Study of Interaction between Hypochlorite Anion and Unilamellar Phosphatidylcholine Liposomes by UV Absorption Spectroscopy

I. N. Yakovenko, A, V. Stefanov, V. E. Formazyuk*, and V. I. Sergienko*

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Absorption of conjugated dienes from autoxidized unilamellar phosphatidylcholine liposomes in 145 mM NaCl and 10 mM Na^+,K^+-phosphate buffer (pH 7.0) decreased only snap increase in the concentration of $HOCl/ClO^-$ (pH 7.0) to 80 μM or more. Absorption of low-reactivity HO_2Cl/ClO_2^- increased during stepwise addition of $HOCl/ClO^-$ into the suspension to a lower final concentration (below 30 μM). These results can be explained by limited permeation of hydrophilic $HOCl/ClO^-$ into the phospholipid bilayer, ion-ion interaction of ClO^- with positively charged phosphatidylcholine heads, and catalysis of the following reaction: $2(HOCl/ClO^-) \rightarrow HO_2Cl/ClO_2^-$.

Key Words: *hypochlorite anion; hypochlorous acid; chloric acid; phosphatidylcholine liposomes; conjugated dienes*

Myeloperoxidase is involved in the synthesis of hypochlorous acid (HOCl), an important factor of the immune defense, or its ionized form hypochlorite anion (ClO^-): $H_2O_2+Cl^- \rightarrow ClO^-+H_2O$ [11]. Myeloperoxidase constitutes 5% neutrophil dry weight. Activated neutrophils secrete not only $HOCl/ClO^-$, but also myeloperoxidase (more than 20% cell level) [12] which is incorporated into biological membranes and damages them by triggering $HOCl/ClO^-$-induced free radical lipid peroxidation (LPO) [5]. $HOCl/ClO^-$ binds to double bonds of unsaturated fatty acids with the formation of chlorohydrin glycol (-CHCl-CHOH-) and oxidizes primary and secondary LPO products [4]. The interaction between $HOCl/ClO^-$ and lipids was studied by electron paramagnetic resonance and luminol-dependent chemiluminescence [4,5].

Here we performed UV spectroscopy study and determined the stoichiometry of interaction between $HOCl/ClO^-$ and lipid conjugated dienes (CD) of autoxidized phosphatidylcholine vesicles.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (EPC) [8] was dispersed in 65 mM Na^+,K^+-phosphate buffer (pH 7.0) by using a UZDN-2 sound disperser at 22 kHz for 15 min until suspension clarification. This indicated that the obtained suspension consists of spherical unilamellar vesicles with a molecular weight of 2×10^6 Da [13]. Studies were performed on liposomal suspensions autoxidized by long-term storage (50 mg/ml buffer for 6 months) at -8°C. Before the experiment, liposomes were diluted with 145 mM NaCl and 10 mM Na^+,K^+-phosphate buffer to a concentration of 0.2 mg/ml.

HOCl was obtained by chemical conversion of CaOCl in the presence of H_3BO_3 [3]. The concentration of HOCl was measured spectrophotometrically taking into account the molar extinction coefficient of ClO^- (pH 12) at 290 nm: $\Sigma_{290}=350\ M^{-1}\times cm^{-1}$ [10]. HOCl was neutralized with NaOH.

NaCl, $Na_2HPO_4\times2H_2O$ (Serva), CaOCl, H_3BO_3, and NaOH (Reakhim) were used. Spectrometry was

Institute of Pharmacology and Toxicology, Ukrainian Academy of Medical Sciences, Kiev; *Institute of Physicochemical Medicine, Russian Ministry of Health, Moscow.
Address for correspondence: vita@finim.msk.ru. Formazyuk V. E.

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performed in a thermostated cuvette using a Perkin Elmer-402 dual-beam spectrophotometer at 37°C. The results were analyzed by Origin 4.0 software.

RESULTS

The characteristic absorption spectrum of the suspension of autoxidized EPC liposomes (0.2 mg/ml) was characterized by elevated absorption of conjugated double bonds (CD) in lipids and carbonyl compounds at λ_{max} of about 232 and 278 nm, respectively (Fig. 1, *a*) [2]. UV absorption of LPO products was non detected 5 min after the addition of HOCl/ClO$^-$ (pH 7.0) to a final concentration of 0.8 mM (Fig. 1, *a*). Differential spectra (after subfraction of the initial absorption of liposome suspension) more accurately show the decrease in proper UV absorption of LPO products (Fig. 1, *b*).

It is known that the absorption of lipid CD (primary products of peroxidation of arachidonic, linoleic, and linoleneic fatty acids) [2,9,14] and polyenic aldehydes (CH_3-$(CH{=}CH)_n$-CHO, intermediate LPO products) with λ_{max} 220, 271, 315, 353, and 378 nm (n=1, 2, 3, 4, and 5, respectively) [1,2] reflects the intensity of free radical LPO processes. However, the absorption of CD partially overlaps a peak (apparent maximum near 205-210 nm) corresponding to nonoxidized lipids. Hence, the intensity of LPO is often estimated from the ratio between absorption maxima at 232 and 210 nm (Klein oxidation index) [9], or by the rise of absorption at 232 nm (for kinetic recordings). Decreased absorption of CD caused by HOCl/ClO$^-$ can provide the basis for a rapid quantitative assay of CD concentration in liposomes. Knowing the molar extinction coefficient for CD ($\Sigma_{232} \approx 2.5 \times 10^4$ $M^{-1} \times cm^{-1}$ [14]) and the maximum decrease in absorption at 232 nm (A232=0.25, Fig. 1, *a, b*), we conclude that the content of CD in liposomes is about 50 nM/mg lipid, i.e., each 25th phosphatidylcholine molecule contains conjugated double bonds.

Studies of the effect of low HOCl/ClO$^-$ concentration on liposomes showed that only certain critical concentration of HOCl/ClO$^-$ in the suspension eliminates UV absorbance corresponding to LPO products: not less than 0.08 mM for $\lambda_{max} \approx 232$ nm and about 0.5

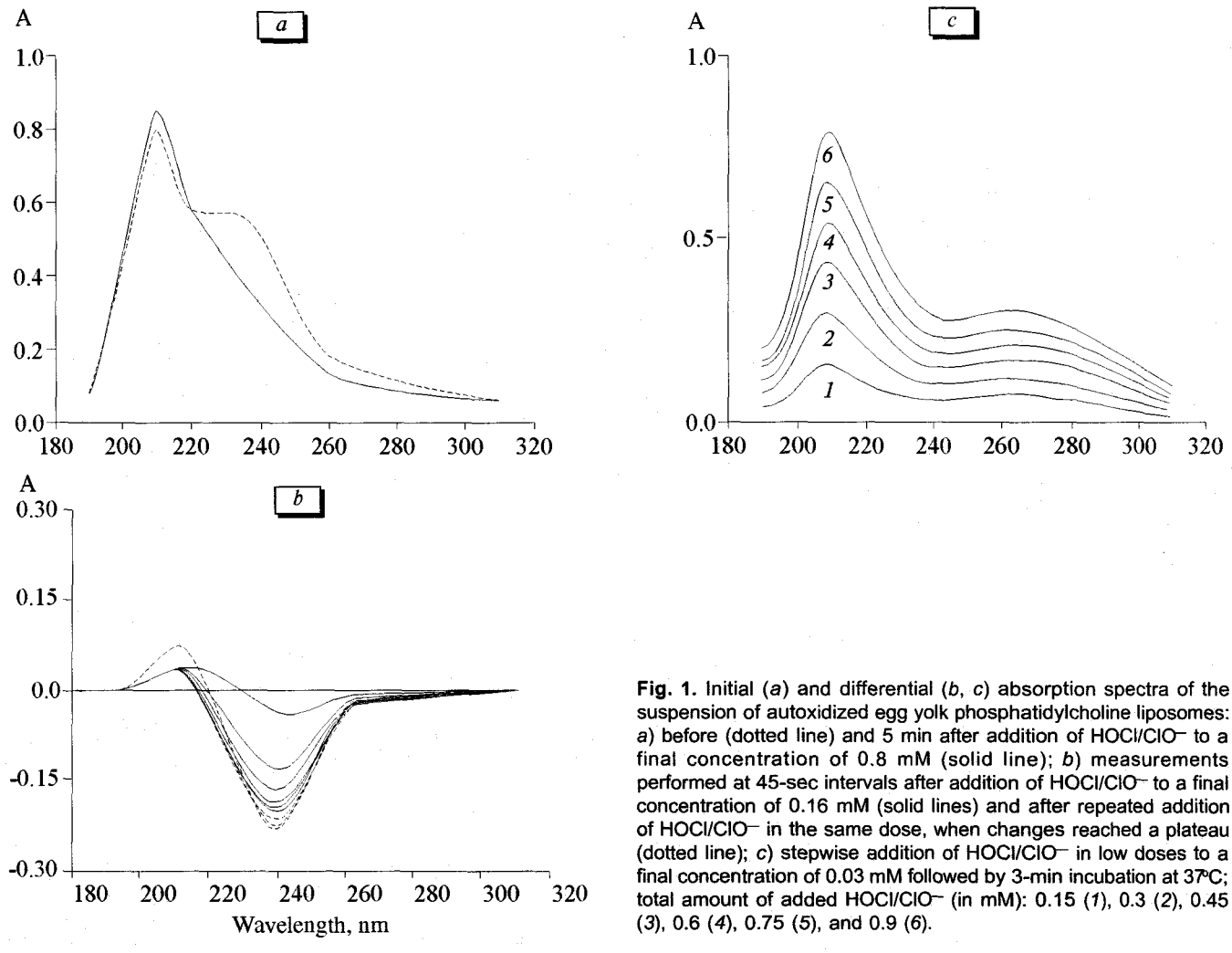

Fig. 1. Initial (*a*) and differential (*b*, *c*) absorption spectra of the suspension of autoxidized egg yolk phosphatidylcholine liposomes: *a*) before (dotted line) and 5 min after addition of HOCl/ClO$^-$ to a final concentration of 0.8 mM (solid line); *b*) measurements performed at 45-sec intervals after addition of HOCl/ClO$^-$ to a final concentration of 0.16 mM (solid lines) and after repeated addition of HOCl/ClO$^-$ in the same dose, when changes reached a plateau (dotted line); *c*) stepwise addition of HOCl/ClO$^-$ in low doses to a final concentration of 0.03 mM followed by 3-min incubation at 37°C; total amount of added HOCl/ClO$^-$ (in mM): 0.15 (*1*), 0.3 (*2*), 0.45 (*3*), 0.6 (*4*), 0.75 (*5*), and 0.9 (*6*).

mM for λ_{max}≈278 nm. Absorption of CD was not detected at a HOCl/ClO$^-$ concentration of 0.16 mM (Fig. 1, *b*); further increase in the content of HOCl/ClO$^-$ to 0.8 mM led to disappearance of absorption at λ_{max}≈ 278 nm (Fig. 1, *a*). Hence, the permeation of HOCl/ClO$^-$ into the lipid bilayer depends on the total content of hydrophilic HOCl/ClO$^-$ in the suspension.

The higher is the concentration of HOCl/ClO$^-$, the greater the probability of its permeation into the lipid bilayer. Addition of small amounts of HOCl/ClO$^-$ into the suspension to a final concentration of 30 μM did not decrease, but even increase absorption of CD at 260 nm. Addition of HOCl/ClO$^-$ into the liposomal suspension to a final concentration of 0.03 M followed by incubation at 37°C for 3 min (time sufficient to stabilize absorption) allowed us to calculate characteristic ClO_2^- spectra from differential UV spectra (after subfraction of the initial absorption of liposomes, Fig. 1, *c*). These data help to elucidate the metabolism of HOCl and ClO$^-$ in living tissues containing low amounts of HOCl/ClO$^-$. Previous studies showed that phosphatidylcholine heads are not oxidized with HOCl/ClO$^-$ compounds [4]. Conversion of HOCl/ClO$^-$ into HO_2Cl/ClO_2^- can be due to the hydrophilicity of HOCl/ClO$^-$ and ion-ion interaction of ClO$^-$ with positively charged phosphatidylcholine heads structured in the lipid bilayer and probably catalyzing the following reaction: 2(HOCl/ClO$^-$)→HO_2Cl/ClO_2^-. It is possible that HO_2Cl/ClO_2^- is also formed at relatively high concentrations of HOCl/ClO$^-$ sufficient for its permeation into the lipid bilayer (Figs. 1, *a*, *b*). However, this process produces no considerable effect on CD absorption due to lower molar absorption of oxidizers (HO_2Cl/ClO_2^-) compared with that of formed LPO products. In this case, the formation of HO_2Cl/ClO_2^- is probably reflected by the rise of absorption at 210 nm (Fig. 1, *a*, *b*).

In animals and humans, HOCl/ClO$^-$-generating myeloperoxidase and polar heads of phosphoglycerides in the membrane lipid bilayer are in peculiar interrelations. The ability of phosphatidylcholine vesicles to convert HOCl/ClO$^-$ into less toxic HO_2Cl/ClO_2^-, which unlike HOCl/ClO$^-$ does not initiate LPO [5], is of considerable importance, because direct intravenous infusions of HOCl/ClO$^-$ compounds are widely used in medical practice. Rapid infusion of these compounds can cause phlebitis and damage to protein-lipid structures in the body, while slow administration is not accompanied by *in vivo* accumulation of HOCl/ClO$^-$ in high concentrations in tissues and does not initiate LPO.

Catalysis of the reaction 2(HOCl/ClO$^-$)→HO_2Cl/ClO_2^- also contributes to positive effects of phosphatidylcholine vesicles in patients with diseases involving neutrophils [7].

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