The Reactions Between HOCl and Differently Saturated Phospholipids: Physiological Relevance, Products and Methods of Evaluation

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Abstract: Phospholipids (PLs) are important constituents of cellular membranes. HOCl is a reactive oxygen species (ROS) generated under inflammatory conditions and capable of reacting with (a) the head group of PLs and (b) the olefinic groups of their fatty acyl residues under the preferred generation of chloramines and chlorohydrines, respectively.

This review discusses these products, their stabilities and *in vivo* relevance. Additionally, important methods (spectroscopic as well as mass spectrometric techniques) to analyze these products are compared.

1. INTRODUCTION

The focus of this paper is the discussion of the reactions between HOCl, an important reactive oxygen species (ROS), and lipids, in particular phospholipids (PLs). PLs occur in large amounts in all cellular membranes but the contribution of the individual PL classes as well as their fatty acyl compositions depend strongly on the species (e.g. human or animal) and the cell type. Additionally, PLs are also major constituents of the lipoproteins of the human blood that are very important for the transport of (apolar) lipids in an aqueous environment. There is large evidence that changes of lipoprotein compositions are responsible for atherosclerotic lesions [1,2].

We will not focus on structural aspects of lipids and their abundance in the human body, but will instead discuss the generation of HOCl, as well as its reactivity with lipids [3].

2. GENERATION OF HOCl, PROPERTIES AND REACTIVITIES

HOCl is generated under *in vivo* conditions from hydrogen peroxide and chloride ions catalyzed by the enzyme myeloperoxidase (MPO) [4].

$$H_2O_2 + Cl^- \rightarrow HOCl + HO^-$$

MPO constitutes about 5% of the total protein mass [4] of neutrophilic granulocytes, important cells of the immune system, and MPO is also present in minor amounts in macrophages. As the number of neutrophilic granulocytes increases massively under inflammatory conditions, the role of MPO and its products are obvious [3]. ROS and particularly HOCl are nowadays assumed to be massively involved in the pathomechanisms of different inflammatory diseases, including atherosclerosis [1], rheumatoid arthritis and cartilage degradation [5], lung infection [6], and many others [3].

HOCl is synthesized technically by introducing chlorine gas to a solution of sodium hydroxide in the cold:

$$Cl_2 + 2\ NaOH \rightarrow NaOCl + NaCl + H_2O$$

HOCl is usually sold as hypochlorite solution because the stability of NaOCl is much higher in comparison to the free acid. However, NaOCl may be easily converted into HOCl by altering the pH as the pk_a value of HOCl is 7.53 [7]. Therefore, there is nearly a 1:1 ratio between NaOCl and HOCl at physiological pH (7.4). This makes the question whether HOCl or NaOCl is the relevant reactive species under *in vivo* conditions less important.

HOCl reacts with virtually all physiologically-relevant molecules, e.g. with proteins, nucleic acids, selected carbohydrates and lipids [8]. Due to its strongly oxidizing and chlorinating ability, hypochlorites are also important constituents of many cleaners or bleachers of the household [9]. Such cleaners are also widely used in hospitals as disinfectants, i.e. as "killers" of bacteria, for instance, *Escherichia coli* [10].

3. THE LARGE VARIABILITY OF LIPIDS

Although lipids are also important for nutrition, the prime importance of lipids is coming from their pivotal role in the cellular membrane. The typical bilayer structure of the membrane is warranted by their high content of amphiphilic lipids [11] (Fig. (**1**)), particularly by phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and cholesterol. In contrast, apolar lipids like cholesteryl esters and triacylglycerols are only minor constituents of the cell membrane, but occur in vast amounts in the lipoproteins of blood [12].

It is important to note that the structural variability of lipids is stemming from differences in the fatty acyl compositions, the different linkage types between the fatty acyl residues and the glycerol backbone (diacyl-, alkyl-acyl- and alkenyl-acyl-) and, finally, the structure of the headgroup. The resulting broad lipid spectrum has hampered detailed lipid analysis over a long time.

Nowadays, however, due to the considerable progress in analytical sciences (in particular regarding mass spectrometry), lipids may be more easily analyzed. Accordingly, "lipidomics" studies are now widely performed [13].

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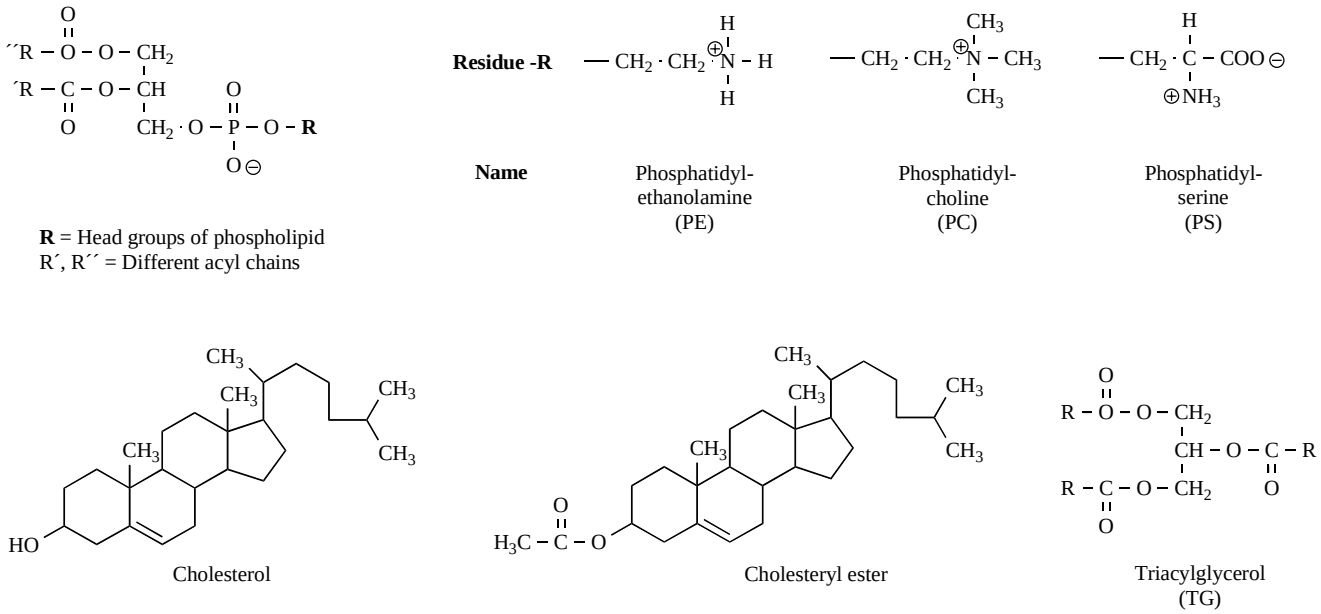


Fig. (1). Structures of selected phospholipids and lipids. Please note that in addition to the headgroup also the linkage between the glycerol and the fatty acyl residues may vary significantly.

4. REACTIONS BETWEEN HOCl AND DIFFERENT LIPID CLASSES

In the next paragraphs some important lipid classes and their reactions with HOCl will be discussed. Due to their importance, the focus will be on PLs present in the cellular membrane.

4.1. Apolar Lipids (Cholesterol, Cholesteryl Esters And Triacylglycerols)

Although even more abundant than PLs, the reactivity of apolar lipids with HOCl was by far less detailed investigated. This is simply caused by a biophysical problem: Apolar lipids do not form a well-defined bilayer structure in water because they are lacking the polar moiety. Therefore, it is much more difficult to obtain significant yields of products because a considerable amount of lipid cannot react with HOCl.

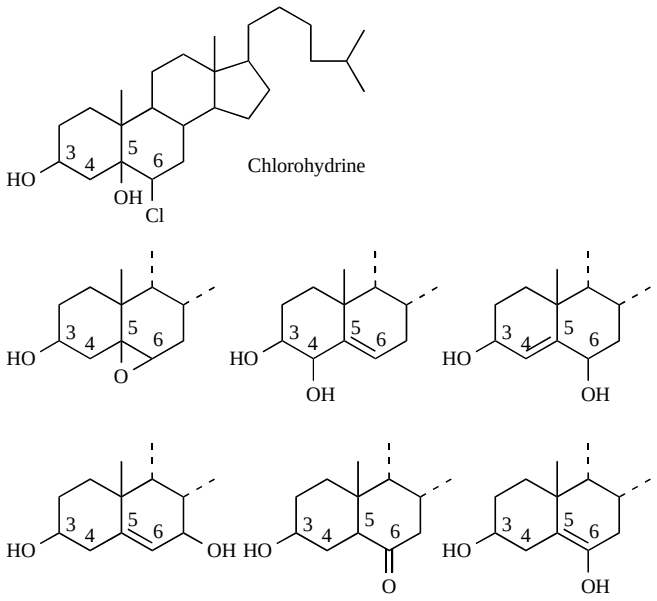


Fig. (2). Proposed structures of reaction products between HOCl and cholesterol.

One possibility to overcome this problem is to entrap a small amount of the apolar lipid in a bilayer of PLs that do not react with HOCl, e.g. a completely saturated PC. Using this approach it could be shown [14] that cholesterol does not give the expected addition products, the chlorohydrins. Instead, the main oxidation products were identified as the epimeric cholesterol 5,6-epoxides and 4-hydroxycholesterol, accompanied by several other hydroxy- and keto-derivatives in smaller yields (cf. Fig. (**2**)).

In contrast, it could be shown in a later study that the reaction of HOCl with cholesterol in a purified liposome system gives the expected α- and β-chlorohydrins [15]. The discrepancy between both studies could be explained by the different applied MS methods: If product analysis is performed by conventional EI MS, chlorohydrins cannot be detected because they are readily converted into their epoxides. This problem may be overcome by using soft-ionization MS (e.g. ESI MS) and will be discussed below in more detail. Again somewhat later, the formation of 6-α-chloro-5-β-cholestane-3-β-5-diol could be clearly proven by NMR spectroscopy [16].

In addition to model systems, cholesterol chlorohydrin formation could also be established on a cellular level: If intact human cells, for instance, red blood cells are exposed to HOCl, cholesterol chlorohydrins are formed. It is nowadays assumed that the formation of cholesterol chlorohydrins is disruptive to cell membranes and destroys the cell by a biophysical mechanism: Winterbourn *et al.* [17] proposed that chlorohydrins, if formed in cell membranes, cause disruption to membrane structures because chlorohydrins are more polar than the parent fatty acyl residues. Therefore they

might be significant in neutrophil-mediated cytotoxicity. Somewhat later it has also been shown that under the influence of HOCl membrane pores are formed in red blood cells [18].

Results consistent with a perturbation of bilayer packing have been also obtained by Drobnies *et al.* [19]: CTP-phosphocholine cytidyltransferase, an integral membrane enzyme which catalyzes a regulatory step in mammalian biosynthesis of phosphocholine, is activated by hypochlorite-oxidized lipids.

4.2. Phosphatidylcholine (PC)

PCs are the so far most established model system to study the reactions between lipids and HOCl from the following four reasons:

1. PCs form well characterized lipid bilayers in water
2. PCs are commercially available with strongly varying fatty acyl residues
3. Exclusively the (unsaturated) fatty acyl residues of PCs react with HOCl, whereas the headgroup is completely inert. Therefore, PCs yield a relatively simple product spectrum.
4. PCs are the most abundant constituents of animal and human cellular membranes.

4.2.1. Generation of Chlorohydrins and Lysolipids

The reaction between the reagent HOCl [20] as well as the complete MPO enzyme system (MPO, H_2O_2 and Cl^-) [21] and PCs with differently unsaturated fatty acyl residues has been comprehensively studied. This reaction leads primarily to the generation of chlorohydrins [22]. The reaction is strongly pH-dependent and the yield of chlorohydrins is favored by lower pH values indicating that HOCl but not ClO^- is the reactive species. Depending on the reaction conditions, glycols, epoxides and dichlorides may also be generated, but in much smaller yields than the chlorohydrins (Fig. (**3**)).

By using isotopically-labeled water (H_2O^{18} and H_2O^{17}) and MALDI-TOF MS it could also be shown that the oxygen present in the chlorohydrin is not derived from HOCl but from the used water. In addition to the chlorohydrin, a second product, where an H atom was replaced by a halogen, could also be detected. These results are consistent with the view that two different ways of stabilization of π-complexes formed after binding of Cl^+ or Br^+ to the double bond exist [22].

Additionally, saturated lysophosphatidylcholines (LPCs) are also important products of the reaction between HOCl and PCs: The yield of LPCs increases if the saturation degree of the applied PC decreases and a mechanism to explain this behavior has been recently proposed [23]: Oxygen and chlorine are rather electronegative elements and weaken the ester bond of lipids by the withdrawal of electrons. Therefore, chlorohydrins are more sensitive to hydrolysis than the original lipids [23].

Chlorohydrins and LPCs were also obtained in significant yields if e.g. lipoproteins from human blood were incubated with HOCl [24]. However, due to the high protein content of lipoproteins and the considerable reactivity of the thiol and amino groups of proteins with HOCl [25] a considerable excess of HOCl was necessary to induce alterations of the lipid constituents [26]. This is a clear indication of the gradual order of reactivity of functional groups with HOCl (SH ~ -S-S- > $-NH_2$ > -C=C-).

4.2.2. Initiation of Peroxidation

In addition to the generation of chlorohydrins, it has also been shown by measuring the production of thiobarbituric acid reactive substances (TBARS) that PLs are peroxidized in the presence of HOCl. Stelmaszynska *et al.* reported that liposomes containing egg yolk PC are oxidized by the $MPO/H_2O_2/Cl^-$ system as well as by stimulated neutrophilic granulocytes [27]. Since virtually no peroxidation occurred in the absence of Cl^- and hydroperoxide formation could be totally inhibited by the HOCl scavenger taurine (2-aminoethanesulfonic acid), it was concluded that HOCl is the oxidizing species. The occurrence of lipid peroxidation was supported by the finding that HNE (4-hydroxy-2-nonenal) [28], another secondary product from the oxidation of oleoyl residues, was formed. Some of the involved radicals could be additionally monitored by ESR spectroscopy [29].

On a simplified model level, it could be shown by using the reaction of t-butyl hydroperoxide with HOCl and subsequent product analysis by NMR that this radical-based mechanism is actually valid [30]. The question if chlorohy-

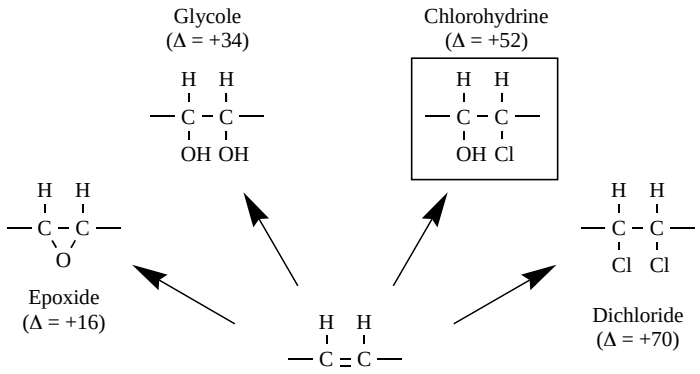


Fig. (3). Potential reaction products of unsaturated phospholipids and HOCl. The indicated numbers give the mass difference in comparison to the educt.

drin formation or peroxidation is more important, is still intensively discussed. However, chlorohydrin formation seems strongly favored [31].

4.2.3. Reactions of Plasmalogens with HOCl

Different membranes, for instance, from stem cells and particularly from animal spermatozoa do not only contain diacyl PLs but possess also significant amounts of alkenyl-acyl PLs, the "plasmalogens". Plasmalogens are nowadays in the focus of research because they exhibit significant antioxidative properties. Accordingly, plasmalogens exhibit also high reactivity with HOCl. For instance, it has been recently shown that plasmalogens react faster with HOCl than diacyl PLs with a comparable fatty acyl composition. This higher reactivity is stemming from the isolated alkenyl-ether residue of plasmalogens. It was demonstrated that beside the lysolipid lacking the fatty acyl residue in *sn*-1 position, considerable amounts of GPC are generated if plasmalogens react with HOCl. Due to the lack of both fatty acyl residues, however, GPC is exclusively detectable in the aqueous phase [32].

4.3. Phosphatidylethanolamine (PE)

In contrast to the comprehensively studied PCs, reactions between HOCl and PE were by far less investigated. This is surprising as the membrane of one of the most common pathogens, *Escherichia coli*, consists primarily of PE, phosphatidylglycerol (PG) and cardiolipin (CL) with PE being the most important constituent [33].

It could be shown by using thin-layer chromatography (TLC), UV spectroscopy and EI MS that mono- and dichloramines are the prime products of the reaction between lipids isolated from *Escherichia coli* and HOCl. Chlorohydrins were only detectable if HOCl is used in vast excess over the PLs [34].

In a very recent paper it was also shown that the products of the reaction between HOCl and PE are capable of reacting with further PLs, particularly PCs, and these products have significant impact on cell metabolism [35], in particular on signal transduction.

It is not surprising that chloramines are the prime reaction products as the amino group of PE exhibits a much higher reactivity with HOCl than the double bonds of the fatty acyl residues [36]. Unfortunately, however, detailed mechanistic studies of PL oxidation are aggravated by their formation of supramolecular aggregates in aqueous solution. Therefore, investigations of the second order rate constants with HOCl were not yet performed with intact PE but only with PE-derived water-soluble fragments, for instance, the phosphorylethanolamine moiety [36]. It may be expected that second order rate constants are much lower in bilayers than in the case of the isolated molecules.

It is well known that during the reaction of PCs with HOCl considerable amounts of the corresponding lysophosphatidylcholines (LPCs) are also generated [23,26] and this was explained by a preferred hydrolysis of the oxidatively-modified fatty acyl residue [23].

However, if this mechanism would be correct, it should apply for all PL classes irrespective of the headgroup. Please note that neither PE nor other PL give rise to lysophospholipids upon HOCl treatment. Therefore, a more detailed study of the reaction between HOCl and the individual PLs is clearly required. This particularly holds for PLs with amino functions as the products derived thereof are not stable and decompose with time. The only totally stable chloramine is derived from taurine, a common model system [37].

4.4. Phosphatidylserine (PS)

The reaction between PS and HOCl was so far not very comprehensively studied [35], whereas investigations of the isolated amino acid serine gave evidence that products similar to PE may be expected because serine undergoes rapid decarboxylation [38].

Nevertheless, PS is often mentioned in the context of HOCl-induced cell damages. A commonly used apoptosis (the "programmed" cell death) assay is based on the binding of a special protein (annexin V) to PS. In a "healthy" cell, there is a pronounced lipid "asymmetry", whereby PS is nearly exclusively located at the inner but not the outer leaflet of the membrane. Under these conditions annexin V is not able to bind to the PS. If the cell is damaged, the asymmetry is not longer adhered and PS is transferred to the outer leaflet resulting in annexin V binding. It could be shown that the extent of annexin V binding correlates with oxidative damages and is strongly enhanced in the presence of HOCl [39]. Other lipid classes and their reactions with HOCl were to these authors knowledge not yet investigated.

5. METHODS OF PRODUCT EVALUATION

Although some important analytical methods to study HOCl-induced effects were already shortly mentioned above, these methods will be more comprehensively discussed in the following chapter. An excellent review is also available in [40].

5.1. Mass Spectrometry (MS)

Although conventional electron impact (EI) MS is less suitable because the intact PLs cannot be analyzed without major degradation, "soft ionization" techniques as electrospray ionization (ESI) [41] and matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) MS [42] are both extremely useful. Although ESI MS seems the method of choice to detect lipid hydroperoxides and chloramines [43], MALDI and ESI seem appropriate tools to study the generation of chlorohydrins from PCs.

As a typical example, the conversion of PC 18:0/18:1 (its monoisotopic mass is 787.6 and the peaks at *m/z* = 788.6 and 810.6 correspond to the H^+ and Na^+ adduct, respectively) into the chlorohydrine under the influence of the HOCl-generating system MPO-H_2O_2-Cl^- is shown in Fig. (**4**). It is evident that chlorohydrine formation can be easily monitored by the increase of the peak at *m/z* = 840.5, which indicates the addition of HOCl to PC 18:0/18:1 (788.6 + 52 (HOCl)). The corresponding Na^+ adduct is, of course, also detectable at *m/z* = 862.5. ESI MS may be used in the same way [44] and was already used, for instance, to monitor the reaction of plasmenylcholine with HOCl [45].

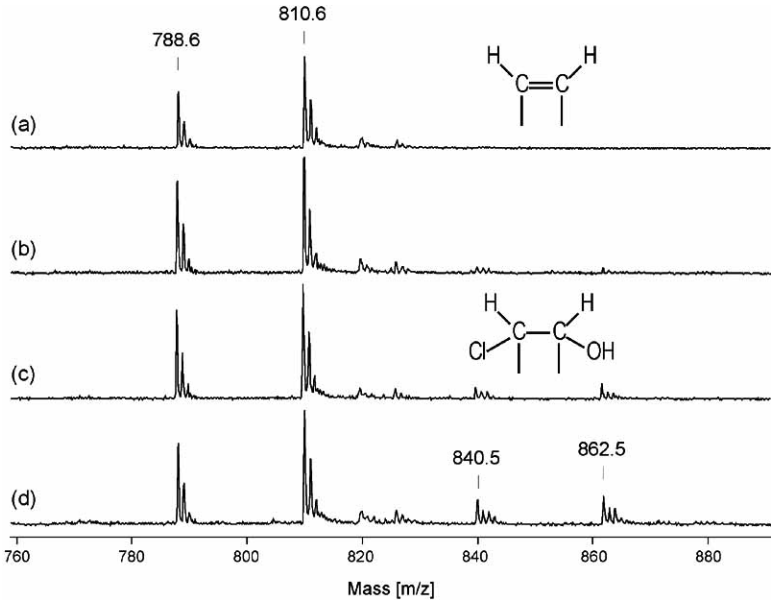


Fig. (4). Changes in the mass spectrum of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (trace a) after incubation with the MPO-H_2O_2-Cl^- system at pH 6.0. PL liposomes (0.03 mg/ml) prepared in 0.14 mol/l NaCl, 50 mmol/l phosphate were incubated with hydrogen peroxide (three additions of 0.1 mmol/l in intervals of 10 min) and MPO (35 nmol/l) for 60 min (b) or 120 min (c). In trace d, all additions of MPO and H_2O_2 have been repeated after one hour. The total incubation time in sample (d) was 2 h. Reprinted with modifications and with permission from Elsevier [20].

5.2. High Resolution NMR Spectroscopy

Although NMR suffers from relatively low sensitivity, it has often been used to study the reaction between HOCl and unsaturated fatty acids or even PLs. ^{1}H NMR was most often used [46] as it is most sensitive. The action of HOCl may be easily monitored by the disappearance of the olefinic and the formation of the typical chlorohydrin resonances that can be easily differentiated from the resonances of simultaneously generated glycols and epoxides [47]. Although ^{13}C NMR should be an even more powerful tool as it enables the differentiation of nearly all carbon atoms along the fatty acyl chain due to its considerable range of chemical shifts, ^{13}C NMR was to these authors knowledge not yet used to a higher extent to study HOCl-induced oxidation reactions of lipids.

Although high resolution ^{31}P NMR is a very useful method in order to differentiate the individual PL species (if aggregation of PL is prevented by a suitable detergent or a solvent mixture [48]), ^{31}P NMR does, unfortunately, not provide convincing information about oxidation products of PLs: The reaction between HOCl and unsaturated PC species leads only to a broadened PC resonance that is not resolved to such an extent that the differentiation of individual products would be possible [24].

5.3. Chemiluminescence

Although chemiluminescence (CL) is not suitable to study the changes of chemical structures, CL is often used as a simple method to investigate the kinetics of the reaction between HOCl and PLs. The advantage of CL in comparison to optical methods is that turbid samples may be also investigated.

Luminol-amplified CL is normally used. The reaction between luminol and HOCl (Fig. (**5**)) is accompanied by the emission of light that can be very sensitively assessed. The second order rate constant of this reaction is 5×10^5 M^{-1} s^{-1}

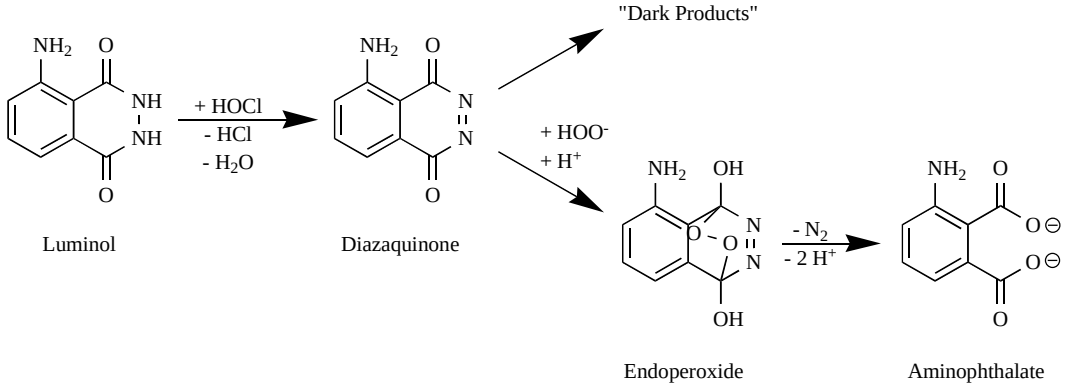
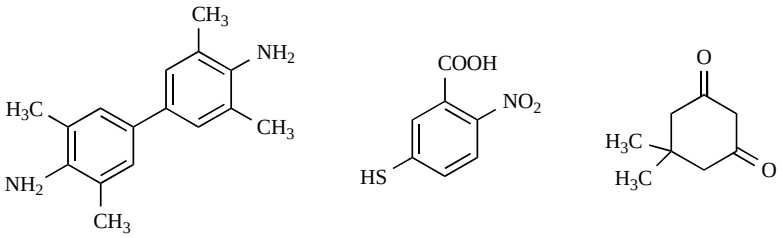


Fig. (5). Proposed reactions of luminol leading to light emission.



a) TMB b) TNB c) Dimedone

Fig. (6). Structures of chemicals frequently used to assay HOCl-induced reactions.

[49]. Light intensity depends on the presence of other compounds (e.g. lipids) competing with luminol for HOCl. If both, the luminol as well as the lipid concentration are known, the second order constant of the reaction between HOCl and the lipid can be easily derived by a simple Stern-Vollmer plot [50].

Second order rate constants of the interaction between HOCl and PL double bonds of about 0.50 M^{-1} s^{-1} could be calculated using this approach [46].

5.4. UV Spectroscopy

The standard method to follow the reaction between HOCl and amines is UV spectroscopy because mono- and dichloramines are characterized by a (weak) absorption in the UV range [51]. Although this approach can be easily used in the case of water soluble compounds, for instance, phosphorylethanolamine or glycero-phosphorylethanolamine (GPE) [36], the reliability of this assay is limited if intact PEs are investigated due to light scattering effects. UV spectroscopy is, however, the method of choice if the reaction between HOCl and small, water soluble compounds as taurine [52] is to be investigated.

The vast majority of UV lipid oxidation assays uses the characteristic absorbance of the reaction products or the educts with selected dyes. The most important ones will be shortly listed:

5.4.1. Determination of the Double Bond Content

This approach is based on the classical determination of the iodine number that is very popular in fat research. The lipid solution is treated with IBr solution and after an incubation of about 1 hour with KI. Immediately after that the concentration of I_3^- is determined spectrophotometrically at 350 nm ($\varepsilon = 2.64\times10^4$ M^{-1} cm^{-1}) [53].

5.4.2. TNB and TMB

These both dyes, 5-thio-2-nitrobenzoic acid (TNB) and 3,3′,5,5′-tetramethylbenzidine (TMB) (Fig. (**6**)) are very useful to detect small amounts of HOCl and, accordingly, for the determination of low activities of MPO. Both assays use the reaction of HOCl with taurine to form a relatively stable chloramine. TNB is bleached in the presence of taurine chloramine and this bleaching effect may be directly used to assess the related HOCl concentration quantitatively [51]. However, as this assay is influenced by the presence of other oxidants, a more reliable assay has been developed on the basis of TMB. Using this approach, taurine chloramine is treated with iodide leading to the formation of ICl that is instantly hydrolyzed to hypoiodous acid:

$$R\text{-}NHCl + H^+ + I^- \rightarrow R\text{-}NH_2 + ICl$$

$$ICl + H_2O \rightarrow HOI + HCl$$

TMB may be used afterwards directly as the chromophore because it is readily oxidized by HOI to a strongly absorbing blue product [54] that allows the determination of μM quantities of HOCl. This assay is about 10 times more sensitive than the TMB assay and orders of magnitude in comparison to the direct measurement of the absorption of HOCl.

5.4.3. Monochlorodimedone

A rather established assay to determine the concentration of HOCl is the chlorination of dimedone (Fig. (**6**)) that is accompanied by an increase in absorption at 290 nm ($\varepsilon = 1.99\times10^4$ M^{-1} cm^{-1}) [55]. However, the contribution of further ROS is so far controversial discussed and the selectivity of the assay questioned.

5.5. Infrared Spectroscopy

Although IR seems highly suitable for the detection of N-Cl vibration bands, there were so far nearly no attempts to study the generation of PE chloramines by means of IR. However, it could be shown that the reaction between e.g. taurine and HOCl is accompanied by the formation of an intense IR band at about 975 cm^{-1} [37], characteristic of N-Cl.

5.6. Chromatographic Methods

Both, HPLC as well as TLC may be used to separate the reaction products between PLs and HOCl. The application of LC/MS has been clearly shown by several groups [56] and it is a considerable advantage of this method that chlorohydrines as well as peroxides are simultaneously detectable. Basically, the same analysis may also be performed by TLC as shown on the example of cholesterol [15] as well as a mixture of PLs isolated from red blood cells [57].

In contrast, the investigation of chloramines derived from PE is much more difficult as the corresponding compounds seem to decompose (at least partially) on the TLC plate as well as the LC column and further efforts are clearly necessary to find more suitable conditions.

6. CONCLUSIONS AND OUTLOOK

The focus of this review was on the reaction between HOCl that is (a) used as an important disinfectant and (b) generated under inflammatory conditions by the enzyme MPO and different lipids. It was shown that lipids with exclusively olefinic residues as HOCl targets yield mainly the chlorohydrins. Due to the stability of these products the reaction may be easily characterized by different methods and even the second order rate constants of the reaction could be determined. Comparable products are obtained if HOCl is generated by the $MPO/H_2O_2/Cl^-$ system mimicking the physiological conditions. This reaction is important for two reasons: (a) Chlorohydrin formation may lead to a destabilization of the cellular membrane and (b) lipid peroxidation may be initiated by the reagent HOCl.

In contrast to the reactions of cholesterol and phospholipids, the reaction between HOCl and lipids with reactive amino residues (e.g. PE and PS) was by far less frequently investigated although these lipids are also very abundant in biological membranes. This might be caused by the larger product variability as reactions with the olefinic as well as the amino functions may occur. Finally, the lability of the chloramines makes analysis much more difficult.

Therefore, further attempts are necessary to study these reactions in more detail. This particularly holds as it has been shown very recently that chlorinated products derived from PE and PS are very important for signal transduction pathways in cells [35].

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LIST OF ABBREVIATIONS

CL	=	Chemiluminescence
ε	=	Extinction Coefficient
EI	=	Electron Impact
ESI	=	Electrospray Ionization
ESR	=	Electron Spin Resonance
GPC	=	Glycero-Phosphorylcholine
GPE	=	Glycero-Phosphorylethanolamine
HNE	=	4-Hydroxy-2-Nonenal
HPLC	=	High Performance Liquid Chromatography
IR	=	Infrared
LC	=	Liquid Chromatography
LPC	=	Lysophosphatidylcholine
MALDI	=	Matrix-Assisted Laser Desorption and Ionization
MPO	=	Myeloperoxidase
MS	=	Mass Spectrometry
m/z	=	Mass over Charge
NMR	=	Nuclear Magnetic Resonance
PC	=	Phosphatidylcholine
PE	=	Phosphatidylethanolamine
PL	=	Phospholipid
ppm	=	Parts per Million
PS	=	Phosphatidylserine
ROS	=	Reactive Oxygen Species
TBARS	=	Thiobarbituric Acid Reactive Substances
TLC	=	Thin-Layer Chromatography
TMB	=	3,3′,5,5′-Tetramethylbenzidine
TNB	=	5-Thio-2-Nitrobenzoic Acid
TOF	=	Time-of-Flight
UV	=	Ultraviolet

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