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The reaction between phosphatidylethanolamines and HOCl investigated by TLC: Fading of the dye primuline is induced by dichloramines

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ABSTRACT

Phosphatidylethanolamines (PEs) and phosphatidylglycerols (PGs) are abundant lipid constituents of the membranes of *Escherichia coli*. The reaction between these lipids and hypochlorous acid (HOCl), an important constituent of disinfectants, was investigated by combined thin-layer chromatography (TLC), mass spectrometry (MS), UV and fluorescence spectroscopy. Primuline is a common dye in lipid research that binds non-covalently to lipids and allows, thus, the direct evaluation of TLC plates by MS. However, primuline staining of the products between PE and HOCl is accompanied by fading of the dye. This only holds if acidic but not alkaline conditions are applied. Using a combination of TLC, UV and fluorescence spectroscopy, it will be shown that dichloramines of PE are responsible for the observed primuline fading. Since dichloramines are slowly converted under alkaline conditions into the nitriles that lack the characteristic UV properties of dichloramines, fading is not observed under alkaline conditions.

1. Introduction

Hypochlorous acid (HOCl) is an important reactive oxygen species (ROS) [1] that is generated under *in vivo* conditions by the enzyme myeloperoxidase (MPO) from H_2O_2 and Cl^- [2]:

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

Due to their strongly oxidizing and chlorinating abilities, HOCl and its salt, NaOCl, are also important constituents of many household cleaning agents with strong bactericidal properties, for instance, against bacteria as *Escherichia coli* [3].

The cellular membrane consists primarily of phospholipids (PL), particularly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In bacteria as *E. coli*, however, the PC moiety is replaced by phosphatidylglycerol (PG). The structures of these three important phospholipid classes are shown in Fig. 1. Please note that the fatty acyl residue in *sn*-1 position is normally a saturated one, while in *sn*-2 it is often unsaturated.

The reaction between HOCl and differently saturated PCs has been already comprehensively studied [4]: this reaction leads (beside smaller amounts of lysophosphatidylcholines [5]) primarily to the generation of chlorhydrines:

$$R\text{–}CH{=}CH\text{–}R' + HOCl \rightarrow R\text{–}CH(Cl)\text{–}CH(OH)\text{–}R' \quad (2)$$

In contrast to PCs, the reactions between HOCl and PEs were by far less detailed investigated. However, it could be shown by using a combination of thin-layer chromatography (TLC), UV spectroscopy and gas chromatography/mass spectrometry (GC/MS) that mono and dichloramines are the prime products if HOCl reacts with lipids isolated from *E. coli* [6]. Additionally, these compounds undergo conversions into nitriles and aldehydes and these reactions may be summarized as follows:

$$R\text{–}CH_2\text{–}NH_2 + HOCl \rightarrow R\text{–}CH_2\text{–}NH\text{–}Cl + H_2O \quad (3)$$

$$R\text{–}CH_2\text{–}NH_2 + 2HOCl \rightarrow R\text{–}CH_2\text{–}NCl_2 + 2H_2O \quad (4)$$

Abbreviations: ε, extinction coefficient; ESI, electrospray; GC, gas chromatography; LPC, lysophosphatidylcholine; MALDI, matrix-assisted laser desorption and ionization; MPO, myeloperoxidase; MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TLC, thin-layer chromatography; TOF, time-of-flight; UV, ultraviolet.

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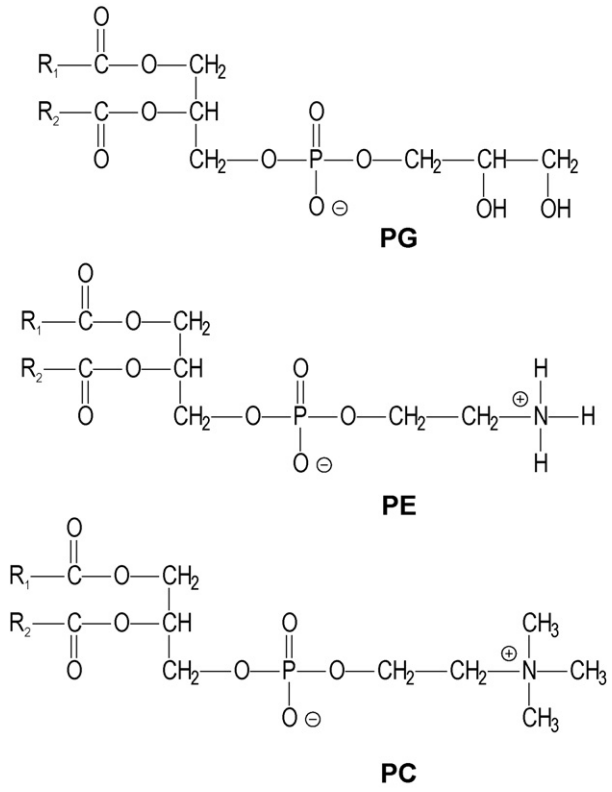

Fig. 1. Comparison of the chemical structures of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). In living systems the fatty acyl residue in *sn*-1 position is normally a saturated and that in *sn*-2 position an unsaturated one. Besides the potential presence of olefinic groups that are capable of reacting with HOCl, the amino group of PE represents an additional reactive functional group.

$$R\text{–}CH_2\text{–}NH\text{–}Cl + H_2O \rightarrow R\text{–}CHO + NH_4Cl \quad (5)$$

$$R\text{–}CH_2\text{–}NCl_2 \rightarrow R\text{–}CN + 2HCl \quad (6)$$

Although the reactions between HOCl and amino groups are much faster than with olefinic groups [7], chlorhydrins may also be formed if an excess of HOCl over the PE is used.

TLC is highly established for the separation of phospholipids and the developed TLC plates may be also directly investigated by mass spectrometry [8]. Although MALDI-TOF MS (matrix-assisted laser desorption and ionization time-of-flight mass spectrometry) MS has certain disadvantages regarding the detection of N-chlorinated products [9], it has the significant advantage that it may be most easily combined with TLC [8].

For this combination, however, staining methods are necessary that do not result in alterations of the molecular weight of the analyte. One very useful dye is primuline [10] because it binds non-covalently to the apolar fatty acyl residues of lipids and does not affect a subsequent MS analysis [11]. Although primuline has been already used in many studies dealing with standard lipids [10,11], it is not yet clear whether this dye may also be used in the presence of potential reactive compounds as chloramines.

Thus, the aim of this paper is the evaluation of potential interactions between primuline and reactive products of the reaction between phospholipids and HOCl. It will be shown that in the presence of PE dichloramines intense fading of the dye occurs, whereas nitriles derived from the chloramines do not interfere with the fluorescence of the dye.

2. Materials and methods

2.1. Chemicals

All isolated phospholipids (1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (DPPE), 1-palmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol (POPG)) as well as an *E. coli* extract were obtained from Avanti Polar Lipids (Alabaster, MA, USA) and used without further purification but diluted with $CHCl_3$ to 0.5 mM solutions. The used *E. coli* extract consisted of PE (67%), PG (23.2%) and cardiolipin (9.8%) as indicated by the supplier.

All chemicals and salts for buffer preparation, all solvents (chloroform, ethanol, methanol, triethylamine, acetic acid, acetonitrile and acetone), taurine (2-aminoethanesulfonic acid) and the primuline dye were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (part of Sigma–Aldrich Chemie GmbH, Taufkirchen) and used as supplied. Sodium hypochlorite was from Sigma–Aldrich (Taufkirchen, Germany). Fifty millimolar phosphate buffer (pH 6) was used for all reactions.

2.2. Incubation of phospholipids with HOCl

An aliquot of the PL of interest (dissolved in $CHCl_3$) was evaporated to dryness. Vesicles were prepared according to [6] by suspending the lipid film in buffer.

A stock solution of NaOCl was kept in the dark at 4 °C. Its concentration was determined at pH 12 using $\varepsilon_{290} = 350\ M^{-1}\ cm^{-1}$ [12] and diluted with buffer immediately prior to use.

All PLs as well as the *E. coli* extract were incubated separately with varying amounts of NaOCl for 30 min at 37 °C and thoroughly shaken. The incubation was stopped by adding chloroform/methanol (1:1, v/v) in the same amount as the aqueous phase to extract the lipids according to Bligh and Dyer [13]. The organic layer was isolated by a Hamilton syringe. One part of the organic layer was immediately analyzed by MALDI-TOF MS and ESI MS as previously described [9], whereas the remaining part was subjected to TLC separation.

2.3. Thin-layer chromatography

Five microliter PL samples (containing about 12 μg PL) were applied using a standard Hamilton syringe on HPTLC silica gel 60 plates (10 cm × 10 cm in size with aluminum backs) (Merck, Darmstadt, Germany) as spots with 1-cm space between the spots and at a distance of 2 cm from the bottom edge of the plate. Plates were developed in commercially available vertical TLC chambers (CAMAG, Switzerland) with chamber saturation using chloroform, methanol, acetic acid (80:12:8, v/v/v) as acidic solvent mixture according to [6] or chloroform, ethanol, water, triethylamine (30:35:7:35, v/v/v/v) as alkaline solvent mixture [8]. The required time of development was about 35 min and the TLC run was performed at room temperature (22 ± 2 °C) and 50 ± 5% relative humidity. Subsequent to development, TLC plates were dried in a stream of warm air. The total length of the run was about 6 cm.

PLs were visualized by spraying with a solution of primuline (Direct Yellow 59) according to [10]. Upon irradiation by UV light (366 nm), individual PLs become detectable as violet spots. TLC plates were scanned by using a videodensitometric device (Biostep GmbH, Jahnsdorf, Germany).

2.4. UV spectroscopy

All UV measurements were performed on a UV–vis Spektrophotometer Hitachi U-2000 in quartz cuvettes. The organic extracts

of phospholipids subsequent to reaction with HOCl and extraction according to Bligh and Dyer [13] were directly used.

2.5. Fluorescence spectroscopy

Fluorescence measurements were performed on a Jobin Yvon Spex FluoroMax 2 spectrofluorimeter (Edison, NJ) at room temperature. Half micro-quartz cuvettes equipped with a magnetic stirrer were used and the sample volumes were 1250 μl. The excitation wavelength was 366 nm. Fluorescence emission spectra were recorded from 380 to 600 nm. The wavelength increment was 1 nm and the integration time was 0.1 s per data point.

3. Results and discussion

PEs and PGs (cf. Fig. 1) are the most relevant phospholipids of *E. coli*. In contrast to PC and PG, PE possesses with its amino residue an additional reactive group. Therefore, even completely saturated PEs react with HOCl under generation of the mono and the dichloramine as the primary and the nitrile as the secondary product [6]. Beside by means of MS [9], the mono and the dichloramine can be easily differentiated by their characteristic UV spectra: the monochloramines show a maximum at about 252 nm ($\varepsilon \approx 429\ M^{-1}\ cm^{-1}$) and the dichloramines at about 300 nm ($\varepsilon \approx 370\ M^{-1}\ cm^{-1}$) [14].

Surprisingly, only a single paper is so far dealing with a detailed analysis of the reaction products between HOCl and PE [6]: *E. coli* lipids were treated with HOCl, the individual lipid classes isolated by TLC and the products subsequently analyzed by GC/MS. For the purpose of comparison, the same approach was used in the present study, but primuline [10] instead of ninhydrine [6] was used for staining the TLC plates.

In Fig. 2, the image of one typical TLC plate is shown. The lane labeled "Cont." corresponds to the pure *E. coli* extract (in the absence of HOCl) and increasing amounts of HOCl were used from the left to the right: in lane 1 a 10-fold and in lane 2 a 20-fold excess of HOCl was used.

Although the disappearance of the PE spot in the presence of increasing amounts of HOCl can be clearly monitored, it is remarkable that "pale" spots are obtained in the case of the reaction products (labeled by "c"). This exclusively holds if TLC is performed under acidic, but not under alkaline conditions (data not shown, cf. below). Please note that the differences in the migration properties (Rf values) between PG and its chlorhydrine (its generation was additionally confirmed by MALDI-TOF MS, data not shown [9]) are very small under the conditions used here. Therefore, a differentiation between products and educts can hardly be made.

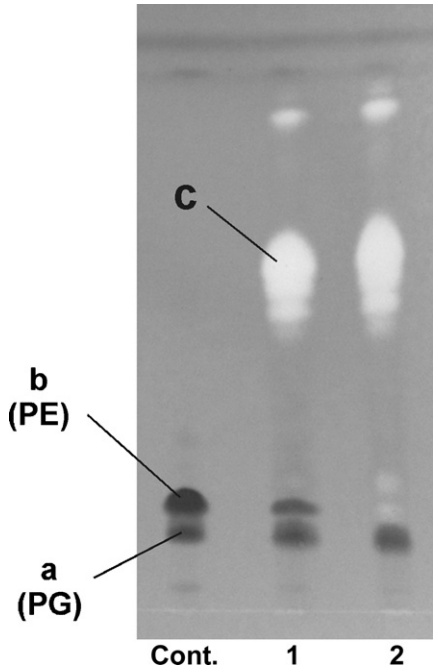

Fig. 2. Typical TLC plate showing *E. coli* phospholipids prior and subsequent to their reaction with HOCl. Chloroform, methanol and acetic acid (80:12:8, v/v/v) were used for development. Spots on the TLC plate were visualized by staining with primuline [10]. The lane termed "Cont." represents the control sample, whereas the lipid mixture in lane 1 was treated with a 10-fold and lane 2 with a 20-fold molar excess of HOCl (pH 6). After incubation (30 min at 37 °C), the lipid suspensions were extracted and the extracts were subjected immediately to TLC.

As PE dichloramines are the prime reaction products of PE if an excess of HOCl is used, it is most likely that these products contribute massively to the fading of the spots on the TLC plate.

Of course, the *E. coli* extract is already a rather complex lipid sample. In order to further clarify the observed fading phenomenon,

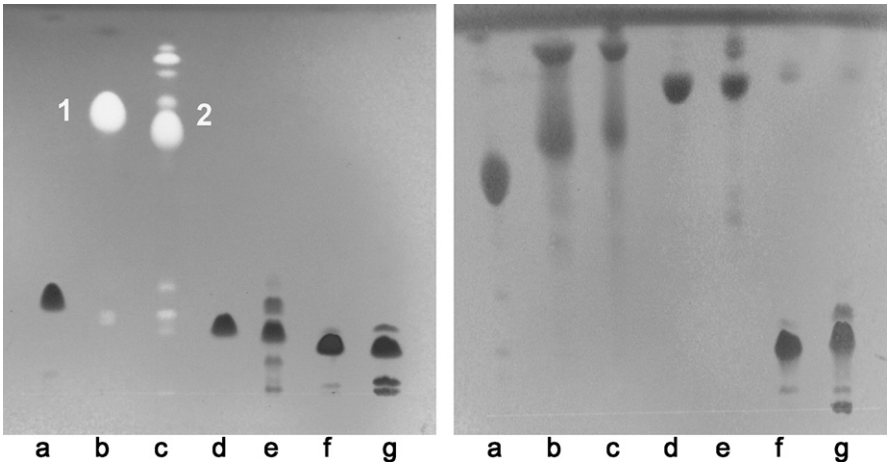

Fig. 3. Typical TLC plates showing selected phospholipids prior and subsequent to reaction with a 10-fold excess of HOCl (pH 6) and developed under different conditions [(a and b) DPPE; (c) POPE; (d and e) POPG; (f and g) POPC]. The lanes at the left represent in each case the pure PL, whereas the lanes at the right represent the PL subsequent to reaction with HOCl. As the fatty acyl compositions of PLs cannot be differentiated under these conditions, only DPPE is shown as reference for PE (a). Chloroform, methanol, acetic acid (80:12:8, v/v/v) were used as acidic conditions (left plate) and chloroform, ethanol, water, triethylamine (30:35:7:35, v/v/v/v) as alkaline (right plate) elution conditions. Spots on the TLC plate were visualized by staining with primuline [10].

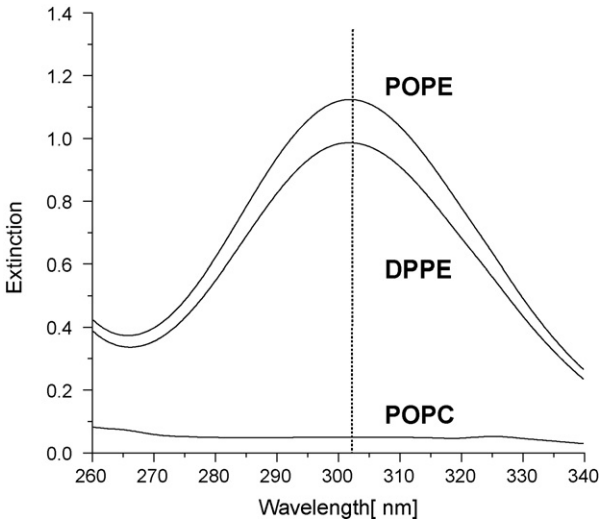

Fig. 4. UV spectra of POPC, DPPE and POPE subsequent to reaction with a 10-fold excess of HOCl. Spectra represent the organic extracts of the corresponding reaction mixtures. Please note that only in the case of the PE species significant absorptions could be detected subsequent to HOCl treatment.

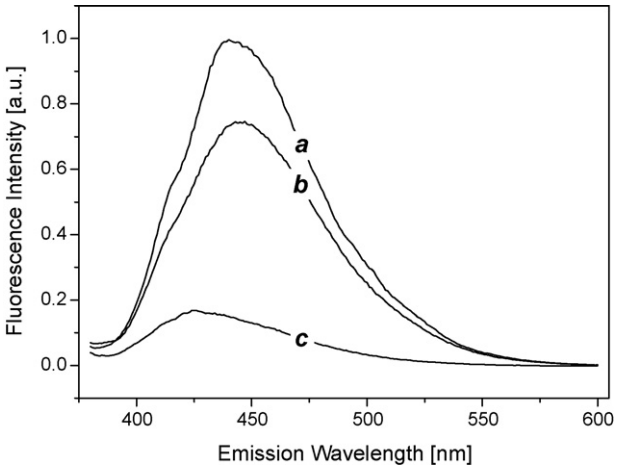

Fig. 5. Fluorescence emission spectra of 2.5 μg/ml primuline in $CHCl_3$ in the absence (a) or in the presence of 5 μl (b) or 20 μl (c) of an organic extract of 1 mg/ml PE dichloramine. The excitation wavelength was 366 nm. All other experimental conditions were as given in Section 2.

some isolated lipids were also treated with an excess of HOCl and subsequently analyzed by TLC.

In Fig. 3 the TLC results of the individual artificial PLs as well as after their reaction with HOCl and subsequent to primuline staining under acidic (left) and alkaline (right) conditions are shown. It is evident that both TLC plates differ not only with regard to separation quality but also in terms of the shape of the spots. The same "fading" effect as already observed with *E. coli* lipids is also obvious if defined PLs are treated with HOCl. It is also evident that subsequent to HOCl treatment there is neither residual DPPE nor POPE. The DPPE gives subsequent to HOCl treatment one intense white spot (3b), whereas the POPE results in several spots (3c). This is as such not surprising as the POPE possesses with its olefinic residue one additional reaction site. The reader should also note that the differences of the Rf values between PG (3d, e) and PC (3f, g), on the one hand, and the corresponding chlorhydrines, on the other hand, are very small under the conditions applied here and, therefore, a differentiation can hardly be made. However, it is again obvious that fading occurs only in the case of the PEs subsequent to reaction with HOCl under acidic, but not alkaline conditions. Accompanying investigations by MS as well as UV spectroscopy have evidenced the generation of dichloramines under these conditions [9]. This applies, however, not only if considerable excesses of HOCl in comparison to the PE are used but also if smaller amounts of HOCl are used (data not shown).

As the dichloramine of DPPE is the major product in the faded spots and could be detected by MS as well as UV spectroscopy (data not shown), it is most probably that this compound contributes primarily to the fading. Fading of the primuline dye is also observed if taurine–dichloramine is applied onto the TLC plate (data not shown).

A potential contribution of the reagent HOCl may be excluded as the DPPE sample was extracted with organic solvents subsequently to reaction with HOCl. Therefore, all residual HOCl is present in the aqueous phase [9].

Another possibility that might explain the observed fading is the direct reaction between the dichloramines and the primuline dye. However, it is very unlikely that this reaction contributes significantly to the observed effects as the fading is also observed (data not shown) in the presence of taurine–dichloramine that is considered as a compound with low reactivity [14,15]. Therefore, it is in our opinion most likely that differences in absorption properties of the individual products contribute to the observed fading.

In order to further confirm this suggestion, UV spectra of DPPE, POPE and POPC subsequent to reaction with HOCl were recorded (Fig. 4). It is obvious that exclusively the products derived from POPE and DPPE exhibit a significant absorption at about 300 nm, while the chlorhydrine of the POPC does not absorb at this wavelength at all. Therefore, the observed absorption is characteristic of the dichloramines [14]. It is, however, not clear whether this property influences the fluorescence properties of primuline.

In order to clarify this question, fluorescence spectra of primuline in the presence of different concentrations of PE dichloramine were recorded (Fig. 5). It is evident that the fluorescence intensity of primuline (5a) decreases if the concentration of PE dichloramine increases (5b, c). Additionally, a slight shift to smaller wavelengths is observed under these conditions.

4. Conclusions

In this paper it was shown that the fluorescence dye primuline is affected by the treatment with PEs in the presence of HOCl. From the obtained data we conclude that PE dichloramines are responsible for the observed fading of the primuline due to their characteristic UV absorption. As neither chlorhydrines nor monochloramines [14] show a significant absorption at this wavelength, their presence does not affect the fluorescence of the primuline. It was not the aim of this study to investigate the mechanisms by which fading occurs in more detail.

It was also shown above that fading is only observed under acidic but not under alkaline conditions. This observation clearly confirms the massive contribution of the dichloramines: it is well known that PE dichloramines decompose massively under alkaline but only to a much lesser extent under acidic conditions into the corresponding nitrile [14]. As nitriles lack the characteristic dichloramine UV absorption, they do not affect the primuline fluorescence and no fading of the dye is observed.

From the obtained data it is concluded that the use of primuline must be regarded with caution in the presence of N-chlorinated products. This particularly holds if fluorescence intensity is used for quantitative evaluations.

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