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Interaction of Exogenous Hypochlorite or Hypochlorite Produced by Myeloperoxidase + H_2O_2 + Cl^- System with Unsaturated Phosphatidylcholines

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Abstract—The interaction between unsaturated phosphatidylcholines and either exogenous or endogenous (produced by the enzyme system involving myeloperoxidase (MPO), H_2O_2, and Cl^-) hypochlorite was studied in multilayer liposomes containing oleic, linoleic, and arachidonic acid residues using MALDI-TOF mass spectrometry. At pH 7.4, hypochlorite reacts with the double bond of the oleic acid residue in 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine producing oleic acid chlorohydrin as the main product. Minor amounts of glycols and epoxides were also detected. The main products of the reaction of hypochlorite with 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine were mono- and di-chlorohydrins of linoleic acid. The signals of monoglycol, epoxide, and glycol- or epoxide-containing monochlorohydrin derivatives were also present in the mass spectrum. The main products of the reaction of hypochlorite with 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine were lysophosphatidylcholine (1-stearoyl-*sn*-glycero-3-phosphocholine) and mono-, di-, and trichlorohydrin. Monoglycol and its derivatives containing one or two chlorohydrin groups were also detected. Along with those, carbonyl compounds (aldehyde and acid) formed as a result of double bond breakage in fifth position of arachidonate were detected. Monochlorohydrin was also found when liposomes comprising 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine were incubated in the presence of enzymatic mixture, MPO + H_2O_2 + Cl^-, at pH 6.0. In the absence of the enzyme or either of its substrates (H_2O_2 or Cl^-) or in the presence of the MPO inhibitor (sodium azide) or hypochlorite scavengers (taurine or methionine), monochlorohydrin formation was not observed. These data confirm the suggestion that just the hypochlorite generated in MPO-catalysis provides for chlorohydrin formation. Thus, the use of MALDI-TOF mass spectrometry has shown, along with chlorohydrins, glycols and epoxides as the products of hypochlorite interaction with unsaturated phosphatidylcholines at physiological pH. It was first determined that hypochlorite breaks double bonds in polyunsaturated phosphatidylcholine and also causes lysophosphatidylcholine formation.

Key words: hypochlorite, myeloperoxidase, phosphatidylcholine, unsaturated lipids, chlorohydrin, glycol, epoxide, lysophosphatidylcholine, MALDI-TOF (matrix assisted laser desorption/ionization time-of-flight) mass spectrometry

Myeloperoxidase (MPO; donor:H_2O_2-oxidoreductase; EC 1.11.1.7) is an enzyme contained in great amounts in neutrophils and monocytes (2-5 and about 0.9% wet weight, respectively) [1, 2]. When the cells become activated, some portion of the enzyme is secreted into the environment, in which MPO not only catalyzes one-electron oxidation of the classic peroxidase substrates, such as aromatic compounds, ascorbate, urate, or peroxides, but also catalyzes two-electron oxidation of chloride to hypochlorous acid (HOCl and its ionized form, hypochlorite, OCl^-)[1] in the following reaction [2, 3]:

$$H_2O_2 + Cl^- \rightarrow H_2O + HOCl. \quad (1)$$

HOCl/OCl^- is a strong oxidizer. It is believed to mediate the main bactericidal function of neutrophils [5,

Abbreviations: MPO) myeloperoxidase; HOCl/OCl^-) hypochlorite; MALDI-TOF (matrix assisted laser desorption/ionization time-of-flight)) mass spectrometry.

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[1] Because the p*K* of hypochlorous acid is 7.5 [4], hereafter and unless otherwise specified we shall use the term hypochlorite to mean the mixture of HOCl and OCl^- that are present together in the environment under study.

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6]. However, hypochlorite is known to express its cytotoxicity not only against bacterial cells, but also against fibroblasts, erythrocytes, platelets, endothelial, and other cells [7-11]. This occurs because of its explicit oxidative and chlorinating ability. $HOCl/OCl^-$ can interact with nucleic acids [12, 13], carbohydrates [14], amino acids [15, 16], proteins [17, 18], and lipids [19-21]. Those reactions are able to initiate *in vivo* nonspecific damage to protein–lipid complexes [22, 23], cells [7-11], and tissues [24] resulting in some cases in pathologic processes [25, 26].

Among possible molecular reactions of hypochlorite with unsaturated lipids, the most probable is a formation of chlorohydrins due to its interaction with double bonds [20, 21, 27-29]:

$$\text{R-HC} = \text{CH-R}_1 + \text{HOCl} \rightarrow \text{R-HC(HO)-CH(Cl)-R}_1, \quad (2)$$

that in alkaline medium undergo dehydrochlorination to form epoxides [30]. Epoxides in turn are quickly hydrolyzed to glycols:

$$\text{R-HC(HO)-CH(Cl)-R}_1 \xrightarrow{-\text{HCl}} \text{R-HC(O)-CH-R}_1 \xrightarrow{+\text{H}_2\text{O}} \text{R-HC(HO)-CH(OH)-R}_1. \quad (3)$$

However, these products (epoxides and glycols) were only found when hypochlorite interacted with an unsaturated bond of cholesterol [20, 31-33]. Alternatively, chlorohydrin isomers were only detected by the methods applied (such as NMR [28], HPLC [30], and various mass-spectrometry techniques [20, 21, 27, 29]) when hypochlorite reacted with aliphatic fatty-acid chains at physiological pH. Only when the pH of reaction mixture was beyond physiological values and approached 8.0, traces of epoxides and glycols began to appear [21, 30]. The methods specified above did not reveal products of free-radical phospholipid destruction; however, some studies show for both $HOCl/OCl^-$ and the system MPO + H_2O_2 + Cl^- induce, at least *in vitro*, peroxidation reactions in an unsaturated lipid [10, 19, 22, 23, 34-37].

With the aim to further investigate the mechanism of reaction between hypochlorite and unsaturated phospholipids, we used in this study a new mass-spectrometry technique, namely MALDI-TOF. As recently reported, this method might be successfully applied to the analysis of lipids [38-42] including phospholipids [38, 39, 41, 42]. In comparison to other methods, MALDI-TOF does not require a preliminary derivatization of substances under study, is simple in use, possesses high sensitivity and specificity, and can detect both lipids and their reaction products directly in an extract.

MATERIALS AND METHODS

Preparing of phospholipid liposomes. Multilayer liposomes were prepared from phosphatidylcholines (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine, or 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine) in 10 mM phosphate, pH 7.4 (or 6.0 in experiments with MPO), containing 0.14 M NaCl, by 30 sec deflocculation of their films formed after their solutions in chloroform were vacuum-evaporated.

Incubation of phospholipids with $HOCl/OCl^-$. Incubation of liposomes with hypochlorite was carried out in the medium containing 0.14 M NaCl and 10 mM phosphate, pH 7.4, at room temperature for 1, 5, 10, 20, 40, 60, 90, and 120 min so that all the incubations were terminated simultaneously. Concentrations of liposomes and hypochlorite are given for each the experiment in the legends for figures. On the incubation completion phospholipids were extracted with the chloroform–methanol (2 : 1 v/v) mixture. An aliquot of the organic phase was used for the MALDI-TOF analysis.

Sodium hypochlorite stock solution was kept in darkness at 4°C. Working solution was prepared immediately before the experiment. Its concentration was determined at pH 12 by spectrophotometry at 290 nm (ε_{290} = 350 $M^{-1} \cdot cm^{-1}$) [4].

Incubation of phospholipids with MPO + H_2O_2 + Cl^-. Liposomes composed by 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (the final concentration was 0.03 mg/ml) were incubated in 50 mM phosphate, pH 6.0, containing 0.14 M NaCl at room temperature for 60 min in the presence of myeloperoxidase (final concentration was 5 μg/ml) and hydrogen peroxide. The latter was added thrice of 0.1 mM 10 min apart. A series of control experiments was conducted in the absence of the enzyme or one of its substrates (H_2O_2 or Cl^-) and also in the presence of MPO inhibitor, sodium azide (10 mM), or hypochlorite scavengers, methionine (10 mM) or taurine (10 mM). After the incubation, phospholipid was extracted with a mixture of chloroform and methanol (2 : 1 v/v) and used for the MALDI-TOF analysis.

Analysis of lipids by MALDI-TOF mass spectrometry. The aliquot (10-50 μl) of organic extract of phospholipid was vacuum-evaporated. The matrix solution (5 μl of 0.5 M 2,5-dihydrobenzoic acid in methanol containing 0.1% trifluoroacetic acid) was added to the sample. The mixture thus prepared (1 μl) was placed onto the sample plate of mass the spectrometer and dried quickly with warm airflow. This procedure provides a homogeneous crystallization of the matrix with lipid resulting in increase in sensitivity and reproducibility of the method [38, 42].

MALDI-TOF measurements were conducted in positive ionization (cationization) mode on a Voyager Biospectrometry workstation (PerSeptive Biosystems,

USA) equipped with a pulse laser (beam wavelength 337 nm). The voltage used was 20,000 V. A signal was accumulated as averaged from 128 individual laser pulses. Relative molecular masses in the range of 450-1500 daltons were detected.

Mass spectrum of phosphatidylcholine (upper spectrum in Fig. 1) is a series of narrow lines, the densest one corresponds to the relative mass (M_r) of molecule composed by the major isotopes (^{12}C, ^{1}H, ^{16}O, ^{14}N, and ^{31}P) plus the mass of ion ionizing this molecule. In our experiments (positive ionization) either H^+ or Na^+ could play the role of ionizing cation, so the line position on the mass spectrum corresponds either $[M_r + H]^+$ or $[M_r + Na]^+$. At the right side of the densest line a trail of damping lines follows; these lines belong to the cationized phospholipid molecules composed not only by the major, but also minor isotopes (preferably ^{13}C and ^{2}H) [38].

Chemicals. The following chemicals were used in this study: 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine, and 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, USA); myeloperoxidase from human neutrophils (Planta GmbH, Austria); sodium hypochlorite (Sigma, USA); 2,5-dihydrobenzoic acid (Aldrich, USA); H_2O_2, methionine, taurine, sodium azide, trifluoroacetic acid, NaCl, $Na_2HPO_4 \cdot 2H_2O$, and all solvents were purchased from Fluka (Switzerland).

RESULTS

Interaction of hypochlorite with 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Figure 1 displays the mass spectra of extracts from liposomes composed of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and incubated with hypochlorite for different periods. The spectrum of intact phospholipid has two lines at 788.6 and 810.6 daltons belonging to the 1-stearoyl-2-oleoyl-*sn*-

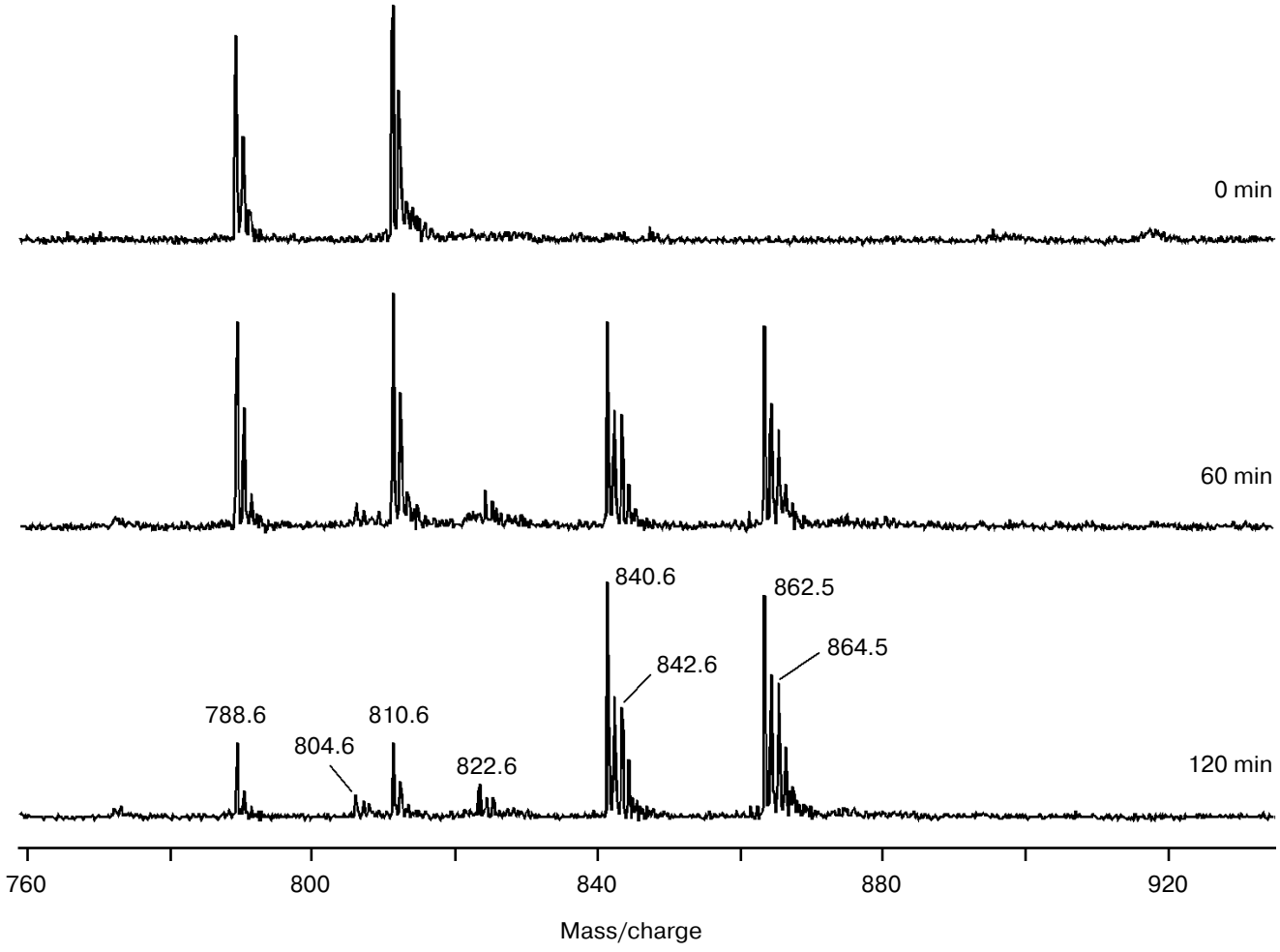

Fig. 1. Mass spectra of lipid extracts from liposomes composed of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine after incubation with hypochlorite. Incubation medium: 10 mM phosphate, pH 7.4, and 140 mM NaCl. Liposomes, 1.3 mM; hypochlorite, 6.5 mM. Temperature 23°C. Indices on the right of curves indicate the incubation terms.

glycero-3-phosphocholine molecules cationized with hydrogen $[M_r + H]^+$ and sodium $[M_r + Na]^+$, respectively. New peaks at 840.6 and 862.5 daltons appear due to the incubation with hypochlorite; these peaks correspond to H^+- and Na^+-adducts of the chlorohydrin produced (see reaction (2)). A specific isotopic distribution of lines also indicates the presence of chlorine in the molecule. The level of components with M_r 842.6 and 864.5 increases distinctly due to the substitution of ^{35}Cl by ^{37}Cl. This occurs because the portion of isotope ^{37}Cl is sufficiently high (24%).

Kinetics of $[M_r + H]^+$ signals for the initial 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and its chlorohydrin are shown in Fig. 2. As can be seen, throughout the incubation of liposomes with hypochlorite the native phospholipid disappears from the reaction mixture and chlorohydrin as the main product appears instead. However, minor peaks at 804.6 and 822.6 daltons (see Fig. 1) are also visible, which correspond to the H^+-adducts of epoxide and glycol formed in position of double bond of oleate according to reaction (3).

Interaction of hypochlorite with 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine. Multilayer liposomes composed by 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine were also incubated with hypochlorite. Figure 3 displays mass spectra of liposome extracts after the incubation. It is seen that the spectrum of intact phospholipid contains two components with M_r 786.6 and 808.6 daltons belonging to the cationized molecules of 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine ($[M_r + H]^+$ and $[M_r + Na]^+$, respectively). The incubation with hypochlorite leads to an appearance of additional lines in the mass spectrum; the densest ones are the lines with masses of 838.5, 860.5, 890.5, and 912.5 daltons. The first two belong to the H^+- and Na^+-adducts of monochlorohydrin. The second two belong to the same adducts of dichlorohydrin, that is when two double bonds are present in the fatty acid chain, both are subjected to the hypochlorite attack.

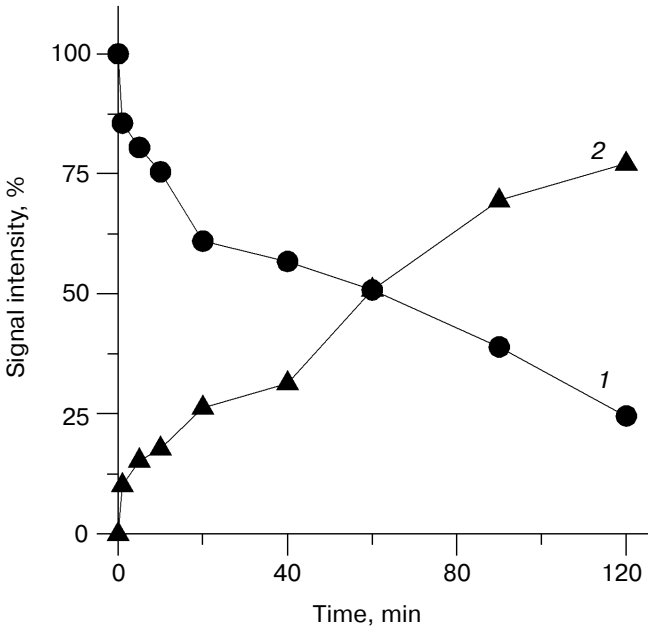

Fig. 2. Changes in the intensity of mass-spectrum signals of protonated 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (*1*) with molecular mass 788.6 daltons (see Fig. 1) and its chlorohydrin (*2*) with molecular mass 840.6 daltons (see Fig. 1) in the course of incubation of phosphatidylcholine-composed liposomes with hypochlorite. The incubation conditions are the same as in the Fig. 1.

Figure 4 shows the kinetic curves of 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine decrease and its mono- and dichlorohydrin production during the incubation of liposomes with hypochlorite. As can be seen, the first minutes are characterized by the fast decrease in the initial phospholipid level (Fig. 4, curve *1*) and a monochlorohydrin production (Fig. 4, curve *2*). About 10 min later dichlorohydrin begins to be also detected (Fig. 4, curve *3*). The level of the latter increases with the passage of time, which is characterized by an increase in signal intensities at 890.5 and 912.5 daltons (see Fig. 3).

Along with this, signals at 820.6 and 872.5 daltons are distinctly visible in the mass spectrum (Fig. 3); these belong, respectively, to the proton adducts of glycol and to a derivative in which molecule chlorohydrin and glycol are present simultaneously. Minor peaks at 802.6 and 854.5 daltons correspond, respectively, to monoepoxide and to a derivative containing both epoxide and chlorohydrin.

Like 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, a specific isotopic distribution is observed for the peaks belonging to the chlorous derivatives of 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine, which is characterized by increased density of the line corresponding to an isotope with mass 2 units exceeding that of the major isotope on the substitution of ^{35}Cl by ^{37}Cl. It is notably obvious for derivatives containing two chlorine atoms, namely dichlorohydrins (Fig. 3, the peaks at 892.5 and 914.5 daltons).

Interaction of hypochlorite with 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine. The same experiments were performed on liposomes composed by 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine. Figure 5 displays the mass spectra of phospholipid extracts prepared just before and 20 and 60 min after the beginning of incubation of liposomes with hypochlorite. The intact phospholipid is characterized by two peaks at 810.6 and 832.6 daltons corresponding to its adducts with proton and Na^+. As follows from Fig. 6, the fast decrease of initial phospholipid is observed just in the beginning of incubation (Fig. 6, curve *1*) with monochlorohydrin formation (Fig. 6, curve *3*). The density of peaks belonging to monochlorohydrin (862.5 and 884.5 daltons for the adducts with H^+ and Na^+, respectively) decreases for long

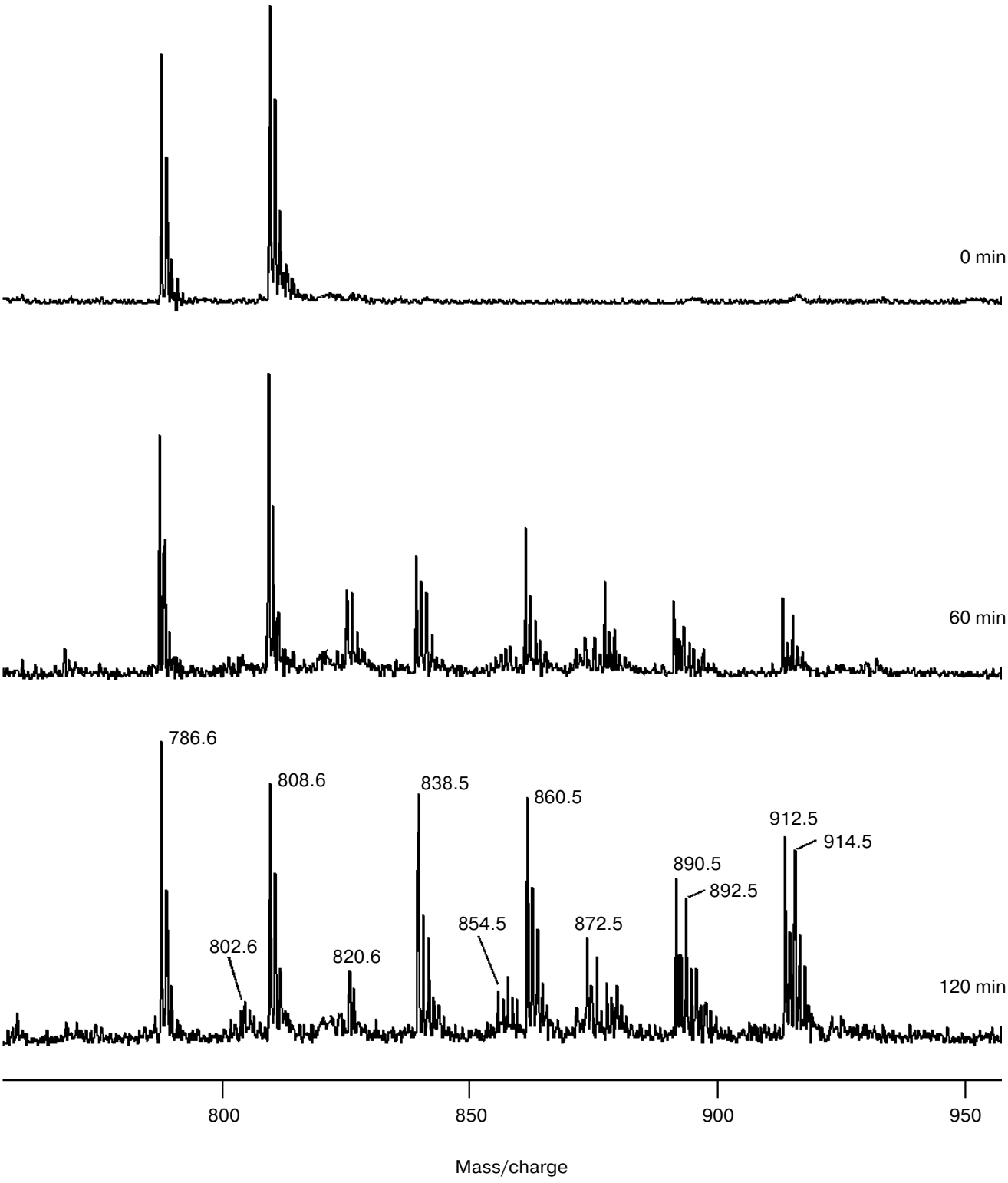

Fig. 3. Mass spectra of lipid extract from liposomes composed of 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine after their incubation with hypochlorite. The incubation medium: 10 mM phosphate, pH 7.4, containing 140 mM NaCl. Liposomes, 1.3 mM; hypochlorite, 6.5 mM. Temperature was 23°C. Indices on the right of curves indicate the incubation terms.

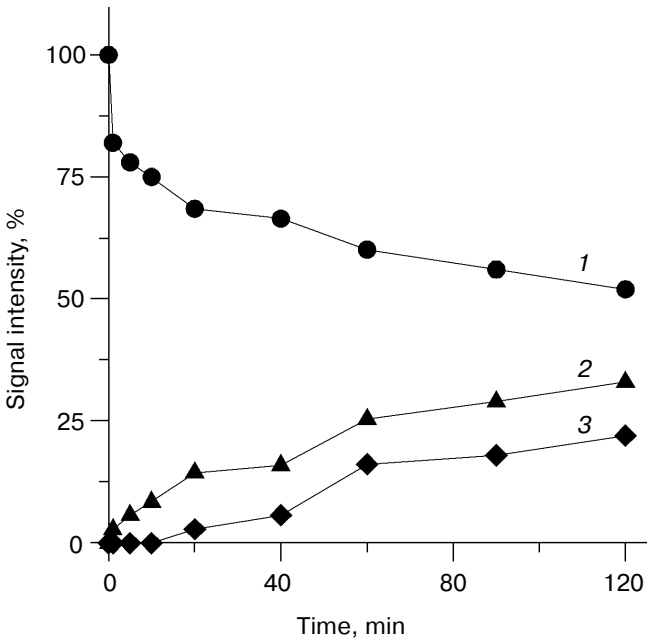

Fig. 4. Changes in the intensity of mass-spectrum signals of protonated 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine (*1*) with molecular mass 786.6 daltons (see Fig. 3), its monochlorohydrin (*2*) with molecular mass 838.5 daltons (see Fig. 3), and dichlorohydrin (*3*) with molecular mass 890.5 daltons (see Fig. 3) in the course of incubation of phosphatidylcholine-composed liposomes with hypochlorite. The incubation conditions were the same as in Fig. 3.

incubation periods, thus indicating that monochlorohydrin virtually disappears from the reaction mixture after 120 min incubation (Fig. 6, curve *3*). This takes place because monochlorohydrins of arachidonate react with hypochlorite to form other derivatives. After 60 min incubation of liposomes with hypochlorite a set of peaks of relatively low intensity is visible in the mass spectrum of the lipid extract, among which are the peaks belonging to dichlorohydrins (914.5 and 936.5 daltons for the adducts of H^+ and Na^+, respectively) and trichlorohydrins (966.5 daltons for the adduct of H^+). Besides, there are the components of H^+-adduct of monoglycol (844.6 daltons) and derivatives containing, along with glycol, mono- (896.5 daltons for the H^+-adduct) and dichlorohydrin (948.5 daltons for the H^+-adduct).

Surprisingly, low-molecular-weight products of the reaction between hypochlorite and 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine were detected (Fig. 5). A large peak at 524.4 daltons was identified as one belonging to the H^+-adduct of lysophosphatidylcholine (1-stearoyl-glycero-3-phosphocholine). The peak at 546.4 daltons belongs to its Na^+-adduct.

Figure 6 (curve *2*) shows kinetics of the signal level at 524.4 daltons in the course of incubation of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine with hypochlorite. In is seen that lysophosphatidylcholine appears in first minutes of the incubation and further accumulates quickly to dominate over all reaction products after 60 min (see Fig. 5).

The signals detected at 622.4, 638.4, and 660.4 daltons suggest the breakage of fatty-acid chain of arachidonate under the action of hypochlorite. The first of them seems to correspond to H^+-adduct of aldehyde formed as a result of arachidonate chain breakage in position 5. The other two belong, respectively, to H^+- and Na^+-adducts of the acid produced from the oxidation of this aldehyde by hypochlorite.

It should be mentioned that so significant amounts of low-molecular-weight products of phosphatidylcholine decomposition were detected only when liposomes composed of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine interacted with hypochlorite. No products of fatty-acid chain breakage were detected both in the case of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine. However, some minor components were present in the mass spectra indicating the presence of trace amounts of corresponding lysophosphatidylcholines (data not shown).

Interaction of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine with the system MPO + H_2O_2 + Cl^-. We also used MALDI-TOF mass spectrometry to study the interaction of the system MPO + H_2O_2 + Cl^- that is known to produce hypochlorite *in vivo* [2, 3, 6] with liposomes composed by 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. The mass spectra of lipid extracts prepared after 60 min incubation are given in Fig. 7. In the absence of MPO (spectrum *1*), two peaks at 788.6 and 810.6 daltons are visible, which belong, respectively, to H^+- and Na^+-adducts of intact 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. The incubation of liposomes for 60 min in the presence of MPO and both its substrates (H_2O_2 and Cl^-) resulted in two new peaks at 840.6 and 862.5 daltons in the mass spectrum (Fig. 7, spectrum *2*), in a similar way as it was observed after the incubation of liposomes composed by 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine with hypochlorite (see Fig. 1). The new signals belong to chlorohydrin ($[M_r + H]^+$ and $[M_r + Na]^+$, respectively). When at least one substrate was absent, we did not observed any signal at 840.6 and 862.5 daltons in the mass spectrum. Figure 7 displays the spectrum (spectrum *3*) detected after the liposomes were incubated with MPO in the absence of chloride. The same result was achieved in the lack of hydrogen peroxide in the incubation medium (data not shown). When either the hypochlorite scavengers (taurine or methionine) (Fig. 7, spectrum *4*; data are presented only for taurine) or MPO inhibitor (sodium azide) (Fig. 7, spectrum *5*) were added to the incubation medium containing the system MPO + H_2O_2 + Cl^-, chlorohydrin production was also inhibited completely.

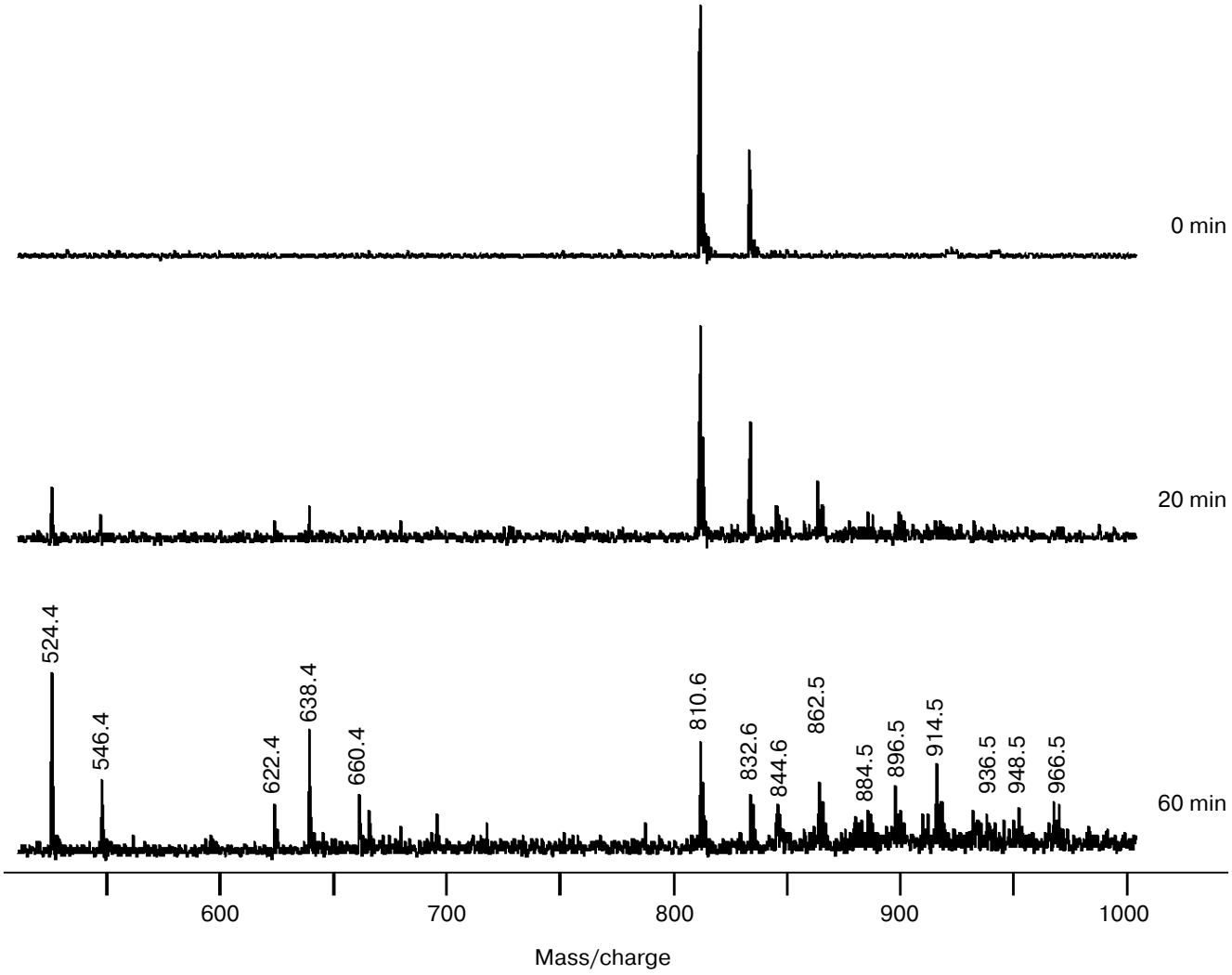

Fig. 5. Mass spectra of lipid extract from liposomes composed by 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine after their incubation with hypochlorite. The incubation medium: 10 mM phosphate, pH 7.4, containing 140 mM NaCl. Liposomes, 2.6 mM; hypochlorite, 25 mM. Temperature was 23°C. Indices on the right of curves indicate the incubation terms.

These data indicate that hypochlorite, both exogenous and produced by the system MPO + H_2O_2 + Cl^-, can react with unsaturated bonds of acyl chains of phospholipids comprising membrane structures.

DISCUSSION

The main biological targets for hypochlorite are commonly supposed to be SH- and NH_2-groups of amino acid residues, tryptophan, and some other low-molecular-weight substances, such as ascorbate, urate, and NADH [15, 16, 43, 44]. Hypochlorite also oxidizes and chlorinates *in vivo* many other functional groups; however, the rate constants of its reactions with the named biologically important compounds have the greatest values (10^4 $M^{-1}\cdot sec^{-1}$ and higher) [16, 45, 46]. For comparison, the rate constant of the reaction between hypochlorite and unsaturated bonds of acyl chains of phospholipids comprising liposomes is as low as 0.5 $M^{-1}\cdot sec^{-1}$ [47]. However, the double bond concentration is high enough in an unsaturated lipid. It is a possible reason by which the reaction of hypochlorite with unsaturated bonds of fatty acid chains occurs not only *in vitro* [20, 21, 27-29], but *in vivo* too [30]. Up to now, various chlorohydrin isomers were only detected as the products of this reaction at physiological pH values [20, 21, 27-30]. And only when the reaction medium was alkaline, epoxides and glycols were observed [21, 30].

Using highly sensitive MALDI-TOF mass spectrometry technique, we first succeeded in detecting at pH 7.4, along with chlorohydrins, also glycols and epoxides as the

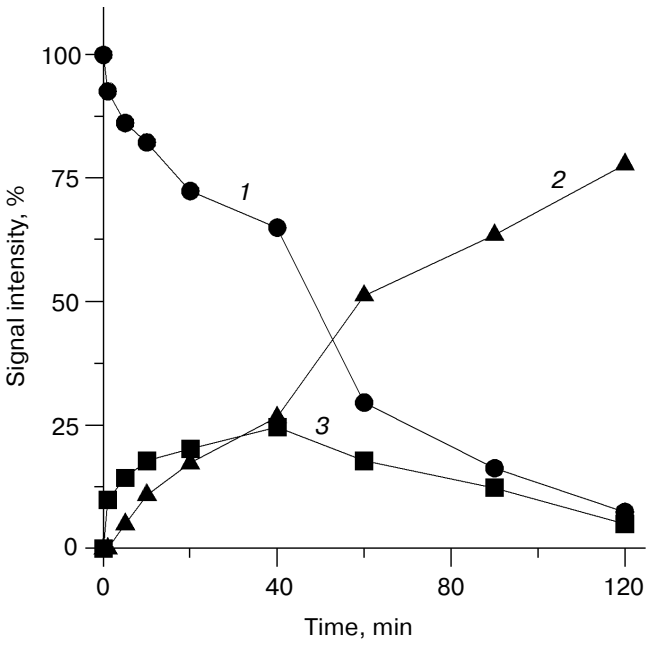

Fig. 6. Changes in the intensity of mass-spectrum signals of protonated 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (*1*) with molecular mass 810.6 daltons (see Fig. 5), its lysophosphatidylcholine (*2*) with molecular mass 524.4 daltons (see Fig. 5), and monochlorohydrin (*3*) with molecular mass 862.5 daltons (see Fig. 5) in the course of incubation of phosphatidylcholine-composed liposomes with hypochlorite. The incubation conditions were the same as in Fig. 5.

products of the reaction of hypochlorite with unsaturated phosphatidylcholines. In particular, chlorohydrin was apparently the main product of the reaction between hypochlorite and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine carrying a single unsaturated bond (signals corresponding to 840.6 and 862.5 daltons in Fig. 1). However, the signals belonging to glycol and epoxide (822.6 and 804.6 daltons, respectively, see Fig. 1) were also detected. So, the order of reactions might be as follows. Initially, hypochlorite forms chlorohydrin by the mechanism of electrophilic addition to a double bond (see reaction equation (2)) [28, 48]. Then the chlorohydrin formed transforms into epoxide due to the reaction of dehydrochlorination. This reaction is apparently a limiting step of the whole process, because it requires an alkaline medium [21, 30]. Additional evidence is that in the mass spectrum a signal belonging to epoxides is of minimum intensity (see Fig. 1). Then in the water solution epoxide, according to the Eq. (3), is hydrolyzed quickly to form a relatively stable glycol.

As for 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine that contains two unsaturated bonds, dichlorohydrins (890.5 and 912.5 daltons, Fig. 3) along with monochlorohydrins (838.5 and 860.5 daltons) were found as the main products of its reaction with hypochlorite. The mass spectrum presented in Fig. 3 also contains the signals of monoglycol (820.6 daltons), epoxide (802.6 daltons), and glycol- or epoxide-containing derivatives of monochlorohydrin (872.5 and 854.5 daltons, respectively). A possible scheme of chemical processes that lead to formation of the above mentioned products in the reaction between hypochlorite and 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine is shown in the Fig. 8. Note that the MALDI-TOF technique does not allow isomers of the same relative molecular mass to be distinguished. So, in Fig. 8 single isomers are only presented as examples.

A diverse set of products was found when we incubated hypochlorite with liposomes composed of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine. In this case, we found, along with mono- (862.5 and 884.5 daltons) and dichlorohydrins (914.5 and 936.5 daltons), also trichlorohydrins (966.5 daltons) as well. The mass spectrum also contained the signals belonging to monoglycol (844.6 daltons) and glycol-containing derivatives of mono- and dichlorohydrin (896.5 and 948.5 daltons, respectively). Because arachidonate has four unsaturated bonds, a diversity of products is formed. It results in significant reduction in peak intensity in the mass spectrum. And because chlorine atoms are abundantly present in the products, the isotopic distribution increases and the spectral lines are broadened. Both facts complicate the detection and identification of minor products. This is a possible reason why we were not able to detect epoxides among the products. One should expect that tetrachlorohydrins and their glycol and epoxide derivatives would be produced in a great excess of hypochlorite.

Particular attention should be directed to the fact that substances with molecular masses less than that of initial phosphatidylcholine were detected among the products of reaction between hypochlorite and 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine. Hypochlorite apparently causes 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine decomposition by two independent mechanisms. The first involves an aldehyde production due to the double bond cleavage in position 5 of arachidonate; this aldehyde is then oxidized by hypochlorite to form an acid (see a scheme on the Fig. 9). In the mass spectrum, the signal corresponding to 622.4 daltons ($[M_r + H]^+$) belongs to the aldehyde and the signals corresponding to 638.4 and 660.4 daltons (H^+- and Na^+-adducts, respectively) belong to the acid (see Fig. 5). It is unclear what mechanism provides the arachidonate bond cleavage. However, many reports [22, 23, 34-37, 47-50] suggest that both hypochlorite and the system MPO + H_2O_2 + Cl^- initiate peroxidation reactions in an unsaturated lipid, so the idea is that the bond cleavage in position 5 of arachidonate might result from those free-radical reactions.

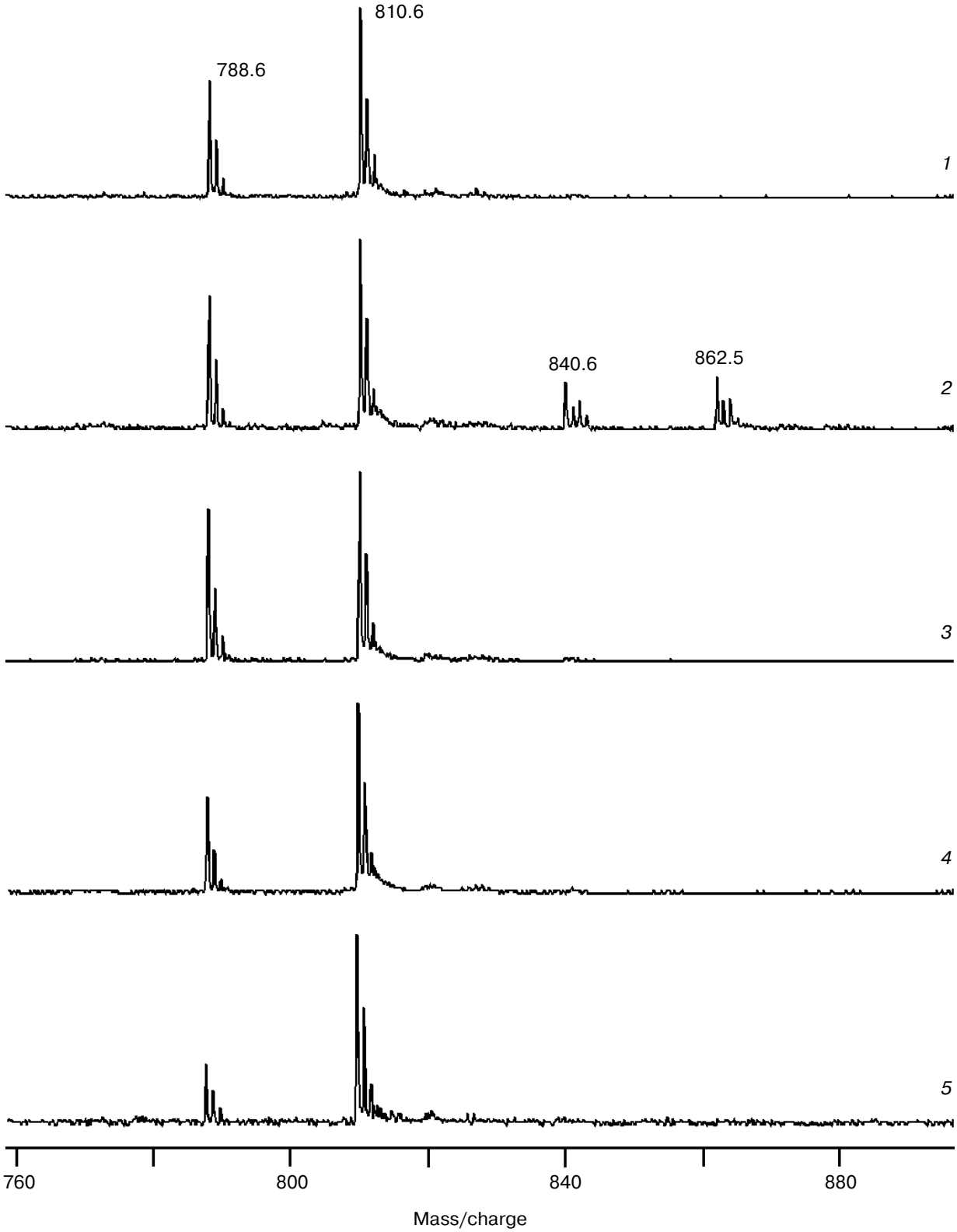

Fig. 7. Mass spectra of lipid extract from liposomes composed by 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine after 60-min incubation in the absence (*1*) or presence (*2-5*) of the system MPO + H_2O_2 + Cl^-. The incubation medium: 50 mM phosphate, pH 6.0, containing 140 mM NaCl. Liposome concentration was 0.03 mg/ml. H_2O_2 was added thrice by 0.1 mM at intervals of 10 min. Temperature was 23°C. *1*) Liposomes; *2*) liposomes + MPO + H_2O_2 + Cl^-; *3*) the same as *2*, but without Cl^-; *4*) the same as *2* + 10 mM taurine; *5*) the same as *2* + 10 mM sodium azide.

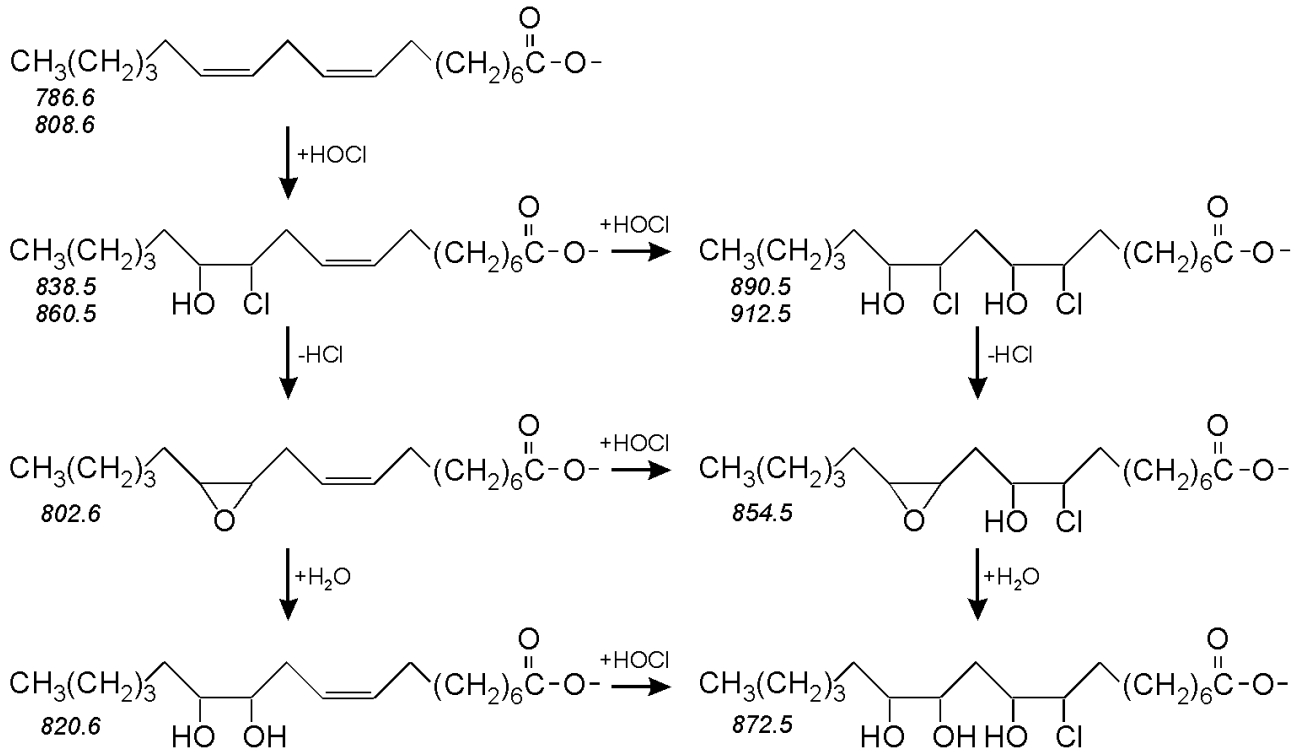

Fig. 8. Scheme of chemical transformations of linoleate comprising 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine in the reaction with hypochlorite. The indexes near the formulae are the molecular masses of corresponding substances in the mass spectrum shown in Fig. 3. The upper (or single) index corresponds to the mass of a molecule cationized by a proton, the lower one corresponds to that cationized by a sodium ion.

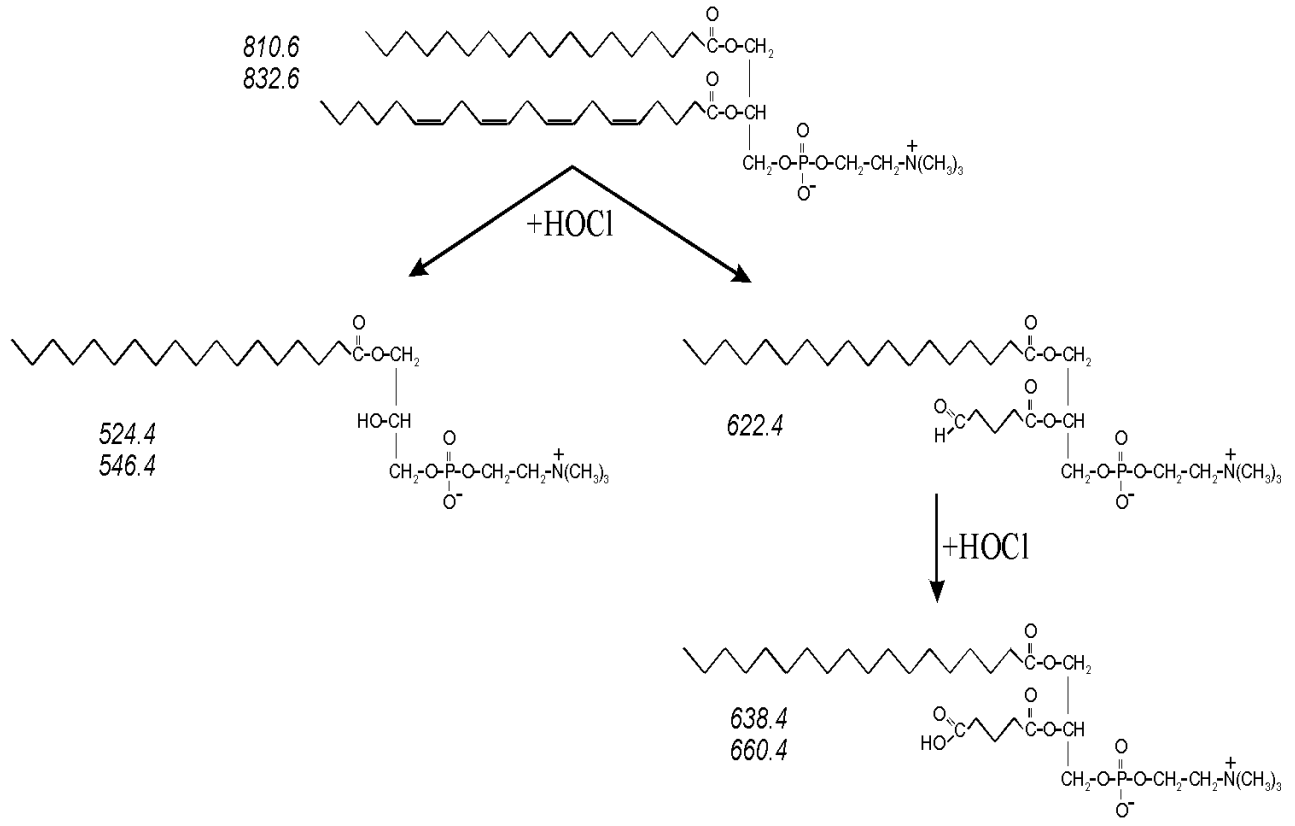

Fig. 9. Scheme of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine decomposition under the action of hypochlorite. The indices near the formulae are the molecular masses of corresponding substances in the mass spectrum shown in Fig. 5. The upper (or single) index corresponds to the mass of molecule cationized by proton and the lower one corresponds to that cationized by sodium ion.

The second mechanism results in lysophosphatidylcholine production (see scheme in Fig. 9). It should be mentioned that minor amounts of lysophospholipids were also produced, when hypochlorite interacted with 1-stearoyl-2-oleoyl- or 1-stearoyl-2-linoleyl-*sn*-glycero-3-phosphocholine. As for the reaction of hypochlorite with 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (see Figs. 5 and 6), lysophospholipid became the main product by the 60th min of incubation. So, hypochlorite can play the role of phospholipase A_2 and convert phosphatidylcholine into lysophosphatidylcholine. This reaction might be of great biological importance because lysophosphatidylcholine is known to control various pathophysiological processes. In particular, lysophosphatidylcholine as one of the main components of oxidized low density lipoproteins induces expression of macrophage- and smooth muscle-derived growth factor [51], stimulates release of adhesive molecules for monocytes and T cells [52], and inhibits both expression of inducible NO-synthase [53] and endothelium-dependent blood vessel relaxation [54], thus providing for the development of atherosclerosis.

The data of our present study suggest that hypochlorite produced by activated neutrophils and macrophages can induce atherosclerosis development not only because it oxidizes low density lipoproteins [10, 22, 23, 55] and they, as a result, begin to undergo an intensive uptake via the scavenger receptors of macrophages and to transform them into foam cells [55, 56], but also because it converts phosphatidylcholine into lysophosphatidylcholine, the known inducer of atherosclerotic blood vessel degeneration.

The experiments have demonstrated that chlorohydrin is produced when liposomes composed of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine are incubated with myeloperoxidase in the presence of both H_2O_2 and Cl^-. Because chlorohydrin production was inhibited completely in the absence of either of the substrates (H_2O_2 or Cl^-) as well as in the presence of MPO inhibitor (sodium azide) or hypochlorite scavengers (taurine or methionine), it seems that it is MPO-produced hypochlorite that causes chlorohydrin production.

In summary, it can be said that the use of MALDI-TOF mass spectrometry technique allows detection, along with chlorohydrins, also glycols and epoxides as the products of the reaction between hypochlorite and unsaturated phosphatidylcholines at physiological pH values. We have shown that hypochlorite causes the cleavage of the double bond in polyunsaturated phosphatidylcholine and leads to lysophosphatidylcholine formation.

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