Effects of the Nitrogen Moieties of Phos-
phatidylethanolamine and Phosphatidylcholine
on the Autoxidation of These Phospholipids in
Aqueous Dispersions

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The effects of nitrogen moieties on the autoxidation of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were studied in Tween 20 micelles at 37°C under air. When the oxidation was initiated by a water-soluble azo compound, the rate of oxidation of PE and PC was proportional to the number of bisallylic hydrogens and to the square root of azo-initiator concentration, and independent of difference in nitrogen moieties of PE and PC. When oxidation was induced by copper (II) ion, the kinetic order in the concentration of copper (II) ion was 0.5, suggesting that the oxidation may thus be initiated by radicals formed in the copper (II) ion-catalyzed decomposition of phospholipid hydroperoxides. The rate of oxidation per the number of bisallylic hydrogens of PE was 6.5-times larger than that of PC, possibly due to the formation of the PE-copper (II) ion complex. The accelerating effect of the ethanolamine moiety on oxidation induced by copper (II) ion was confirmed by the finding that the oxidation of PC was promoted by the addition of non-oxidizable dimyristoyl-PE but not dimyristoyl-PC.

1 Introduction

Phospholipids are particularly susceptible to oxidation since they contain polyunsaturated fatty acids as phosphoglyceride esters and its oxidation proceeds by a free radical chain mechanism. Therefore, the rate of the oxidation of phospholipids is dependent on the rate constants of chain initiation, propagation, and termination. The oxidizability of phospholipid is dependent on the compositions of the polyunsaturated fatty acids because that of polyunsaturated fatty acid is very high due to the high number of the ratio of the rate constant for propagation to the square root of that for termination1)-3). It is also suggested that the nitrogen-containing components such as choline, ethanolamine and serine change the rate of oxidation4)-6). For example, Corliss and Dugan4), and Tsai and Smith5) reported the possibility of the changes in the rate of chain initiation. Moreover, Morita and Fujimaki6) noted phosphatidylethanolamine (PE) to be more sensitive to oxidation induced by copper (II) ion than phosphatidylcholine (PC). Their data, however, are not quantitative, because the rate of chain initiation was not regulated and the microenvironments of PE and PC should be very different since PC forms liposomal bilayer in

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water dispersion while PE forms hexagonal II phase\(^7\). Furthermore, the compositions of the fatty acids of PE or PC were not determined. Here we report the autoxidation of PE and PC in Tween 20 micelles initiated by a water–soluble azo compound or induced by cupric ion to elucidate the effects of nitrogen moieties on lipid peroxidation.

2 Experimental

2-1 Materials

Soybean PE (purity 98 %) was obtained from Sigma Chemical Co., Ltd. (U.S.A.) and purified as reported previously\(^8\). Soybean PC (purity 99 %) obtained from Sigma Chemical Co., Ltd. was purified by high performance liquid chromatography (HPLC) using Inertsil PREP-SIL column (20 mm \(\phi \times 250 \text{ mm}\)) and methanol as a column and an eluent, respectively. The flow rate was 10 mL/min and the retention time of PC was 17.5 min. These purifications were necessary to remove antioxidants from the commercial phospholipids. The compositions of fatty acids of PE and PC measured by gas-liquid chromatography after methanolysis with tetramethylammonium hydroxide in methanol were as follows: PE, palmitate 30.6, stearate 2.8, oleate 7.3, linoleate 53.9, and linolenate 5.4 mol %; PC, palmitate 16.2, stearate 4.3, oleate 11.5, linoleate 62.6, and linolenate 5.4 mol %. Tween 20 and copper (II) sulfate were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). 2,2’-Azobis (2-amidinopropane) dihydrochloride (AAPH) used as the water-soluble radical initiator was supplied from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). 2,6-Di-t-butyl-4-methylphenol (BMP) was re-crystallized from methanol. Dimyristoyl PC (14:0 PC) and dimyristoyl PE (14:0 PE) were obtained from Sigma Chemical Co., Ltd. 0-Phosphorylethanolamine was obtained from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan).

2-2 Procedure

The water dispersions of phospholipids were prepared as follows using Tween 20 as the nonionic surfactant \(^4\),\(^9\). Appropriate amounts of PE and/or PC in methanol and Tween 20 in chloroform (and, if necessary, BMP, 14:0 PC or 14:0 PE) were placed in glass flask. The solvents were removed by a water aspirator using a rotary evaporator and then by a vacuum pump. An aqueous solution of AAPH or copper (II) sulfate (and, if necessary, O-phosphorylethanolamine) in phosphate buffer (5 mM, pH 7.4) was added to the flask. After shaking the mixture vigorously, it was transferred into a reaction vessel. Oxidations were carried out at 37 °C in air. The rates of oxidations of PE and PC were followed by the formation of conjugated diene using HPLC monitored at 234 nm as reported previously\(^8\). HPLC conditions for oxidation of the mixture of PE and PC were as follows: column, Inertsil PREP-SIL (4.6 mm \(\phi \times 250 \text{ mm}\)) + Unisil Q NH2 (4.6 mm \(\phi \times 250 \text{ mm}\)); eluent, methanol/40 mM aqueous NaH2PO4 (90/10 by volume); flow rate, 1.0 mL/min; detection, absorbance at 234 nm. Under these conditions, the hydroperoxides of PE and PC were eluted at 6.2 and 8.7 min, respectively. The concentration of hydroperoxides at the start of a run was estimated by HPLC/isoluminol assay\(^10\) as 0.8 \(\mu\) M for PE or 7.9 \(\mu\) M for PC.

![Graph](https://via.placeholder.com/150)

**Fig. 1** Formation of conjugated diene during the oxidation of 0.31 mM PE (○) and 0.31 mM PC (■) in 0.25 % Tween 20 micelles at 37 °C initiated with 0.50 mM AAPH.
Results and Discussion

3.1 Oxidation initiated by AAPH

The AAPH-initiated oxidation of PE and PC in Tween 20 micelles proceeded smoothly without any induction period as shown in Fig. 1. Chain lengths for the oxidation of PE and PC exceeded 11 (see Tables 1 and 2), indicating both PE and PC to be oxidized by a free radical chain mechanism.

The autoxidation of organic substrates proceeds by the following mechanism: 1,7

Initiation:

\[
A-N=N-A \rightarrow 2eA \cdot + (1-e)A-N=N-A \tag{1}
\]

\[
A \cdot + O_2 \rightarrow AO_2 \cdot \tag{2}
\]

Propagation:

\[
AO_2 \cdot + LH \rightarrow AOOH + LO_2 \cdot \tag{3}
\]

Termination:

\[
2 LO_2 \cdot \rightarrow \text{nonradical products} \tag{6}
\]

where \(A-N=N-A\) and LH are azo initiators and substrates, respectively. The rates of chain initiation \(R_i\) and chain propagation \(R_p\) for the above system can be represented by eqs. 7 and 8, respectively:

\[
R_i = 2e \cdot k_d \left[ A-N=N-A \right] \tag{7}
\]

\[
R_p = k_p / (2k_t)^{1/2} \cdot R_i^{1/2} \left[ LH \right] \tag{8}
\]

where \(e, k_d, k_p\) and \(k_t\) are the efficiency of chain initiation and rate constants for the decomposition of azo compounds, propagation step and termination step, respectively. The rate of chain initiation was measured by the conventional method\(^{1,8,11}\) using eq. 9 from the induction period \((t_{inh})\):

\[
R_i = 2 \left[ BMP \right] / t_{inh} \tag{9}
\]

observed by the addition of a chain-breaking antioxidant, BMP. In the oxidations of both PE and PC, \(e \cdot k_d\) for AAPH at 37°C was \(4.9 \times 10^{-7} \, \text{s}^{-1}\), suggesting that PE and PC are located in a similar microenvironment in the micelle.

Table 1 shows the results of oxidations of PE and PC at various concentrations of AAPH. The numbers in the last two rows in Table 1 are essentially constant for PE and PC, indicating both kinetic orders in the rate of chain initiation to be 0.5. The effects of variation in the concentrations of LH were determined using non-oxidizable 14:0 PC, where total amounts of two lipids were fixed and only relative amounts were changed as shown in Table 2. The near

### Table 1

<table>
<thead>
<tr>
<th>Run No.</th>
<th>PE/μmol</th>
<th>PC/μmol</th>
<th>[AAPH]/mM</th>
<th>(R_i \times 10^5) /mol/L-oil(^a) per s</th>
<th>(R_p/R_i^{1/2} [LH]) /mol/L-oil per s</th>
<th>Kinetic chain length</th>
<th>(R_p/R_i^{1/2} [LH]) /((s\cdot mol/L-oil)^{-1/2}) per active (H^b)</th>
<th>(R_p/R_i^{1/2} [LH]) /((s\cdot mol/L-oil)^{-1/2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td></td>
<td>0.25</td>
<td>1.02</td>
<td>0.053</td>
<td>36</td>
<td>0.021</td>
<td>0.019</td>
</tr>
<tr>
<td>2</td>
<td>3.1</td>
<td></td>
<td>0.50</td>
<td>1.28</td>
<td>0.047</td>
<td>22</td>
<td>0.018</td>
<td>0.019</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td></td>
<td>0.75</td>
<td>1.66</td>
<td>0.050</td>
<td>19</td>
<td>0.019</td>
<td>0.020</td>
</tr>
<tr>
<td>4</td>
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<td></td>
<td>1.00</td>
<td>1.78</td>
<td>0.046</td>
<td>15</td>
<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>5</td>
<td>3.1</td>
<td></td>
<td>1.50</td>
<td>1.98</td>
<td>0.042</td>
<td>11</td>
<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>6</td>
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<td></td>
<td>0.25</td>
<td>1.06</td>
<td>0.056</td>
<td>37</td>
<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>7</td>
<td>3.1</td>
<td></td>
<td>0.50</td>
<td>1.54</td>
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<tr>
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<td></td>
<td>0.75</td>
<td>2.00</td>
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<tr>
<td>9</td>
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<td>1.00</td>
<td>2.15</td>
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<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>10</td>
<td>3.1</td>
<td></td>
<td>1.50</td>
<td>2.65</td>
<td>0.057</td>
<td>15</td>
<td>0.019</td>
<td>0.019</td>
</tr>
</tbody>
</table>

\(a\) Oil volume was calculated using the values assumed to be 0.650 L-oil/mol for PE and 0.658 L-oil/mol for PC. The oil volume of Tween 20 was estimated to be 6.69 μL.

\(b\) Number of bisallylic hydrogens in one molecule.
constancy of the numbers in the last two row indicates kinetic orders in PE and PC to be 1. Thus, the oxidation of PE and PC in Tween 20 micelles follows the usual kinetic rate law and the oxidation rate can be represented by eq. 8. Barclay et al. reported the same kinetic rate law is applicable to the autoxidation of linoleate in sodium dodecyl sulfate micelles. Since the number of bisallylic hydrogens (active H) in one molecule of PE and PC are 2.59 and 2.94, the oxidizabilities \( \frac{k_p}{(2k_r)^{1/2}} \) for PE and PC per active H are 0.017 and 0.018 (s·mol/L·oil)^{-1/2}, respectively, as shown in Tables 1 and 2. The equality of these values suggests that differences in nitrogen moieties between PE and PC have no effect on the oxidation rates. This was confirmed by the findings that the values for oxidizability per active H of the mixtures of PE and PC were almost equal to that of PE or PC, and that the ratios of the oxidation rate of PE to that of PC were essentially identical with the ratios of the sum of active H of PE to that of PC (Table 3). This was the case in the oxidation of PE and PC in organic solvents.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>2</th>
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<th>12</th>
<th>7</th>
<th>13</th>
<th>14</th>
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<tbody>
<tr>
<td>PE/µmol</td>
<td>3.1</td>
<td>2.5</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[PE]/mol/L-oil</td>
<td>0.36</td>
<td>0.29</td>
<td>0.22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC/µmol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.1</td>
<td>2.5</td>
<td>1.9</td>
</tr>
<tr>
<td>[PC]/mol/L-oil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.36</td>
<td>0.29</td>
<td>0.22</td>
</tr>
<tr>
<td>14 : 0 PC/µmol</td>
<td>-</td>
<td>0.63</td>
<td>1.25</td>
<td>-</td>
<td>0.63</td>
<td>1.25</td>
</tr>
<tr>
<td>( R_p \times 10^6 ) /mol/L-oil per s</td>
<td>1.28</td>
<td>1.14</td>
<td>0.76</td>
<td>1.54</td>
<td>1.26</td>
<td>0.87</td>
</tr>
<tr>
<td>( R_i \times 10^6 ) /mol/L-oil per s</td>
<td>0.57</td>
<td>0.58</td>
<td>0.58</td>
<td>0.57</td>
<td>0.58</td>
<td>0.58</td>
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</table>

Kinetic chain length

<table>
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<th>11</th>
<th>12</th>
<th>7</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_p/R_i^{1/2} ([LH]) ) ( /([s·mol/L·oil]^{-1/2}) ) per active H</td>
<td>0.047</td>
<td>0.052</td>
<td>0.046</td>
<td>0.057</td>
<td>0.058</td>
<td>0.053</td>
</tr>
</tbody>
</table>

3.2 Oxidation induced by cupric ion

The oxidations of PE and PC induced by copper (II) ion were carried out in 5 mM phosphate buffer (pH 7.4). The oxidation of PE proceeded much faster than that of PC as shown in Fig. 2. In this system, the oxidation is assumed to be initiated by the radicals formed in the decomposition of hy-
The presence of hydroperoxides was

\[ \text{LOOH} + \text{Cu}^{II} \xrightarrow{k_1} \text{LO}_2^\cdot + \text{Cu}^{I} + \text{H}^+ \]  

confirmed by HPLC/isoluminol assay\(^10\).

The rate of chain initiation in this system \((R_i)\) was calculated from the observed rate of oxidation \([R_p, \text{obs.}]\) and the \(k_p/(2 k_t)^{1/2}\) value obtained in the previous part of this study using eq. 8. BMP was not used for the determination of \(R_i\) because it is expected to be dependent on hydroperoxide concentration as shown in eq. 12. Even when copper (II) ion was not added, considerable rates of oxidations of PE and PC were observed. Therefore, the rate chain initiation in the absence of copper (II) ion \((R_{i0})\) was calculated similarly using eq. 8. The rate of chain initiation due to the addition of copper (II) ion \([R_{i, \text{net}}]\) was calculated by subtraction of \(R_{i0}\) from \(R_i\), and then the rate of oxidation ascribed to the addition of copper (II) ion \([R_p, \text{net}]\) was calculated using eq. 8.

When we want to compare the susceptibilities of phospholipids to oxidation induced

\[ \begin{align*}
R_i & = 2 k_1 \left[ \text{LOOH} \right] \left[ \text{Cu}^{II} \right] \\
R_p & = k_p / (2 k_t)^{1/2} R_i^{1/2} \left[ \text{LH} \right] \\
& = k_p (k_1/k_t)^{1/2} \left[ \text{LOOH} \right]^{1/2} \left[ \text{Cu}^{II} \right]^{1/2} \left[ \text{LH} \right]
\end{align*} \]

where \(k_1\) is the rate constant for reaction 10.

**Table 4**  Effects of variation in the concentration of copper (II) ion on the oxidation of 0.31 mM PE and 0.31 mM PC in the presence of 0.02 mM conjugated diene in 0.25 % Tween 20 micelles at 37°C induced by copper (II) ion.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE/μmol</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PC/μmol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>[Cu^{II}]/μM</td>
<td>0</td>
<td>1.0</td>
<td>3.0</td>
<td>5.0</td>
<td>0</td>
<td>1.0</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>(R_p, \text{obs.}) \times 10^6) /mol/L-oil per s</td>
<td>2.9</td>
<td>5.1</td>
<td>7.2</td>
<td>9.3</td>
<td>0.72</td>
<td>0.96</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>(R_i \times 10^6) /mol/L-oil per s</td>
<td>2.9(^b)</td>
<td>9.0</td>
<td>18.0</td>
<td>30.5</td>
<td>0.12(^c)</td>
<td>0.22</td>
<td>0.41</td>
<td>0.55</td>
</tr>
<tr>
<td>(R_i, \text{net}) \times 10^6) /mol/L-oil per s</td>
<td>–</td>
<td>6.2</td>
<td>15.1</td>
<td>27.7</td>
<td>–</td>
<td>0.10</td>
<td>0.29</td>
<td>0.43</td>
</tr>
<tr>
<td>(R_p, \text{net}) \times 10^6) /mol/L-oil per s</td>
<td>–</td>
<td>4.2</td>
<td>6.6</td>
<td>8.9</td>
<td>–</td>
<td>0.63</td>
<td>1.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^{a}\) See footnote \(^a\), Table 1.

\(^{b}\) The value of \(R_{i0}\) for the oxidation of PE.

\(^{c}\) The value of \(R_{i0}\) for the oxidation of PC.
by the copper (II) ion, it is necessary to compare the rates of oxidation at the same concentration of hydroperoxides. Therefore, we selected 0.02 mM for conjugated diene concentration (about 5% conversion of the active methylene groups). The concentration of conjugated diene would be almost identical to that of hydroperoxides since the major polyunsaturated fatty acid contained in PE or PC used in this study was linoleate. The results were summarized in Table-4. Interestingly, the rate of oxidation of PE was much faster than that of PC. Since the oxidizabilities for PE and PC were almost the same as described before, the rate of chain initiation should be accelerated in the oxidation of PE. This may be explained by the formation of a complex between copper (II) ion and PE: this complex should increase the copper (II) ion concentration on the surface of PE micelle and the possibility of interaction of copper (II) ion and hydroperoxides which exist in the micelle. In fact, the high complex formation constant for O-phosphorylethanolamine and copper (II) ion is reported as 10^{6.61}.

Plots of the rate of oxidation against the square root of the concentration of copper (II) ion in the oxidation of PE and PC are shown in Fig.-3. Good linear correlations observed indicate that the rate of oxidation is proportional to the square root of the concentration of copper (II) ion in each oxidation, and these results are consistent with the relationships expected from eq. 13. The ratio of the slopes was 5.7, and hence the rate of oxidation per active H of PE is 6.5 (≈5.7 × 2.94/2.59) times as large as that of PC. If the difference in the rate of oxidation depends only on the difference in the concentration of copper (II) ion on the surface of each micelle, the concentration of cupric ion on the surface of PE micelle is estimated to be more than 40-fold compared with that of PC micelle.

In order to confirm the effect of the nitrogen moieties on the copper (II) ion induced oxidations of PE and PC, the oxidation of PC in the presence of 14:0 PE, PC, or O-phosphorylethanolamine was carried out (Fig.-4). The addition of 14:0 PE accelerat-

![Fig.-3](image1)

**Fig.-3** Plots of $R_p$ against $[Cu^{II}]^{1/2}$ in the oxidation of 0.31 mM PE (●) and 0.31 mM PC (■) in the presence of 0.02 mM conjugated diene in 0.25 % Tween 20 micelles at 37°C.

![Fig.-4](image2)

**Fig.-4** Formation of conjugated diene during oxidation of 0.25 mM PC mixed with 0.06 mM 14:0 PE (●) or 14:0 PC (■) and 0.31 mM PC in the presence (○) or absence (□) of 0.5 mM O-phosphorylethanolamine in Tween 20 micelles at 37°C induced by 3.0 μM copper (II) ion.

The data of the formation of conjugated diene of 0.31 mM PC were corrected for difference in the number of bisallylic hydrogens.
ed the copper (II) ion-induced oxidation of PC, whereas the addition of 14:0 PC did not. Furthermore, the addition of O-phosphorylethanolamine suppressed the oxidation, suggesting that the effective concentration of cupric ion for the decomposition of hydroperoxides would be reduced due to the formation of O-phosphorylethanolamine-copper (II) ion complex.

In summary, the azo compound-initiated oxidation of PE and PC in Tween 20 micelles follows the usual kinetic rate law and no effect of nitrogen moieties on the rate of oxidation was observed, suggesting that PE and PC have the same oxidizability if the content of bisallylic hydrogens is the same. On the other hand, large differences in chain initiation were suggested in copper (II) ion-induced oxidations of PE and PC probably because of the interaction of PE and copper (II) ion.

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References