot 4H_2O$, $Ce(NH_4)_2(NO_3)_6$, and NaCl were purchased from Reakhim (Russia). *tert*-Butyl hydroperoxide and all solvents applied for UV-spectrometry were purchased from Fluka (Switzerland).

In some experiments, we used hypochlorite synthesized electrochemically from 0.9% NaCl solution on an EDO-3 unit [20] for 30 min at 1 A. The electrode contained titanium micropromoted with platinum. The concentration of OCl^- was determined from its absorption at 290 nm and pH 12 taking the extinction coefficient ε_{290} = 350 $M^{-1} \cdot cm^{-1}$ [12].

EPR experiments. Short-living radicals produced in the reaction of hypochlorite with organic hydroperoxide were measured by the spin-trapping method. EPR spectra were recorded at room temperature on an E-4 radio spectrometer (Varian, USA) equipped with a computer. Samples for the EPR spectrum recording were prepared as follows: spin trap (20 mM), $(CH_3)_3COOH$ (1-20 mM), and hypochlorite (0.1-1 mM) were added into the detection medium (50 mM phosphate, pH 7.4), mixed quickly and placed into the resonator of the radio spectrometer in a flat quartz cell. EPR spectra were recorded under the following conditions: magnetic intensity 0.33 T; microwave power 10 mW; modulation amplitude 0.05 mT; and response time 0.03 sec. Constants a_H^β and a_N characterizing the hyperfine splitting on the nuclei of hydrogen β-atom and nitrogen atom, respectively, and ΔH_{PP} characterizing the spectral line width were used as the spectral parameters of spin adducts.

The software and database of the spectral parameters of EPR spin adducts were acquired from the Internet (http://epr.niehs.nih.gov) and used for the calculation of constants of the EPR spectrum hyperfine splitting and identification of spin adducts detected. The software description and details of its use for EPR spectrum modeling are described elsewhere [21]. The software enables the creation of a calculated spectrum from the measured empiric parameters of the experimental EPR spectrum, optimization of the calculated spectrum by its comparison with the experimental one, and thus more precise determination of the values of hyperfine splitting constants (to compare with the constant values determined empirically).

Chemiluminescence was measured on a CLM-3 device connected with the MacLab interface and MacIntosh computer using the Chart program at 23°C and pH 7.4. The following systems were tested: $(CH_3)_3COOH$ (22.2 mM) + hypochlorite (4 mM); $(CH_3)_3COOH$ (22.2 mM) + $Ce(NH_4)_2(NO_3)_6$ (4 mM), and $(CH_3)_3COOH$ (22.2 mM) + Fe^{2+} (4 mM).

Variational statistic methods were used for the **statistical data processing**. The significance of the differences was evaluated by Student's *t*-test. The measured values were taken as significant, when $p < 0.05$.

RESULTS AND DISCUSSION

Detection of radicals in the reaction of hypochlorite with $(CH_3)_3COOH$. Hypochlorite is known to initiate free-radical LP [5-11]. At present, the body of data indicates that the reaction of hypochlorite with organic hydroperoxides that are always present in some amounts in unsaturated lipids, may be an initiation step in hypochlorite-induced LP [11, 13-15]. A set of experimental data indirectly indicating free radicals, such as peroxyl and/or alkoxyl radicals, are formed in the course of this reaction [11, 14, 15, 17, 19]. However, this supposition has not yet been proven experimentally.

We employed the EPR spin-trapping method for the detection of free radicals generated in the reaction of hypochlorite with $(CH_3)_3COOH$. The latter was chosen as a model for organic hydroperoxides.

The first experiments showed that EPR spectra given in the Figs. 1a and 2a are detected when hypochlorite is added to $(CH_3)_3COOH$ in the presence of spin traps 4-POBN and PBN, respectively. Interestingly, when hypochlorite is added to the spin traps in the absence of $(CH_3)_3COOH$, EPR spectra are also detected (Figs. 1b and 2b), but their hyperfine splitting constants are different. These data suggest that hypochlorite interacts directly with the spin traps 4-POBN and PBN, thus forming spin adducts. Particularly, the interaction of hypochlorite with 4-POBN gives a spin adduct with the following parameters: $a^{\beta}_{H} = 0.148$ mT; $a_N = 1.537$ mT; and $\Delta H_{PP} = 0.042$ mT; and in the case of PBN these parameters are: $a^{\beta}_{H} = 0.190$ mT; $a_N = 1.558$ mT; and $\Delta H_{PP} = 0.074$ mT.

However, when hypochlorite was added to the trap in the presence of $(CH_3)_3COOH$, other adducts, along with those specified above, were also detected in EPR spectra and characterized by different hyperfine splitting constants: $a^{\beta}_{H} = 0.233$ mT; $a_N = 1.484$ mT; $\Delta H_{PP} = 0.063$ mT for 4-POBN and $a^{\beta}_{H} = 0.360$ mT; $a_N = 1.547$ mT; $\Delta H_{PP} = 0.063$ mT for PBN. Figure 1a displays the EPR spectrum that is a superposition of the signals from two spin adducts; one of them forms from the direct interaction of hypochlorite with the spin trap (4-POBN), the second one is a result of the interaction of hypochlorite with $(CH_3)_3COOH$. The evaluated values of spectral parameters allowed the simulation of the recorded EPR spectra with the computer program offered in [21]. These spectra are displayed in the Figs. 1 and 2 (spectra (c) and (d)). One can see that the model spectra are essentially identical to the experimental ones, thus confirming our hypothesis on the existence of two spin adduct types when hypochlorite is added to $(CH_3)_3COOH$ in the presence of spin traps.

These results give evidence for the interaction of hypochlorite with $(CH_3)_3COOH$ leading to the formation of an unknown radical that reacts with a spin trap (4-POBN or PBN) and forms a spin adduct possessing certain spectral parameters. Our further attention was attracted mainly to this spin adduct. First, we attempted to study its stability and possible conversion into other spin adducts. To do this, we used the spin trap 4-POBN and determined both the EPR signal intensities and hyperfine splitting constant values at defined intervals. Kinetics curve of the EPR signal intensity for the radical

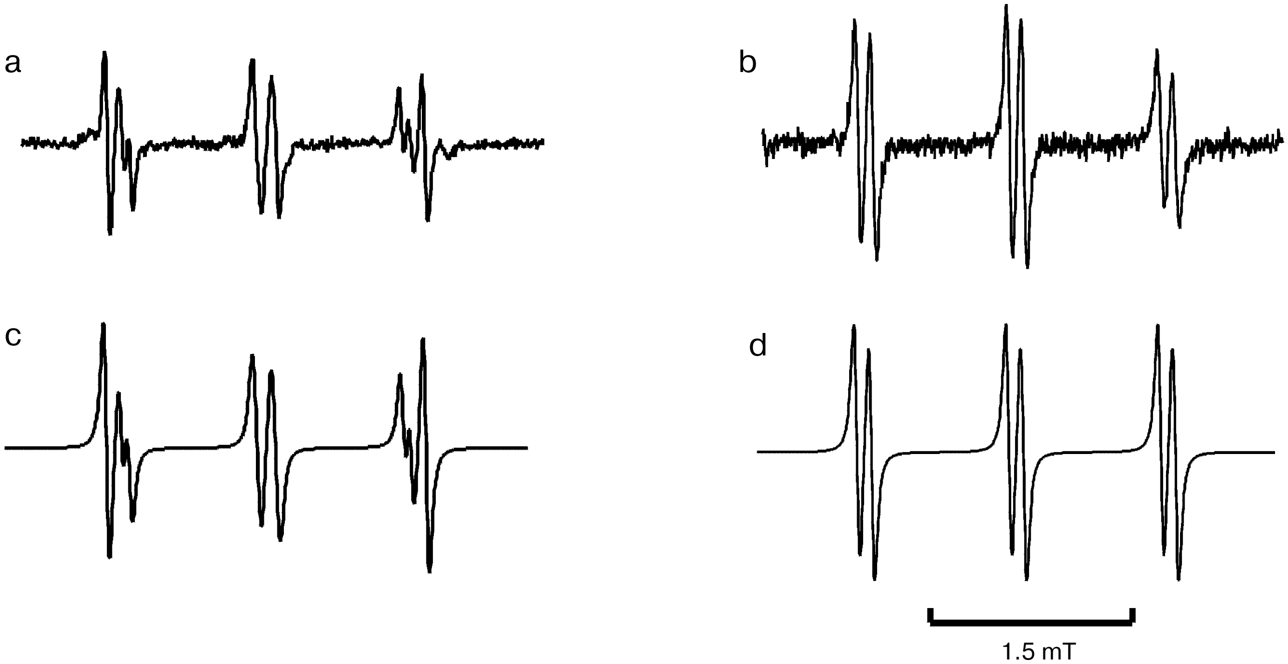

Fig. 1. EPR spectra recorded in the system $(CH_3)_3COOH + HOCl/OCl^-$ in the presence of the spin trap 4-POBN and their computer-aided simulation. Measurements were conducted at room temperature in 50-mM phosphate, pH 7.4. a) 4-POBN (20 mM) + $(CH_3)_3COOH$ (5 mM) + $HOCl/OCl^-$ (1 mM); c) computer-aided simulation of the spectrum (a); b) 4-POBN (20 mM) + $HOCl/OCl^-$ (1 mM); d) computer-aided simulation of the spectrum (b).

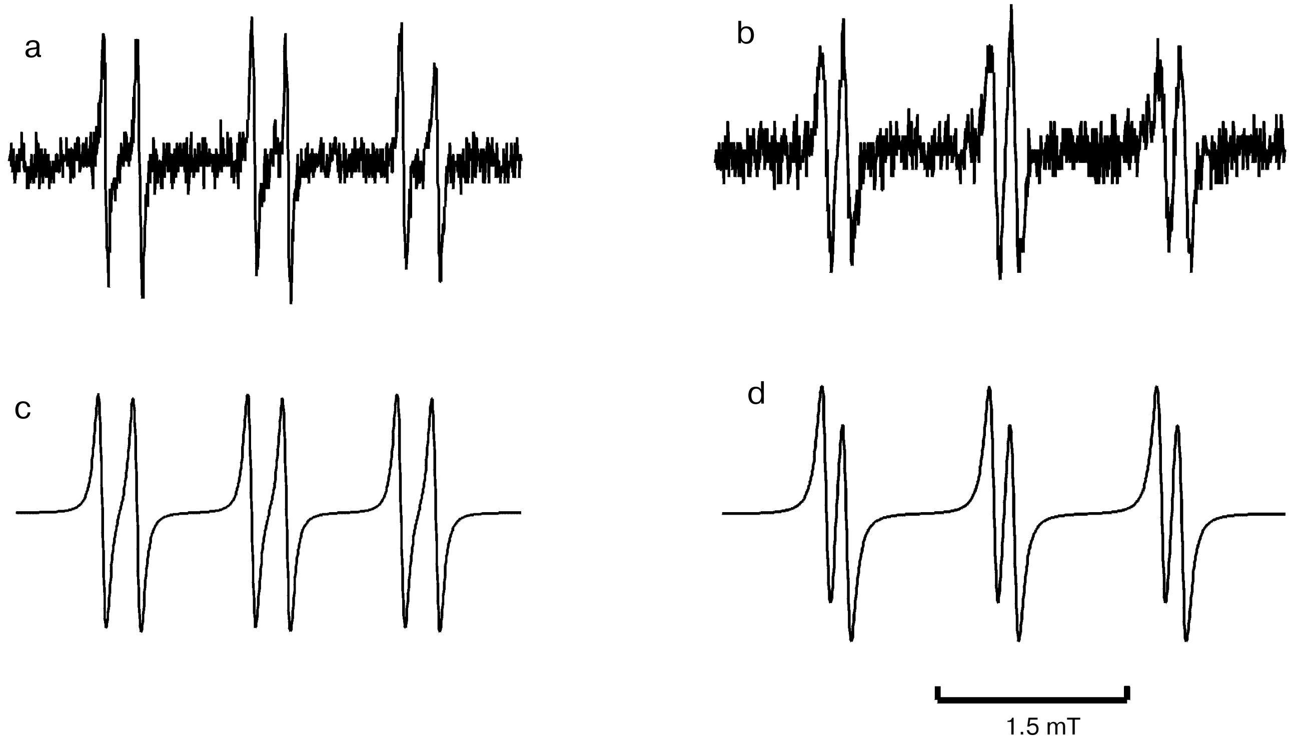

Fig. 2. EPR spectra recorded in the system $(CH_3)_3COOH$ + HOCl/OCl^- in the presence of the spin trap PBN and their computer-aided simulation. Measurements were conducted at room temperature in 50-mM phosphate, pH 7.4. a) PBN (20 mM) + $(CH_3)_3COOH$ (3 mM) + HOCl/OCl^- (1 mM); c) computer-aided simulation of the spectrum (a); b) PBN (20 mM) + HOCl/OCl^- (1 mM); d) computer-aided simulation of spectrum (b).

spin adduct generated in the HOCl/OCl^- + $(CH_3)_3COOH$ system is given in the Fig. 3. Obviously, the spin adduct formed is relatively unstable and disappears in time. However, as seen from the experiment, the constants of hyperfine EPR splitting remain unchanged. Evidently, the primary spin adduct does not turn into another spin adduct. Considering these kinetic results we further recorded all spectra not later than 2-3 min after hypochlorite was added to the mixture of $(CH_3)_3COOH$ with a trap, when the EPR signal intensity was high enough.

If our hypothesis is correct, that is two spin adducts, namely the adduct formed from the direct interaction of hypochlorite with a trap and the adduct due to by free radical formation in the reaction of hypochlorite with $(CH_3)_3COOH$, are actually produced upon the addition of hypochlorite to $(CH_3)_3COOH$ in the presence of spin trap, we might expect that as hypochlorite and $(CH_3)_3COOH$ concentration ratio changes, the corresponding EPR signal intensity ratio for these adducts would change too.

The results of our experiments are shown in the Figs. 4 and 5. Figure 4 shows that the intensity of signal from the spin adduct of radical resulting from the interaction of hypochlorite with $(CH_3)_3COOH$ increases as $(CH_3)_3COOH$ concentration increases at constant concentration of hypochlorite (curve *1*). The signal intensity of spin adduct resulting from the direct interaction

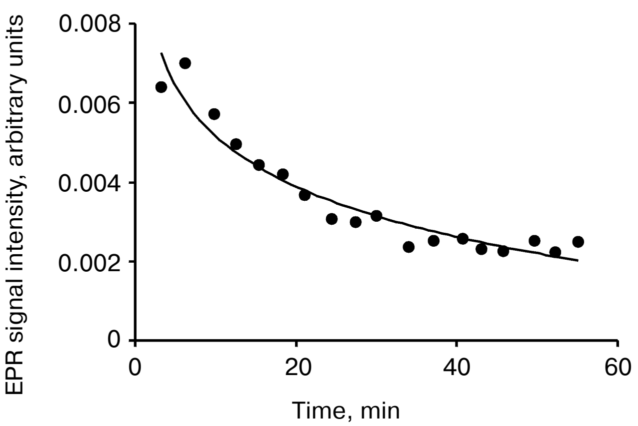

Fig. 3. EPR signal kinetics for the spin adduct with the hyperfine splitting constants $a^{\beta}_{H} = 0.233$ mT; $a_N = 1.484$ mT; $\Delta H_{PP} =$ 0.063 mT, which is produced from the interaction of hypochlorite with $(CH_3)_3COOH$ in the presence of spin trap 4-POBN. Measurements were conducted at room temperature in 50 mM phosphate, pH 7.4. The specimen composition was: 4-POBN (20 mM) + $(CH_3)_3COOH$ (5 mM) + HOCl/OCl^- (1 mM).

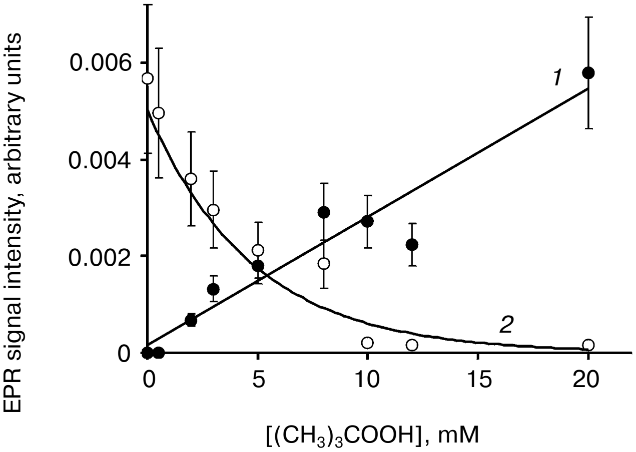

Fig. 4. Effect of $(CH_3)_3COOH$ on the intensity of EPR signals of spin adducts detected in the system $(CH_3)_3COOH$ + $HOCl/OCl^-$ in the presence of the spin trap, 4-POBN. Measurements were conducted at room temperature in 50 mM phosphate, pH 7.4. The specimen composition was: 4-POBN (20 mM) + $(CH_3)_3COOH$ + $HOCl/OCl^-$ (1 mM). *1*) Spin adduct of radical produced in the reaction of hypochlorite with $(CH_3)_3COOH$ (hyperfine splitting constants are: a^β_H = 0.233 mT; a_N = 1.484 mT; ΔH_{PP} = 0.063 mT); *2*) spin adduct produced on the interaction of hypochlorite with the spin trap 4-POBN (hyperfine splitting constants are: a^β_H = 0.148 mT; a_N = 1.537 mT; and ΔH_{PP} = 0.042 mT).

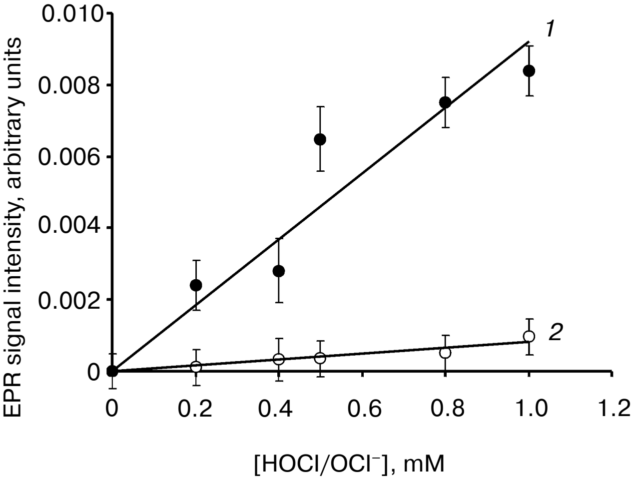

Fig. 5. Effect of hypochlorite on the intensity of EPR signals of spin adducts detected in the system $(CH_3)_3COOH$ + $HOCl/OCl^-$ in the presence of the spin trap 4-POBN. Measurements were conducted at room temperature in 50 mM phosphate, pH 7.4. The specimen composition was: 4-POBN (20 mM) + $(CH_3)_3COOH$ (20 mM) + $HOCl/OCl^-$. *1*) Spin adduct of radical produced in the reaction of hypochlorite with $(CH_3)_3COOH$ (hyperfine splitting constants are: a^β_H = 0.233 mT; a_N = 1.484 mT; ΔH_{PP} = 0.063 mT); *2*) spin adduct produced on the interaction of hypochlorite with the spin trap 4-POBN (hyperfine splitting constants are: a^β_H = 0.148 mT; a_N = 1.537 mT; and ΔH_{PP} = 0.042 mT).

between hypochlorite and trap decreases concurrently (curve *2*). This phenomenon is due to the decrease in hypochlorite portion reacting with trap, because its enlarged amount is spent on the interaction with $(CH_3)_3COOH$ as its concentration increases.

The signal intensity changes are shown in Fig. 5 for the spin adducts of 4-POBN studied at constant $(CH_3)_3COOH$ concentration and increasing hypochlorite concentration. The increasing hypochlorite concentration obviously results in substantial enhancement of the signal from radical spin adduct due to the interaction between hypochlorite and $(CH_3)_3COOH$. The signal intensity from the adduct due to the direct interaction of hypochlorite with the trap moderately rises concurrently as well.

These results confirm our conclusion that two spin adducts are formed in the system $(CH_3)_3COOH$ + $HOCl/OCl^-$ + spin trap; one of them is a product of direct interaction of hypochlorite with a trap, the second one is formed in the reaction of the trap with a radical produced from the interaction of $(CH_3)_3COOH$ with $HOCl/OCl^-$.

Identification of free radicals formed in the reaction of hypochlorite with $(CH_3)_3COOH$. The formation of either alcoxyl ($(CH_3)_3CO^\bullet$) or peroxyl ($(CH_3)_3COO^\bullet$) radicals would be expected when hypochlorite interacts with $(CH_3)_3COOH$. Alcoxyl radical is known to be generated on the reduction of organic hydroperoxide with Fe^{2+} [16, 22]:

$$(CH_3)_3COOH + H^+ + Fe^{2+} \rightarrow Fe^{3+} + (CH_3)_3CO^\bullet + H_2O. \quad (1)$$

It is also known that Ce^{4+} can oxidize $(CH_3)_3COOH$ to peroxyl radical [22]:

$$(CH_3)_3COOH + Ce^{4+} \rightarrow Ce^{3+} + (CH_3)_3COO^\bullet + H^+. \quad (2)$$

Considering this fact and using the spin trap 4-POBN, we attempted to detect EPR spectra of spin adducts produced in the course of known reactions (1) and (2) and to compare EPR spectral parameters of these adducts with those of spin adducts formed in the reaction of hypochlorite with $(CH_3)_3COOH$.

The hyperfine splitting constants of EPR spectra we recorded are given in Table 1. We succeeded in detecting a spin adduct formed in reaction (2) with the following spectral parameters: a^β_H = 0.233 mT; a_N = 1.510 mT; and ΔH_{PP} = 0.063 mT. Another spin adduct with hyperfine splitting constants: a^β_H = 0.276 mT; a_N = 1.633 mT; and ΔH_{PP} = 0.085 mT was detected in reaction (1) in the system Fe^{2+} + $(CH_3)_3COOH$.

A comparison of these spectral parameters to those of spin adduct produced from the interaction of hypochlorite with $(CH_3)_3COOH$ showed that the spectral parameters of the adduct we found are nearly identical to those of the adduct formed in reaction (2) and differ from

Table 1. Spectral parameters of spin adducts of radicals formed in the reaction of $(CH_3)_3COOH$ with Fe^{2+}, Ce^{4+}, or $HOCl/OCl^-$ in the presence of spin trap 4-POBN

System	Radical formed	EPR spectral parameters of spin adduct		
		a_H^β, mT	a_N, mT	ΔHpp, mT
$Fe^{2+} + (CH_3)_3COOH$	$(CH_3)_3CO^\bullet$	0.276 ± 0.009	1.633 ± 0.007	0.085 ± 0.008
$Ce^{4+} + (CH_3)_3COOH$	$(CH_3)_3COO^\bullet$	0.233 ± 0.008	1.510 ± 0.009	0.063 ± 0.007
$HOCl/OCl^- + (CH_3)_3COOH$	$(CH_3)_3COO^\bullet$	0.233 ± 0.007	1.484 ± 0.015	0.063 ± 0.009

Note: Reactions were conducted at room temperature in 50 mM phosphate, pH 7.4. Concentrations (mM): 4-POBN, 20; $(CH_3)_3COOH$, 20; Fe^{2+}, 3; Ce^{4+}, 5; $HOCl/OCl^-$, 1.

Table 2. Spectral parameters of spin adducts of radicals formed in the absence or presence of ethanol in the Fenton reaction and in the reactions of $(CH_3)_3COOH$ with Ce^{4+} or $HOCl/OCl^-$

System	Radical formed	EPR spectral parameters of spin adduct		
		in the absence of ethanol	in the presence of ethanol	mT
$Fe^{2+} + H_2O_2 + CH_3CH_2OH$	$CH_3{}^\bullet CHOH$	0.170 ± 0008	0.275 ± 0.011	a_H^β
		1.537 ± 0.008	1.611 ± 0.010	a_N
		0.064 ± 0.009	0.085 ± 0.009	ΔHpp
$Ce^{4+} + (CH_3)_3COOH + CH_3CH_2OH$	$(CH_3)_3COO^\bullet$	0.233 ± 0.008	0.233 ± 0.012	a_H^β
		1.510 ± 0.009	1.510 ± 0.007	a_N
		0.063 ± 0.007	0.053 ± 0.008	ΔHpp
$HOCl/OCl^- + (CH_3)_3COOH +$	$(CH_3)_3COO^\bullet$	0.233 ± 0.007	0.233 ± 0.008	a_H^β
$+ CH_3CH_2OH$		1.484 ± 0.015	1.490 ± 0.008	a_N
		0.063 ± 0.009	0.053 ± 0.011	ΔHpp

Note: Reactions were conducted at room temperature in 50 mM phosphate, pH 7.4. Concentrations (mM): 4-POBN, 20; $(CH_3)_3COOH$, 20; Fe^{2+}, 3; H_2O_2, 3; Ce^{4+}, 5; $HOCl/OCl^-$, 1; ethanol, 1000.

those of the spin adduct formed in reaction (1). From these data, it can be concluded that the radical generated in the course of the reaction we studied between hypochlorite and $(CH_3)_3COOH$ is a peroxyl radical.

Alcoxyl and hydroxyl radicals, due to their high reactivity, can abstract the hydrogen atom from the ethanol molecule to form the hydroxy ethyl radical. Peroxy radical possesses relatively low reactivity [23] and cannot participate in the reaction or do this very slowly, hence, hydroxy ethyl radical adduct can be scarcely detected in the reaction mixture in which peroxyl radicals are generated.

Taking this fact into account, we used ethanol as an intermediate acceptor for the *tert*-butoxyl radicals. The results of these experiments are presented in Table 2, from which it is obvious that in the case of the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^\bullet OH + OH^-, \qquad (3)$$

as it could be expected, the spin adduct of hydroxyl radical was detected. Another spin adduct, namely hydroxy ethyl radical adduct with the following spectral parameters: $a_H^\beta = 0.275$ mT; $a_N = 1.611$ mT; and $\Delta H_{PP} = 0.085$ mT

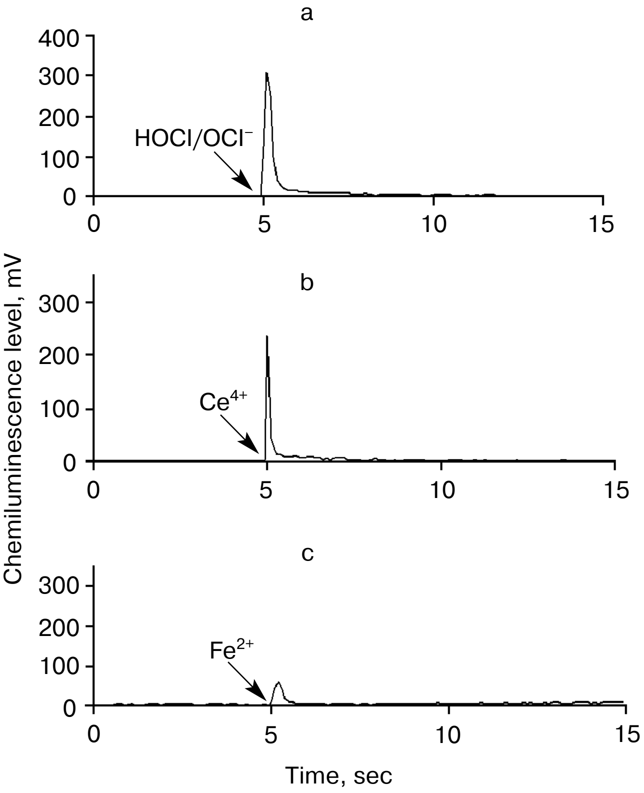

Fig. 6. Chemiluminescence flash after the addition of 4 mM $HOCl/OCl^-$ (a), Ce^{4+} (b), or Fe^{2+} (c) to $(CH_3)_3COOH$ (22.2 mM). Arrows indicate moments when $HOCl/OCl^-$, Ce^{4+}, and Fe^{2+}, respectively, were added. Measurements were conducted at room temperature in 5.0 mM phosphate, pH 7.4.

was detected on the excess of ethanol in the reaction mixture.

We examined the reaction $Fe^{2+} + (CH_3)_3COOH$, in which alcoxyl radical or, in the presence of ethanol as the intermediate acceptor, hydroxyl radical is to be formed, and found that the spectral parameters of the spin adduct of hydroxyl radical (a_H^β = 0.275 mT; a_N = 1.611 mT; and ΔH_{PP} = 0.085 mT; see Table 2, Fenton reaction in the presence of ethanol) are little different from those of the adduct of *tert*-butoxyl radical (a_H^β = 0.276 mT; a_N = 1.633 mT; and ΔH_{PP} = 0.085 mT; see Table 1, reaction (1)).

EPR spectra of a practically identical spin adducts were recorded (see Table 2) on addition of Ce^{4+} to $(CH_3)_3COOH$ in the presence or absence of ethanol. This is because peroxyl radical generated in the reaction studied (2) cannot detach the hydrogen atom from ethanol, hence, the formation of hydroxy ethyl radical is impossible.

Similar results were obtained on addition of hypochlorite to $(CH_3)_3COOH$ in the presence or absence of ethanol. A spin adduct generated in this case was similar in its parameters to spin adduct formed from the interaction between Ce^{4+} and $(CH_3)_3COOH$ (see Table 2). Ethanol had no effect on the spectral parameters of this adduct. This fact indicates that the same radical, namely peroxyl radical, is formed both in the reaction of hypochlorite with $(CH_3)_3COOH$ and in the reaction of Ce^{4+} with $(CH_3)_3COOH$.

Thus, the data again confirm our conclusion that a radical of peroxyl nature is produced in the course of the reaction between hypochlorite and organic hydroperoxide.

Vladimirov et al. demonstrated repeatedly [16, 24] that reactions in which peroxyl radicals are generated are accompanied by ultra-low glow, chemiluminescence that is due to the recombination of these radicals. If peroxyl radical is actually produced in the system under study ($(CH_3)_3COOH + HOCl/OCl^-$), this reaction is to be accompanied by a chemiluminescence flash.

Using the chemiluminescence technique, we studied the reaction of hypochlorite with $(CH_3)_3COOH$. The results were compared to those obtained on addition of Ce^{4+} to $(CH_3)_3COOH$ (in this case, peroxyl radical is generated, see reaction (2)) or Fe^{2+} to $(CH_3)_3COOH$ (in this case, alcoxyl radical is formed, see reaction (1)).

Chemiluminescence kinetics curves for the reactions studied are displayed in Fig. 6. It was shown in control experiments that an addition of $(CH_3)_3COOH$ or hypochlorite to the phosphate buffer solution (pH 7.4) that served as the reaction medium was not accompanied by any chemiluminescence flash (data not shown). However, a bright chemiluminescence flash was detected on addition of hypochlorite into $(CH_3)_3COOH$ solution, as seen from the Fig. 6a. A similar result was obtained on addition of Ce^{4+} to the $(CH_3)_3COOH$ solution (see Fig. 6b). On the other hand, an addition of Fe^{2+} to $(CH_3)_3COOH$, although accompanied by chemiluminescence, the flash was substantially weaker than in the case of the two reactions described previously (Fig. 6c). This flash may be, at least in part, due to the recombination of

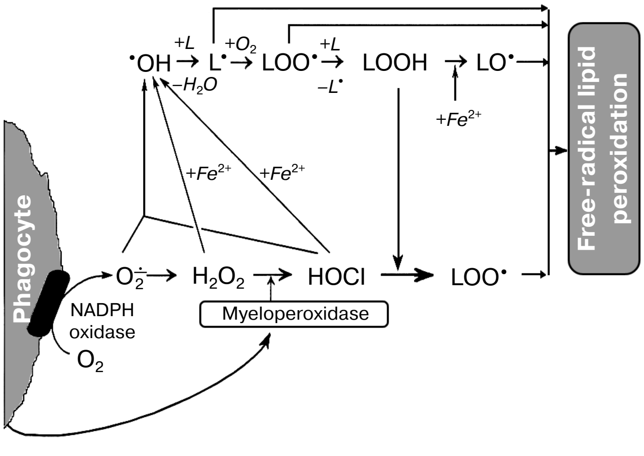

Fig. 7. Scheme for an involvement of hypochlorite produced by activated phagocytes in the initiation of free-radical lipid peroxidation. See explanations in the text.

tert-butyl peroxyl radicals generated from the interaction between *tert*-butoxyl radicals (generated in turn during the course of reaction (1)) at $(CH_3)_3COOH$ excess according to the following reaction:

$$(CH_3)_3CO^\bullet + (CH_3)_3COOH \rightarrow (CH_3)_3COO^\bullet + (CH_3)_3COH.$$

Thus, there were systems generating expectedly peroxyl radicals, in which chemiluminescence flashes of high intensity were detected. This fact confirms our proposition on peroxyl radical generation in the system $HOCl/OCl^- + (CH_3)_3COOH$.

The results of present study using the spin trap method as well as the chemiluminescence technique show that the peroxyl radical as a free-radical intermediate is produced in the reaction of hypochlorite with $(CH_3)_3COOH$ and possibly in its reactions with other organic hydroperoxides; this radical is a typical initiator of free-radical lipid peroxidation processes [16, 24].

One can suggest that this reaction is one of the most probable mechanisms initiating free-radical LP that occurs when hypochlorite interacts with unsaturated lipids comprising natural protein–lipid complexes, such as lipoproteins and biological membranes. One of the possible mechanisms of hypochlorite participation in free radical modification of these natural protein–lipid complexes can be presented by the scheme shown in the Fig. 7. Phagocyte stimulation is accompanied by the activation of NADPH-oxidase, a membrane-bound enzyme catalyzing superoxide anion radical ($^\bullet O_2^-$) generation. The radical dismutates spontaneously or under the action of another enzyme, superoxide dismutase, to hydrogen peroxide (H_2O_2). Myeloperoxidase, one further enzyme catalyzing hypochlorite formation using H_2O_2 and Cl^- as substrates is secreted into the extracellular medium when phagocytes are activated. Each of the mentioned active oxygen forms (such as H_2O_2, $^\bullet O_2^-$, and HOCl) can be a source of extremely reactive hydroxyl radical $^\bullet OH$ as a result of reaction (3) as well as reactions [25-27]:

$$HOCl + {}^\bullet O_2^- \rightarrow {}^\bullet OH + O_2 + Cl^-;$$

$$HOCl + Fe^{2+} \rightarrow {}^\bullet OH + Fe^{3+} + Cl^-.$$

The radical $^\bullet OH$ detaching the hydrogen atom from an unsaturated lipid (L) generates alkyl radical ($L^\bullet$). The radical $L^\bullet$ can initiate free-radical LP reactions, or, alternatively, can react with an oxygen molecule to form peroxyl radical ($LOO^\bullet$), from which, in turn, hydroperoxide (LOOH) originates. The hydroperoxide can be reduced by Fe^{2+} into alcoxyl radical ($LO^\bullet$), or, alternatively, it can be oxidized by hypochlorite into peroxyl radical. Both radicals give birth to free-radical chain LP. Thus, hypochlorite like divalent ferrous ions, through the conversion of hydroperoxide molecule into free radical (peroxyl in this case), can play an important role in the intensification of LP reactions on the stage of chain branching. This process can be of great pathophysiological importance, because oxidative modification of biological membranes and blood lipoproteins plays an important role in initiation of various pathological processes, particularly, oxidative lipoprotein modification can lead to the development of early stages of arteriosclerosis [28-30].

This study was supported by the Russian Foundation for Basic Research (grant No. 02-04-49276) and the Deutsche Forschungsgemeinschaft (grant AR 283/5).

REFERENCES

1. Klebanov, G. I., Vladimirov, Yu. A., Benov, L. C., and Ribarov, S. R. (1988) *Byul. Eksp. Biol. Med.*, **105**, 674-677.
2. Hiramatsu, K., Rosen, H., Heinecke, J. W., Wolfbaur, G., and Chait, A. (1987) *Arteriosclerosis*, **7**, 55-60.
3. Panasenko, O. M., Vol'nova, T. V., Osipov, A. N., Azizova, O. A., and Vladimirov, Yu. A. (1991) *Biomed. Sci.*, **1**, 581-589.
4. Scaccini, C., and Jialal, I. (1994) *Free Rad. Biol. Med.*, **16**, 49-55.
5. Evgina, S. A., Panasenko, O. M., Sergienko, V. I., and Vladimirov, Yu. A. (1992) *Biol. Membr. (Moscow)*, **9**, 946-953.
6. Stelmaszynska, T., Kukovetz, E., Egger, G., and Schaur, R. J. (1992) *Int. J. Biochem.*, **24**, 121-128.
7. Panasenko, O. M., Evgina, S. A., Aidyraliev, R. K., Sergienko, V. I., and Vladimirov, Yu. A. (1994) *Free Rad. Biol. Med.*, **16**, 143-148.
8. Panasenko, O. M., Arnhold, J., Schiller, J., Arnold, K., and Sergienko, V. I. (1994) *Biochim. Biophys. Acta*, **1215**, 259-266.
9. Panasenko, O. M., Evgina, S. A., Driomina, E. S., Sharov, V. S., Sergienko, V. I., and Vladimirov, Yu. A. (1995) *Free Rad. Biol. Med.*, **19**, 133-140.
10. Panasenko, O. M., and Arnhold, J. (1996) *Biol. Membr. (Moscow)*, **13**, 89-99.
11. Panasenko, O. M. (1997) *Biofactors*, **6**, 181-190.
12. Morris, J. C. (1966) *J. Phys. Chem.*, **70**, 3798-3805.
13. Panasenko, O. M., Arnhold, J., Vladimirov, Yu. A., and Sergienko, V. I. (1995) *Biochemistry (Moscow)*, **60**, 1079-1086.
14. Arnhold, J., Panasenko, O. M., Schiller, J., Arnold, K., Vladimirov, Yu. A., and Sergienko, V. I. (1996) *Z. Naturforsch.*, **51c**, 386-394.
15. Panasenko, O. M., Arnhold, J., Vladimirov, Y. A., Arnold, K., and Sergienko, V. I. (1997) *Free Rad. Res.*, **27**, 1-12.
16. Vladimirov, Yu. A., and Archakov, A. I. (1972) *Lipid Peroxidation in Biological Membranes* [in Russian], Nauka, Moscow.
17. Panasenko, O. M., Arnhold, J., and Schiller, J. (1997) *Biochemistry (Moscow)*, **62**, 951-959.
18. Kanofsky, J. R. (1989) *Chem.-Biol. Interact.*, **70**, 1-28.
19. Panasenko, O. M., and Arnhold, J. (1999) *Free Rad. Res.*, **30**, 479-487.
20. Vasil'ev, Yu. B., Sergienko, V. I., Grinberg, V. A., and Martynov, A. K. (1990) *Advances in Science and Technology. Electrochemistry and Medicine* [in Russian], Vol. 31, VINITI, Moscow, pp. 10-54.
21. Duling, D. R. (1994) *J. Magn. Reson.*, **B 104**, 105-110.

22. Osipov, A. I. (1983) *EPR Studies of Free Radicals Generated on Peroxidation*: Candidate's dissertation [in Russian], Moscow State University, Moscow.
23. Sies, H., Stahl, W., and Sundquist, A. R. (1992) *Ann. N. Y. Acad. Sci.*, **669**, 7-20.
24. Vladimirov, Yu. A., Olenev, V. I., Suslova, T. B., and Cheremisina, Z. P. (1980) *Adv. Lipid Res.*, **17**, 173-249.
25. Candeias, L. P., Patel, K. B., Stratford, M. R., and Wardman, P. (1993) *FEBS Lett.*, **333**, 151-153.
26. Candeias, L. P., Stratford, M. R., and Wardman, P. (1994) *Free Rad. Res.*, **20**, 241-249.
27. Osipov, A. N., Yakutova, E. Sh., and Vladimirov, Yu. A. (1993) *Biofizika*, **38**, 390-396.
28. Panasenko, O. M., and Sergienko, V. I. (1993) *Biol. Membr. (Moscow)*, **10**, 341-382.
29. Panasenko, O. M., Azizova, O. A., and Vladimirov, Yu. A. (1992) in *Soviet Medical Reviews. Section B: Physicochemical Aspects of Medicine Reviews* (Lopukhin, Yu. M., ed.) Harwood Academic Publishers, New York, Vol. 3, Pt. 3, pp. 1-74.
30. Berliner, J. A., and Heinecke, J. W. (1996) *Free Rad. Biol. Med.*, **20**, 707-727.