OXYGEN ABSORPTION AT THE PROCESS OF THE DEGRADATION OF LINOLEIC ACID HYDROPEROXIDES

Kazuki Kanazawa,¹ Tomohiko Mori, and Setsuro Matsushita²

Research Institute for Food Science, Kyoto University, Kyoto
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Oxygen was absorbed during the process of decomposition of LAHPO to their SP. The oxygen absorption by LAHPO as well as oxidizing LA was retarded by the addition of antioxidants. The kinetics studies of oxygen absorption reaction were carried out using a polarographic electrode when LAHPO was in excess or by a respirometer when oxygen was in excess. In both cases, the reaction followed the first-order kinetics. Total amount of the absorbed oxygen by LAHPO was 1.26 moles per mole of LAHPO, that is, the stoichiometric ratio of the reaction of LAHPO with oxygen was 1 to 1. When LAHPO was incubated under oxygen-free atmosphere in the presence of a catalyst, the decomposition of LAHPO was remarkably reduced. Thus, the oxygen absorption at the degradation steps of LAHPO was due to LAHPO itself and not to SP, and LAHPO was decomposed by the oxygen action. A possible scheme containing linoleic acid dihydroperoxide which was a reaction intermediate at the degradation process of LAHPO was discussed.

The radical mechanism of fatty acid autoxidation has been thoroughly studied (1-3), but the origin of the initial free radicals necessary to begin the autoxidation process in fatty acids has not been clarified (4-6). The oxidation propagation in the presence of air is usually assumed to be initiated by the breakdown of hydroperoxides which are regenerated during the chain reaction.

The hydroperoxides are decomposed to SP (carbonyl compounds, alcohols, acids, etc.). These products are highly susceptible to further oxidation (7, 8). The complicated and dynamic nature of SP makes the quantitative study of their

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¹ Present address: Department of Agricultural Chemistry, Kobe University, Kobe.

Abbreviations: LAHPO, linoleic acid hydroperoxides; SP, secondary degradation products; LA, linoleic acid; POV, peroxide value; TLC, thin-layer chromatography; BHA, butylated hydroxyanizole.
occurrence in the autooxidizing lipids very difficult. The toxicity of hydroperoxides and their SP has been studied (9, 10), and both compounds have been shown to be toxic, but the mechanisms involved are quite different (11, 12). To solve these problems, the mechanism of LAHPO decomposition to form SP must be made clear. The autoxidation mechanism of LA has been proposed by BOLLAND (13) and BATEMAN (14) on the basis of the basic autoxidation scheme of hydrocarbon. On the other hand, JOHNSTON et al. (15) indicated that LAHPO consumed 1 mole of oxygen, but did not describe the mechanism within the process of the degradation of LAHPO. Given these considerations, an attempt was made to elucidate kinetically the degradation mechanism of LAHPO.

EXPERIMENTAL

LA was purchased from Tokyo Kasei Co., and LA-1-14C (35.0 mCi per mmole) was obtained from Daiichi Chemical Co.

Preparation of LAHPO. LA was autoxidized, and LAHPO was fractionated as described in a previous paper (11). Autoxidized LA was subjected to silica gel column chromatography, and the LAHPO fraction was further purified (12) by preparative TLC (Merck silica gel PF254 containing CaSO4) using ethyl ether: n-hexane: acetic acid (60: 40: 1.5) as the solvent system.

Determination of the purity of LAHPO. The purified LAHPO was characterized by the POV method (16), UV spectrophotometry (17) and carboxyl titration with tetra-n-butylammonium hydroxide (18). From the above determinations, the purity of LAHPO was estimated to be between 92 to 97%. However, LA or SP could not be detected on TLC.

SP. SP was obtained from the ethyl ether eluate of the same column as described above. The concentration of SP was expressed as acetaldehyde (19). The fractionated SP was free of LAHPO or LA as determined by TLC.

Labelled LAHPO. In the presence of 4 ml of carrier LA, 0.2 mCi of LA-1-14C was autoxidized, fractionated and purified by the same method as for LAHPO. The purified LAHPO-1-14C obtained was 1.65 x 10^4 cpm per μmole (0.5 μCi/μmole)

The concentration and purity of labelled LAHPO were determined by the same procedures as described above.

TLC. TLC was used (Merck silica gel plate No. 5721), for the detection of LA, LAHPO or SP. The solvent system used was the same as shown in the method for LAHPO. The spots were detected by exposing the plates to iodine vapour, or spraying 5% phenylhydrazine in 0.1 N HCl methanol solution. With the phenylhydrazine reagent, orange red colour was developed by LAHPO, and yellow red colour was developed by SP.

Determination of oxygen absorption in aqueous emulsion. Twenty milliliters of oxygen-saturated water containing FeSO_4 (2 x 10^{-6} M) was taken into a beaker
and was placed in a water bath at 37°C, and was stirred continuously with a magnetic stirrer (20). While oxygen absorption was determined, the reaction mixture was kept exposed to atmosphere. However, for determining the rate constants of oxygen absorption reaction, the reaction mixture was covered over with liquid paraffin to prevent from atmospheric oxygen before the insertion of a polarographic electrode (Beckman oxygen analyzer Model 39550). After stabilization, 0.5 ml of LAHPO or SP methanol solution was added into water layer by injection. The rate of oxygen absorption was recorded with polarography (Yanaco Model P8). Oxygen-saturated water was obtained by bubbling air for 3 hr and oxygen-free water was prepared by adding sodium sulfite.

Determination of oxygen absorption by a respirometer. Gilson differential respirometer Model GRP 14 was used. The main chamber contained 1.5 ml of LAHPO emulsion and the side chamber contained 0.5 ml of FeSO₄ solution. After stabilization, the catalyzer solution was mixed into LAHPO emulsion and oxygen consumption was read (21). The reaction temperature was kept at 37°C and the chamber was continuously shaken.

Method of removing oxygen from Thumberg tube. The sample was put in a Thumberg tube and the catalyzer solution was taken into the side arm. The tube was cooled in liquid nitrogen and evacuated with a vacuum pump. Then, the tube was warmed at room temperature and argon gas was introduced. These procedures were repeated several times.

Measurement of radioactivity. The incubated LAHPO-1-14C was developed on TLC in parallel. One of these was detected with iodine vapour and the other was detected by phenylhydrazine reagent as shown in the method for TLC. After the iodine was evaporated out from the former plate, the spot and residual parts were scraped, and the radioactivity was measured with Packard scintillation counter Model 2002. The scintillator used consisted of 4 g of POPOP and 0.1 g of PPO in 1 liter solution of toluene: dioxane: ethyl cellosolve (1:1:1) containing 75 g of naphthalene. Packard thixotropic gel powder (CAB-O-SIL) was added to the sample solution before counting.

RESULTS

Dissolved oxygen absorption by LAHPO

LA, LAHPO and SP were emulsified in the oxygen-saturated water containing ferrous sulfate, and oxygen absorption was determined as described in "Experimental" (Fig. 1). LAHPO absorbed dissolved oxygen. LAHPO was very reactive even under low oxygen pressure. The absorption rate of LA was fast at the initial stage. But after 50% of the dissolved oxygen was consumed, the rate became low and was similar to the dissolution rate of the atmospheric oxygen into the reaction mixture. Thus, LAHPO was more reactive with dissolved oxygen than LA. SP also absorbed a little dissolved oxygen, but the direct comparison
Fig. 1. Dissolved oxygen consumption determined by polarographic oxygen analyzer. The experimental conditions are shown in "Experimental." The final concentration of LAHPO (——), and LA (—), were $5 \times 10^{-8}$ M, and that of SP (-----) was $10^{-2}$ M as acetaldehyde (or $3 \times 10^{-3}$ M by POV).

The rate of oxygen absorption with the rate of LAHPO or LA may be difficult because of SP being a mixture of aldehydes, ketones, alcohols, acids, etc. BHA or uric acid was added to the reaction mixture. Figures 2A and B show the effect of the antioxidants on the oxygen absorption of LA and LAHPO, respectively. The oxygen absorption by LAHPO as well as that of LA was reduced by the addition of antioxidants (22). These results suggest that the oxygen absorption by LAHPO depends on a radical reaction.

**Rate of oxygen absorption measured by polarography**

Oxygen absorption was determined by polarography under the conditions described in "Experimental." The oxygen absorption curves were expressed in the following formulas.

The rate of the reaction can be expressed as the rate of decrease of the concentration of oxygen $[O_2]$ at any time $t$. If the substance chosen is a reactant, the rate $v = -d[O_2]/dt$. It is clear that the reaction involves two reactants, LAHPO and oxygen, from the result in Fig. 1. Therefore, the rate can be expressed in relation to the concentration of the reactants by the equation $-d[O_2]/dt = k[LAHPO]^m[O_2]^n$, where $k$ is the rate coefficient, $m$ and $n$ are constants. The reaction is determined to be of the $m$th order with respect to LAHPO, and the $n$th order with respect to oxygen. If one reactant is in excess of the other, the rate equation can be expressed in a simple form. About 4.5 μmoles of dissolved oxygen is contained in the reaction mixture. LAHPO is in large
Fig. 2. Effects of antioxidants to the oxygen absorption by LA (A) and LAHPO (B). The experimental conditions are the same as in Fig. 1 except adding antioxidants. — - - , addition of $5 \times 10^{-4}$ M uric acid, -----, addition of $5 \times 10^{-5}$ M BHA, ----, no addition.

excess of oxygen (Fig. 3A). The other reactant $[O_2]$ falls with time. The decrease of $[O_2]$ is equal to $([O_2]_0 - [O_2]_t)$. Then the rate equation of the differential form is $-d[O_2]/dt = k'([O_2]_0 - [O_2])^n$, where $[O_2]_0$ is the concentration of dissolved oxygen at the initial stage, and $[O_2]_t$ is that of time $t$. Here, if $n = 1$, the above formula integrates and the constant of the integration is denoted by the initial concentration of oxygen at time $t = 0$, so that $\ln ([O_2]_0/([O_2]_0 - [O_2]_t)) = k't$. The procedure for testing the applicability of this equation to the reaction would be to determine $k'$ at various time intervals during the course of the reaction. Thus, when the time is plotted versus $\log [O_2]/([O_2]_0 - [O_2]_t)$, $k'$ can be obtained from Fig. 3A. The oxygen absorption reaction by LAHPO follows the first-order with respect to oxygen in the above assay system. On the other hand, when LAHPO and dissolved oxygen were in equimolar proportion, the reaction follows the second order (Fig. 3B). If 1 mole of oxygen is consumed by 1 mole of LAHPO, the decrement of LAHPO equals the decrease of dissolved oxygen. When the time is plotted versus $[O_2]_0/[O_2]_0([O_2]_0 - [O_2]_t)$, $k$ can be obtained from Fig. 3B. The oxygen absorption reaction by LAHPO contains no subsequent reaction steps.
268 K. KANAZAWA, T. MORI, and S. MATSUSHITA

Fig. 3. The kinetics curve of the oxygen absorption by LAHPO determined by polarographic oxygen analyzer when LAHPO was in excess of dissolved oxygen (A) and LAHPO and oxygen were in an equimolar proportion system (B). — — 100 μmoles of LAHPO (The rate coefficient of the reaction is 0.469 min⁻¹). — — 4.5 μmoles of LAHPO (0.00838 min⁻¹). 4.5 μmoles of dissolved oxygen was contained in these systems.

Rate of oxygen absorption measured by a respirometer

When the other reactant, oxygen, is present in large excess, the kinetics order was observed by a respirometer as described in “Experiment”. The decrease of [LAHPO] corresponded with the decrement of [O₂] (Fig. 3B). Therefore, if m = 1, log [O₂]₀/[O₂]₀−[O₂]ᵣ can be plotted versus time in the same way as described in the polarographic result. The rate constant k was determined in different concentrations of catalyzer (Fig. 4). The reaction followed the first order with respect to LAHPO in the assay system. To determine whether the other reaction steps are involved in the reaction or not, the half-period method was used with the catalyzer being kept constant. Since the half-period method involves the measurement of the time taken for one-half of
the original reacting substance to disappear, the value of the half-period is related to the rate constant. The rate coefficients were nearly equal to 0.0249 min⁻¹, even when LAHPO concentration at the beginning of the reaction was changed to 4/5, 3/5 or 2/5 (Fig. 5). The reaction of LAHPO with oxygen corresponds to an elementary reaction.

Fig. 4. The kinetics curve of the oxygen consumption by LAHPO determined by a respirometer. The concentration of LAHPO at the beginning of the reaction was 10.7 μmoles. The concentration of catalyzer (FeSO₄) was changed as follows. —×—, 4 × 10⁻² M (The rate coefficient is 0.0295 min⁻¹); —○—, 10⁻² M (0.0153); —△—, 10⁻³ M (0.00390); —□—, catalyzer free (0.000991). The gas phase volume of the reaction chamber of the respirometer was 25 ml.

Overall oxygen absorption

For the stoichiometric determination, the total amount of oxygen consumption was measured with a respirometer by the same procedure in Fig. 4. One mole of LA absorbed 2 moles of oxygen or more and 1 mole of LAHPO absorbed 1 mole of oxygen or more in all cases whether the catalyzer was added or not (Table 1). The stoichiometric ratio of oxygen to LAHPO seems to be 1 to 1.

On the other hand, LAHPO decomposes to SP with oxygen absorption. LAHPO was incubated in a respirometer, oxygen consumption by LAHPO was measured and the products were detected by TLC (Fig. 6). The number of spots of SP increased, while LAHPO spots reduced on TLC as the reaction proceeds.
The kinetics curve of the oxygen absorption by LAHPO determined by a respirometer. When the concentration of the catalyst (FeSO₄) was constant, 3.5 × 10⁻⁴ M, the concentration of LAHPO at the beginning of the reaction was changed as follows, _×_, 10.7 μmoles (rate coefficient is 0.0287 min⁻¹); _○_, 8.6 μmoles (0.0252); _□_, 6.4 μmoles (0.0232); _△_, 4.3 μmoles (0.0240). The slope of the curve is 0.0249 min⁻¹.

Table 1. Total oxygen absorption by LAHPO.

The oxygen absorption was determined by a respirometer as described in the text.

<table>
<thead>
<tr>
<th>Concentration of sample at the beginning of the reaction</th>
<th>Concentration of FeSO₄</th>
<th>Reaction time (min)</th>
<th>μmoles of consumed O₂/μmoles of added sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.7 μmoles of LAHPO</td>
<td>2 × 10⁻² M</td>
<td>1380</td>
<td>1.26</td>
</tr>
<tr>
<td>6.0 μmoles of LAHPO</td>
<td>5 × 10⁻³ M</td>
<td>1085</td>
<td>1.42</td>
</tr>
<tr>
<td>6.0 μmoles of LAHPO</td>
<td>10⁻⁸ M</td>
<td>1085</td>
<td>1.25</td>
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<tr>
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<td>5 × 10⁻⁴ M</td>
<td>1415</td>
<td>1.07</td>
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<tr>
<td>8.0 μmoles of LAHPO</td>
<td>0</td>
<td>2040</td>
<td>1.02</td>
</tr>
<tr>
<td>5.52 μmoles of LA b</td>
<td>2 × 10⁻² M</td>
<td>1000</td>
<td>2.36</td>
</tr>
</tbody>
</table>

  a The reaction mixture was incubated until the oxygen absorption reached the maximum.
  b LA was purified by TLC and the concentration was determined by titrating with tetra-n-butylammonium hydroxide (18).

Degradation of LAHPO under oxygen-free condition

Clarifying the oxygen absorption reaction by LAHPO is an elementary reaction, one reactant, LAHPO, was incubated in the absence of the other re-
OXYGEN ABSORPTION BY LINOLEIC ACID HYDROPEROXIDES

Fig. 6. Degradation of LAHPO with the increase of oxygen absorption. LAHPO was incubated in a respirometer, and at certain intervals, LAHPO and their SP were extracted from the reaction mixture with ethyl ether several times. The ether layer was applied on a TLC qualitatively.

actant, oxygen, at 37°C for 1 hr. The LAHPO remained intact, when LAHPO was incubated under an oxygen-free system (Fig. 7). The results in Table 2 suggest a little degradation of LAHPO. But, apparently, as the concentration of LAHPO at the initial stage increased, the degradation percentage of the incubated LAHPO decreased remarkably. The LAHPO may not be decomposed without oxygen and the reaction of LAHPO with oxygen contains an elementary process.

DISCUSSION

LAHPO obviously absorbed oxygen (Fig. 1). Besides, LAHPO was more reactive than LA or SP even under low oxygen pressure. The oxygen absorption by LAHPO as well as that of LA might be a radical reaction (Figs. 2A and B). For making the reaction mechanism of LAHPO with oxygen clear, the rate coefficients were determined in a steady state treatment by the addition of a suitable amount of catalyzer and before the termination reaction started. When one reactant was isolated by the other reactant being present in excess, the rate of the reaction was observed by the decrease of the oxygen concentration. These oxygen absorption curves were adapted to the simple formulas as described in
Table 2. LAHPO left after incubating for 1 hr under oxygen-free atmosphere at 37°C. The experimental conditions are shown in the text.

<table>
<thead>
<tr>
<th>μmoles of LAHPO</th>
<th>Oxygen</th>
<th>Incubation (hr)</th>
<th>LAHPO left (%)&lt;sup&gt;a&lt;/sup&gt; Exp. I</th>
<th>LAHPO left (%)&lt;sup&gt;a&lt;/sup&gt; Exp. II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity check&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>93.6</td>
<td>89.5</td>
</tr>
<tr>
<td>6.5</td>
<td>+</td>
<td>1</td>
<td>20.9</td>
<td>21.0</td>
</tr>
<tr>
<td>6.5</td>
<td>—</td>
<td>0</td>
<td>89.5</td>
<td>87.0</td>
</tr>
<tr>
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<td>—</td>
<td>1</td>
<td>67.6</td>
<td>65.2</td>
</tr>
<tr>
<td>13.0</td>
<td>—</td>
<td>1</td>
<td>76.3</td>
<td>72.4</td>
</tr>
<tr>
<td>15.2</td>
<td>—</td>
<td>1</td>
<td>88.0</td>
<td>87.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Radioactivity at the region of Rf 0.35–0.48/total radioactivity applied on TLC.

<sup>b</sup> Authentic sample of labelled LAHPO as shown in Fig. 7.

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Fig. 7. Degradation of LAHPO by incubating under oxygen-free atmosphere. After the gas phase of Thumberg tube was exchanged with argon gas as described in Experimental, the tube was incubated at 37°C for 1 hr and was cooled in liquid nitrogen. Two ml of argon-saturated ethyl ether was introduced to the tube. Then, air in the tube was exchanged with argon. The content of the tube was melted at room temperature, and the reaction products were extracted into ether layer by shaking. The ethyl ether layer was quantitatively subjected to TLC in an argon-saturated chamber. After development, the spots on the plates were detected as shown in “Experimental.” 1, authentic sample of LAHPO; 2, 6.5 μmoles of LAHPO was incubated for 1 hr in air; 3, 4 and 5, 6.5, 13.0 and 15.2 μmoles of LAHPO, respectively, was incubated at 37°C for 1 hr under oxygen-free atmosphere.

“Results.” The reaction followed the first order with respect to LAHPO or oxygen (Figs. 3A and 4). By stoichiometric analysis, 1 mole of LA consumed 2 moles of oxygen or more and 1 mole of LAHPO absorbed 1 mole of oxygen or
OXYGEN ABSORPTION BY LINOLEIC ACID HYDROPEROXIDES

more (Table 1). LA produced LAHPO and SP, and LAHPO yielded SP during the reaction. These products also absorbed oxygen (Fig. 1). The decrease of [LAHPO] or the increase of [SP] could not be measured. But Fig. 6 suggests the changes of [LAHPO] and [SP] as the reaction proceeded. The ratio of oxygen to LAHPO is considered to be 1 to 1.

Even when a reaction rate varies with the reactant concentration in accordance with the equation, it may be difficult to decide whether it is an elementary reaction or not. But in the equimolar proportion system of LAHPO and oxygen, the reaction follows the second order (Fig. 3B). The rate coefficients of the reaction were usually kept constant in all cases by the half-period method (Fig. 5). Furthermore, the rate of complex reaction will not fit in the results in Figs. 3A and 4. Thus, the reaction of LAHPO with oxygen seems to contain only one reaction step.

LAHPO has been reported to be polymerized even under nitrogen atmosphere (15). If the reaction is a complex reaction, oxygen is absorbed by SP, and LAHPO is decomposed by autolysis without oxygen action. Under this experimental condition, it is practically impossible to eliminate oxygen completely from the reaction system. But when the remaining oxygen was consumed by LAHPO, the residual LAHPO might not be decomposed. Given these considerations, the effect of an increased concentration of LAHPO at the beginning of the reaction was observed. The above supposition was supported (Table 2). When LAHPO was incubated at 37°C for 1 hr in the medium containing the catalyzer, the amount of remaining LAHPO approached that of no-incubation as the concentration of LAHPO at the initial stage was increased. However, a very small amount of oxygen left is not considered constant from the calculation of the absolute quantity of the degraded LAHPO (Table 2). It may be difficult to say that no autolysis of LAHPO occurs by the radicals produced by the catalyzer. But the percentage of the degradation of LAHPO was remarkably reduced without oxygen (Table 2). The rate of the reaction was measured under oxygen-containing atmosphere under similar conditions. Moreover, INGLETT et al. (23) indicated that no radical existed in an oxidizing system of LAHPO under a nitrogen atmosphere. For these reasons, it can be considered that the reaction of LAHPO with oxygen is an elementary reaction and the oxygen is absorbed to LAHPO itself and not to SP on the autoxidation system containing oxygen.

Water which works as an inhibitor (24, 25) did not influence the reaction velocity. At low oxygen pressure, metal catalyzers such as Cu2+ and Fe3+ accelerate the termination of radicals more specifically than the oxygen absorption by LAHPO. But a metal catalyzer such as Fe2+ does not have this function (26). Such new spots were not found on TLC of the incubated LAHPO (Fig. 7). Carboxyl group of LAHPO generates CO2 by the action of metal catalyzers (27), and labelled LAHPO may become nonradioactive products. But such spots were not detected by TLC (Fig. 7).
From the foregoing observations, it is considered that the LAHPO itself reacted with oxygen and that the LAHPO was very stable in the presence of metal catalyzer or by heating in the absence of oxygen. Thus, oxygen played the part of radical initiator (28, 29) as well as reactant.

From the results obtained above, a possible mechanism containing linoleic acid dihydroperoxide can be proposed (Fig. 8). The pathways, A, B, C and D, have already been ascertained or suggested (7, 30–32), and the pathway E is proposed from the present experiments. The pathways F, G and H could be suggested by these overall considerations. The compound 'OOLOOH may be very unstable and may easily decompose. The supposition in this scheme may explain the fact that many kinds of products are detected in the autoxidation of fatty acids (33) as in the case of hydrocarbons (34). But the detailed mechanism of autoxidation of lipids must be ascertained more specifically by the structural analysis of the SP or by the analysis of the terminal products containing volatile compounds (35–37).

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OXYGEN ABSORPTION BY LINOLEIC ACID HYDROPEROXIDES

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