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The Role of Iron in Peroxidation of Polyunsaturated Fatty Acids in Liposomes

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This work investigated iron-catalyzed lipid oxidation in marine phospholipid liposomes. Oxygen consumption was used as a method to study lipid oxidation at pH 5.5 and 30 °C. The relationship between consumed oxygen and amount of peroxides (PV) and thiobarbituric reactive substances (TBARS) formed showed that both Fe^{2+} and Fe^{3+} catalyzed lipid oxidation. When Fe^{2+} was added to liposomes at a concentration of approximately 10 μM, an initial drop in dissolved oxygen (oxygen uptake rate >258 μM/min), followed by a slower linear oxygen uptake (oxygen uptake rate 4–6 μM/min), was observed. Addition of Fe^{3+} induced only the linear oxygen uptake. The initial fast drop in dissolved oxygen was due to oxidation of Fe^{2+} to Fe^{3+} by preexisting lipid peroxides (rate 79 μM Fe^{2+}/min). Fe^{3+} is reduced by peroxides to Fe^{2+} at a slow rate (0.25 μM Fe^{3+}/min at 30 °C) in a pseudo-first-order reaction. The redox cycling between Fe^{2+} and Fe^{3+} leads to an equilibrium between Fe^{2+} and Fe^{3+} resulting in a linear oxygen uptake. During the linear oxygen uptake, the interaction of Fe^{3+} with lipid peroxide is the rate-limiting factor. Both alkoxy and peroxy radicals are formed by breakdown of peroxides by Fe^{2+} and Fe^{3+}. These radicals react with fatty acids giving lipid radicals reacting with oxygen.

KEYWORDS: Iron; oxidation; phospholipids; liposomes

INTRODUCTION

Liposomes made from marine phospholipids have a high potential as an oral supplement for long-chain polyunsaturated fatty acid due to the observed higher lipid bioavailability from liposomes compared to fish oil (*1*). They also have potential as an α-tocopherol supplement (*2*) and as delivery systems in pharmacology (*3*). However, high susceptibility to oxidation limits the utilization of marine phospholipids both in processed foods and as nutritional supplements. Lipids dispersed in an aqueous matrix may be in contact with a variety of water-soluble components including transition metals. Among the transition metals, iron is one of the most important prooxidants for lipid oxidation (*4*). Understanding iron-mediated lipid oxidation will therefore help to make oxidatively stable marine phospholipid liposomes.

Ferrous iron can promote lipid oxidation by: the formation of reactive hydroxyl radicals in Fenton reactions, breakdown of preformed lipid hydroperoxides, or generation of H_2O_2 consumed by the Fenton reaction (*5*). As almost all lipids contain at least traces of peroxides, studies of lipid oxidation in aqueous colloidal systems suggest that, during the propagation step, the interaction between lipid hydroperoxides (LOOH) located at or near the droplet surface and transition metals, originating in the aqueous phase, is the most common cause of oxidative instability (*6–8*). In several studies (*7, 9*) it was observed that when peroxides were removed, iron did not induce lipid peroxidation. LOOH-dependent initiation has been proposed to occur through two pathways (*2*): (a) LOOH breakdown by Fe^{3+} and subsequent hydrogen abstraction by $LOO^{\bullet}$ (reactions 1 and 2)

$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + H^{+} + Fe^{2+} \quad (1)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (2)$$

or (b) LOOH breakdown by Fe^{2+} to free radicals (reactions 3 and 4)

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \quad (3)$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet} \quad (4)$$

Lipid radical ($L^{\bullet}$) can react with diradical triplet oxygen (O_2) to form peroxy radical ($LOO^{\bullet}$) (*10*). The $LOO^{\bullet}$ formed can react further in reaction 2 and form lipid radical ($L^{\bullet}$). Hence a single formed $L^{\bullet}$ can result in conversion of several fatty acid side chains into lipid hydroperoxides, accompanied by oxygen consumption. The decomposition of peroxy radicals ($LOO^{\bullet}$) also results in the accumulation of short-chain lipid peroxidation end products, mostly aldehydes (*11*).

A possible circulation between Fe^{2+} and Fe^{3+} through peroxide decomposition reactions 1 and 3 was proposed by

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Pokorny (*12*) to be an important source of free radicals, but this circulation theory does not seem to have attracted much attention.

Several studies have investigated iron-induced oxidation in liposomes, and different oxidation kinetics have been described. In a few studies, a lag period without accumulation of lipid peroxidation products was observed. This lag period was hypothesized to be the time necessary to reach a 1:1 ratio between Fe^{2+} and Fe^{3+}, which is required to initiate oxidation (*13*). Driomina et al. (*14*), Tampo and Yonaha (*15*), and Tang et al. (*16*) proposed that reaction between Fe^{2+} and lipid peroxide was responsible for the initiation of lipid peroxidation and that lipid peroxidation could only start when the concentration of Fe^{2+} was reduced to a critical value. Before reaching this critical value it was proposed that Fe^{2+} interacted with lipid radicals. In a few studies it was observed that oxidation initiated by Fe^{2+} had two phases: a fast phase taking place in a short period followed by a slower oxidation phase (*17–21*). However, the mechanism of oxidation in these two phases was not clarified. Chaiyasit et al. (*21*) observed a rapid decomposition of cumene hydroperoxide followed by small changes in peroxide value after addition of Fe^{2+} in hexadecane emulsions. The changes in rate of peroxide depletion were explained by depletion of Fe^{2+} with the resulting Fe^{3+} being unable to significantly promote peroxide decomposition. Fukuzawa et al. (*9*) explained the oxidation induced by Fe^{2+} to be initiated by peroxide breakdown to alkoxy radical accompanied by oxygen consumption. The rapidly reached constant rate was explained by the limiting availability of LOOH.

The objective of this work has been to study the role of iron in peroxidation of phospholipids in liposomes. Oxygen uptake by liposomes was used to continuously monitor peroxidation of unsaturated fatty acids. The oxidation kinetics and oxidation pathways during iron-induced oxidation of unsaturated fatty acids have been investigated.

MATERIALS AND METHODS

Chemicals. 2-Morpholinoethanesulfonic acid (MES), 2,2-dipyridyl, triphenylphosphine (TPP), $FeSO_4$, $FeCl_3$, butylated hydroxytoluene, thiobarbituric acid, and 1,1,3,3-tetraethoxypropane (TEPE) were from Sigma-Aldrich; *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) and cumene hydroperoxide (CumOOH) were from Fluka; ethylenediaminetetraacetic acid (EDTA) and $(NH_4)_2Fe(SO_4)_2$ (titrisol) were from Merck.

Phospholipids. Phospholipids used in the experiments were isolated from North Atlantic cod (*Gadus morhua*) roe. Before extraction, the cod roe was kept at –40 °C.

The extraction of total lipids was performed according to the method of Bligh and Dyer (*22*). Phospholipids were isolated from total lipids using the acetone precipitation method (*23*), with a few modifications as described by Mozuraityte et al. (*17*). The fatty acid composition of the phospholipids was analyzed by gas chromatography of fatty acid methyl esters, and the lipid classes were determined by thin-layer chromatography as described in Mozuraityte et al. (*18*). Isolated phospholipids contained 98 ± 2% of phospholipids, traces of cholesterol, and unknown compounds. In average, the isolated phospholipids contained 30 ± 4% saturated, 29 ± 5% monounsaturated, and 41 ± 9% polyunsaturated fatty acids. DHA made up 26 ± 9% and EPA made up 11 ± 2% of total fatty acids.

Preparation of Liposomes. Liposomes were made as described by Mozuraityte et al. (*17*). Chloroform solution of phospholipids was evaporated to dryness with a stream of nitrogen gas. The residual solvent was completely evaporated under vacuum. The liposomes which were prepared with *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) or triphenylphosphine (TPP) were prepared from phospholipids that had been mixed with either TPP or DPPD in chloroform and then dried under nitrogen. Phospholipids were sonicated in a 5 mM MES buffer, pH 5.5, with an MSE ultrasonic disintegrator Mk2 (MSE Scientific instruments, Sussex, England) at a lipid concentration of 30 mg/mL. The lipid concentration in the liposome solution was 6 mg/mL in all experiments if nothing else is mentioned. To verify the experiment temperature, the temperature was measured directly in the cell. All experiments were performed at 30 °C.

Oxidation Experiments. The consumption of dissolved oxygen by liposomes in a closed, stirred, water-jacketed cell was used as a measure of lipid oxidation. The concentration of dissolved oxygen was measured continuously by a polarographic oxygen electrode (Hansatech Instrument Ltd., Norfolk, U.K.). When measuring dissolved oxygen concentration, the background oxygen uptake rate was observed for 4–6 min before Fe^{2+} or Fe^{3+} injection. Twenty microliters of $FeSO_4$ or $FeCl_3$ working solution was injected through a capillary opening in the cell to catalyze lipid oxidation in 1 mL of liposome solution. A stock solution of $FeSO_4 \cdot 7H_2O$ (99.5%, Merck) or $FeCl_3$ (15 and 30 mM) in 0.5 N HCl was prepared fresh every month. The working solution (40 times diluted Fe^{2+} or Fe^{3+} stock solution in 5 mM MES buffer, pH 5.5) was prepared daily. The duration of experiments for oxygen uptake was approximately 20 min.

For studies of the relationship between oxygen uptake, PV, and TBARS, the experiments were performed in a closed test tube with a screw cap and rubber septum. The vessel, with a glass ball inside to facilitate mixing, was completely filled with liposome solution and kept in a water bath at 30 °C. To catalyze the lipid oxidation, Fe^{2+} or Fe^{3+} working solution with a final concentration of 7.5 μM for Fe^{2+} and 15 μM for Fe^{3+} in the liposome solution was injected. After the desired reaction time, the oxidation was stopped by the injection of a solution of EDTA (0.75 mM in 5 mM pH 5.5 MES buffer) with a final concentration of 15 μM. After oxidation, the concentration of dissolved oxygen in the liposome solution was measured. Seven milliliters of the liposome solution was used for fat extraction and subsequent PV measurements. For analyses of the peroxide value (PV) in liposomes prepared with triphenylphosphine (TPP) the fat was extracted before measurement of PV. Chloroform/methanol (1:1) was used for the extraction of lipids from liposomes. Peroxide value (PV) was analyzed by the ferric thiocyanate method as described by the International Dairy Federation (*24*) and modified by Ueda et al. (*25*) and Undeland et al. (*26*). Cumene hydroperoxide was used as standard for quantitative peroxide analyses. The analysis was performed in triplicate.

Thiobarbituric acid reactive substances (TBARS) were analyzed in the water phase as described by McDonald and Hultin (*27*) using 0.2 mL of liposome solution, 0.8 mL of H_2O, and 2 mL of TBAR reagent. The absorbance values were compared to a standard curve prepared with 1,1,3,3-tetraethoxypropane for the calculation of TBARS concentrations. The analysis was performed in triplicate.

Determination of the Oxidation of Fe^{2+}. Fe^{2+} concentration measurements were performed as described by Fukuzawa and Fujii (*28*). Forty microliters of 375 mM 2,2-dipyridyl (final concentration, 5 mM) was added to 3 mL of liposome solution, and the absorbance was measured at 520 nm. Fe^{2+} concentration was calculated using an extinction coefficient of 9600 cm^{-1} M^{-1}. The extinction coefficient was obtained from a standard curve based on $(NH_4)_2Fe(SO_4)_2$ (titrisol) in MES buffer.

Determination of the rate of Fe^{2+} production during reduction of Fe^{3+} was performed by following the increase in absorbance due to production of the 2,2-dipyridyl−Fe^{2+} complex as a function of time. 2,2-Dipyridyl solution was added to the samples after different oxidation times, mixed with 2,2-dipyridyl, and quickly transferred to the cuvette, and the increase in absorbance was recorded. The concentration of Fe^{2+} at the time of withdrawal of the sample was found by extrapolation back to zero time. The rate of Fe^{3+} reduction was calculated from the increase in the absorbance of the 2,2-dipyridyl−Fe^{2+} complex.

Microsoft Excel was used for data processing and statistical analyses. The significance level was set at 95% ($p = 0.05$).

RESULTS AND DISCUSSION

Oxidation Catalyzed by Fe^{2+} and Fe^{3+}. Oxidation of polyunsaturated fatty acids (PUFAs), induced by Fe^{2+} and Fe^{3+}, was performed in a closed system containing a solution of liposomes. Both Fe^{2+} and Fe^{3+} induced lipid oxidation of cod

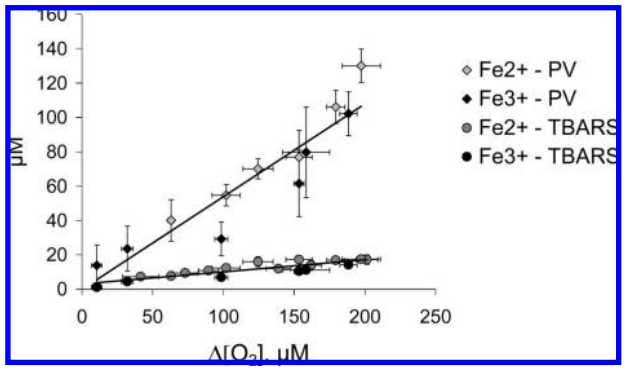

Figure 1. Concentration of formed lipid peroxide (PV) and thiobarbituric reactive substances (TBARS) as a function of consumed oxygen during the oxidation of unsaturated fatty acids. The concentrations of Fe^{2+} and Fe^{3+} used to initiate oxidation were 7.5 or 15 μM. The phospholipid concentration in liposomes was 9 mg/mL. Each point represents an average of four determinations, and standard deviation is given as error bars.

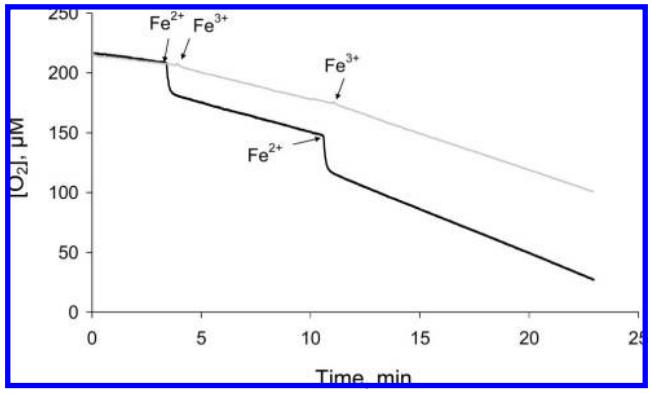

Figure 2. Concentration of dissolved oxygen in liposome solution after addition of Fe^{2+} or Fe^{3+}. Each addition increased the iron concentration by 7.5 μM. The phospholipid concentration in the liposome solution was 6 mg/mL.

roe phospholipids in the liposomes. The iron-induced oxidation was measured as consumption of dissolved oxygen accompanied by an increase in peroxides (PV) and thiobarbituric acid reactive substances (TBARS) (**Figure 1**). Data from the lipid oxidation experiments induced by Fe^{2+} and Fe^{3+} were pooled together, and a significant linear relationship ($p < 0.05$) between consumed oxygen and amount of PV and TBARS was observed, indicating that both Fe^{2+} and Fe^{3+} ions catalyzed the same type of oxidation. Low concentrations of Fe^{2+} or Fe^{3+} (i.e., 7.5 μM) resulted in consumption of all dissolved oxygen (at 30 °C, ~230 μM) and formation of ~100 μM PV (calculated with cumene hydroperoxide as a reference) and ~20 μM TBARS, indicating that a single iron molecule leads to formation of several molecules of peroxides and TBARS (**Figure 1**).

Initial Drop in Dissolved Oxygen. When Fe^{2+} was added to liposomes to catalyze lipid oxidation, an *initial drop* in concentration of dissolved oxygen was observed; this was followed by a slower *linear decrease* in concentration of dissolved oxygen (**Figure 2**). The initial drop in dissolved oxygen was proportional to the added Fe^{2+} concentration ($p < 0.05$) (**Figure 3**). The amount of oxygen consumed during the initial drop was observed to be 5.6 ± 0.5 times higher than the amount of added iron (**Figure 3**). As a linear relationship between consumed oxygen vs peroxide formation was observed (**Figure 1**), this indicates that also during the initial drop a single

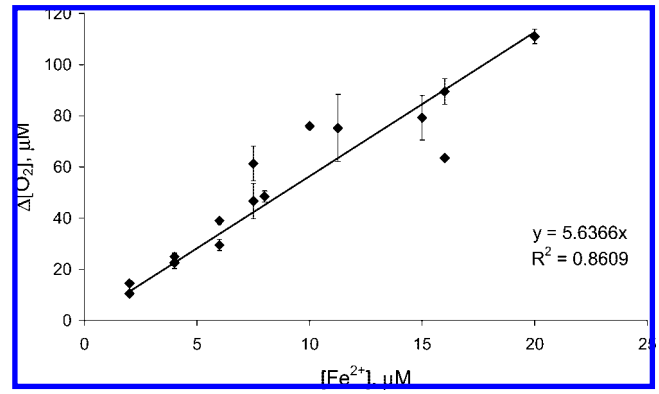

Figure 3. Decrease in concentration of dissolved oxygen $\Delta[O_2]$ observed immediately after addition of Fe^{2+} to a liposome solution (6 mg/mL) for different amounts of added Fe^{2+}.

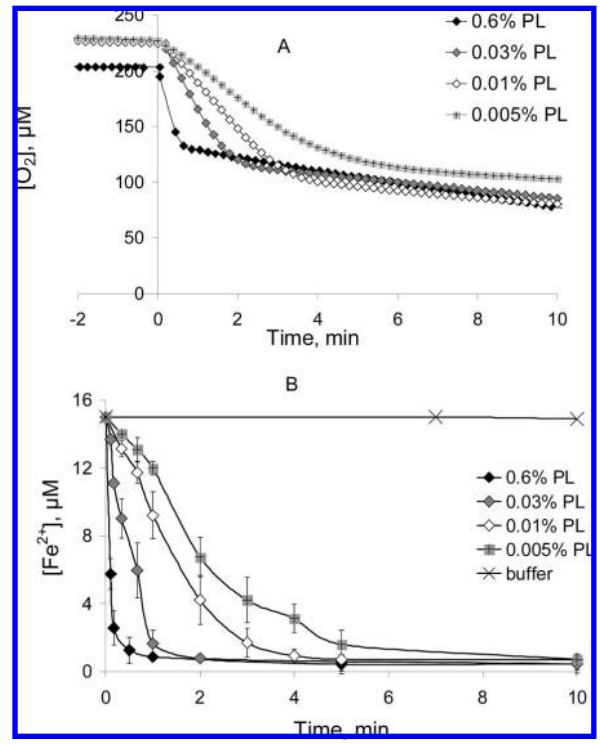

Figure 4. Changes in concentration of dissolved oxygen as a function of time with low concentrations of phospholipids (**A**) and the change in the concentration of Fe^{2+} in the same experiments (**B**). The concentration of added iron was 15 μM Fe^{2+}.

Fe^{2+} ion led to consumption of several oxygen molecules and generation of several peroxides.

Repetitive addition of Fe^{2+} to the same liposome solution induced repetitive initial oxygen drops proportional to the added Fe^{2+} concentration (7.5 μM), which shows that the initial drop in oxygen concentration is an equilibrium reaction involving Fe^{2+}. However, when Fe^{3+} was added to the liposome solution, the initial drop in dissolved oxygen concentration was hardly detectable (**Figure 2**). To measure the rate of the initial drop in dissolved oxygen, the concentration of phospholipids in the liposome solution was reduced. The rate of oxygen consumption during the initial drop was clearly dependent on the phospholipid concentration in the liposome solution (**Figure 4A**) being slower at lower lipid concentration.

After addition of Fe^{2+} to liposomes, a fast decrease in Fe^{2+} concentration during the initial oxygen uptake phase was observed (**Figure 4B**) which was due to oxidation of Fe^{2+} to

Table 1. Concentration of Lipid Peroxides and Rate of Oxygen Uptake and Fe^{2+} Oxidation during the Initial Drop in Dissolved Oxygen Observed upon Addition of Fe^{2+} in Liposomes with Different Phospholipid (PL) Concentrations[a]

concn		rate, μM/min	
[PL], %	[PV], μM	O_2 uptake	Fe^{2+} oxidation
0.600	36.2 ± 4.1	247.0 ± 21.2	75.1 ± 6.2
0.030	1.8 ± 0.2	68.1 ± 0.6	12.8 ± 0.8
0.010	0.6 ± 0.1	39.2 ± 2.8	5.4 ± 0.8
0.005	0.3 ± 0.0	27.4 ± 0.9	4.2 ± 0.3
0	0	0	0

[a] 15 μM Fe^{2+} was used to catalyze the oxidation. Results are means ± SE ($n = 3$).

Fe^{3+}. No changes in Fe^{2+} concentration were observed in buffer only, which proves that oxygen itself did not oxidize Fe^{2+} at pH ~5.5. Reduced Fe^{2+} oxidation rate was observed in liposome solutions with low phospholipid concentration, indicating that phospholipids were the rate-limiting factor at the low lipid concentrations used (**Table 1**). Peroxides are the proposed substrates for initiation of iron-induced lipid oxidation according to the peroxide-dependent oxidation mechanism (*6–8*). At the initiation of oxidation, in a liposome solution with a phospholipid concentration of 6 mg/mL, the peroxide concentration was 36.2 ± 4.1 μM (**Table 1**). When the lipid concentration in the liposome solution was lowered (from 6 to 0.05 mg/mL), the concentration of peroxide (substrate for reaction 1) and/or allylic center of unsaturated fatty acids (substrate for reactions 3 and 4) available per Fe^{2+} unit probably became limiting, resulting in a lower oxidation rate of phospholipids and Fe^{2+}.

To determine the importance of preexisting lipid peroxides on iron-induced oxidation, triphenylphosphine (TPP) was added to the phospholipids to reduce the peroxide value prior to the formation of liposomes. Triphenylphosphine (TPP) breaks down peroxides to alcohols (*7*). The effectiveness of TPP in reduction of the PV value was determined by analyzing the PV value in phospholipids extracted from the liposomes. When different concentrations of TPP (0, 100, 200, and 400 μM) were added to the phospholipids, peroxide concentrations of 36.2, 29.8, 23.6, and 22.8 μM, respectively, were measured in the liposome solution (6 mg/mL phospholipids). A surplus of 400 μM TPP was used to remove the peroxides; however, peroxides were still measured in liposomes made with the highest TPP concentration. Ohyashiki et al. (*29*) also found that an added surplus of TPP only gave a partial reduction of the PV values. As the decrease in PV value was asymptotically by the increase in TPP concentration, this could be explained by selective decomposition of peroxides by TPP, so that only TPP-reactive lipid peroxides are broken down. Another explanation could be that some peroxides were produced during liposome preparation or during phospholipid extraction for PV measurement. Inhibition of oxygen uptake was observed in liposomes treated with TPP (**Figure 5**). No initial oxygen consumption was observed at concentrations of 200−400 μM TPP. At the same concentrations of TPP the linear oxygen uptake was also completely inhibited. These results suggest that TPP-reactive lipid peroxides are necessary to initiate lipid peroxidation by Fe^{2+}, which is in agreement with the findings of Fukuzawa et al. (*7*) and Tadolini et al. (*30*).

Constant Oxygen Uptake Rate. During the initial drop in dissolved oxygen, Fe^{2+} is oxidized to Fe^{3+}. When the Fe^{2+} concentration reaches a constant level (~1 μM), a constant oxygen uptake is observed (**Figure 4**). When EDTA was added at double the concentration of Fe^{2+}, the initial drop or the

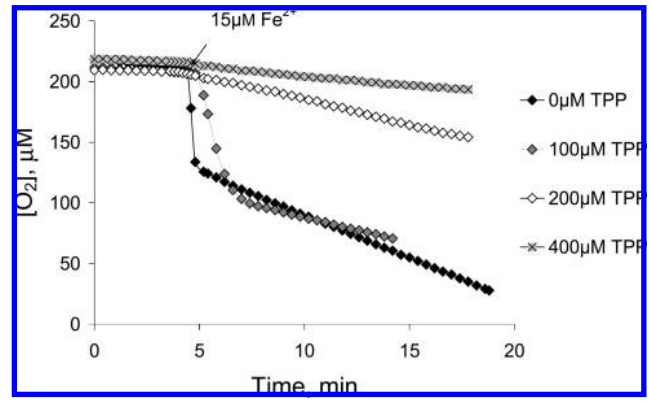

Figure 5. Changes in concentration of dissolved oxygen in liposome solution (6 mg/mL) with different concentrations [0–400 μM or 0–66 μmol/g phospholipids (PL)] of triphenylphosphine (TPP). To catalyze the reaction, 15 μM Fe^{2+} was used.

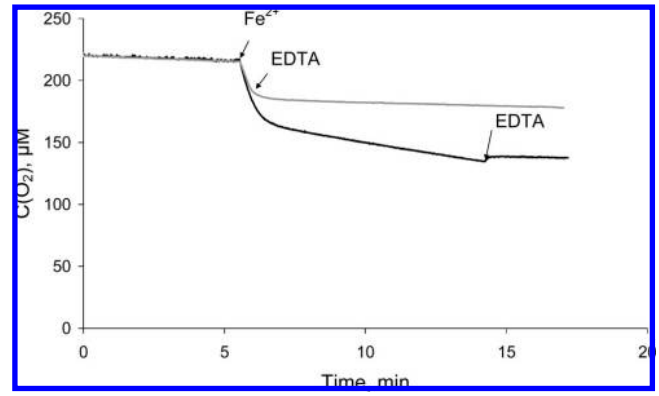

Figure 6. Effect of addition of 15 μM EDTA on the initial and linear oxygen uptake phase induced by 7.5 μM Fe^{2+}. The phospholipid concentration in the liposome solution was 0.3 mg/mL.

constant oxygen uptake was immediately stopped (**Figure 6**). This shows that iron ions are intimately involved in the oxidation reaction, continuously producing radicals resulting in oxidation measured both as the initial drop and as the constant oxygen uptake.

Because Fe^{2+} reacts irreversibly and rapidly with 2,2-dipyridyl forming a pink-colored complex, this can be used to measure the changes in Fe^{2+} concentration during oxidation (**Figure 7A**). When Fe^{2+} was added to liposomes already containing 2,2-dipyridyl, the added concentration of Fe^{2+} was measured (**Figure 7B**, oxidation time 0 s). This indicates that 2,2-dipyridyl complexes Fe^{2+} and prevents oxidation of Fe^{2+} to Fe^{3+} (**Figure 7B**). When 2,2-dipyridyl was added to the liposomes after different durations of oxidation induced by Fe^{2+}, the concentration of the Fe^{2+}−2,2-dipyridyl complex increased with time until the concentration of the Fe^{2+}−2,2-dipyridyl complex reached the initial added concentration of Fe^{2+} (**Figure 7B**). This shows that the increase in concentration of the Fe^{2+}−2,2-dipyridyl complex was due to continuous formation of the Fe^{2+}−2,2-dipyridyl complex, at the same rate as formation of Fe^{2+} from Fe^{3+} according to reaction 1. The increase in formation of Fe^{2+}−2,2-dipyridyl during the first 20 min after addition of 2,2-dipyridyl to the liposome solution was used to calculate the rate of reduction of Fe^{3+} to Fe^{2+}. The initial rate of reduction of Fe^{3+} to Fe^{2+} varied from 0.15 to 0.22 μM/min at room temperature and ~0.25 μM/min at 30 °C (**Table 2**) and was dependent on Fe^{3+} concentration, following a pseudo-first-order reaction. The rate constant was 0.015 ± 0.001 min^{-1} at 20 °C and 0.020 ± 0.001 min^{-1} at 30 °C. The

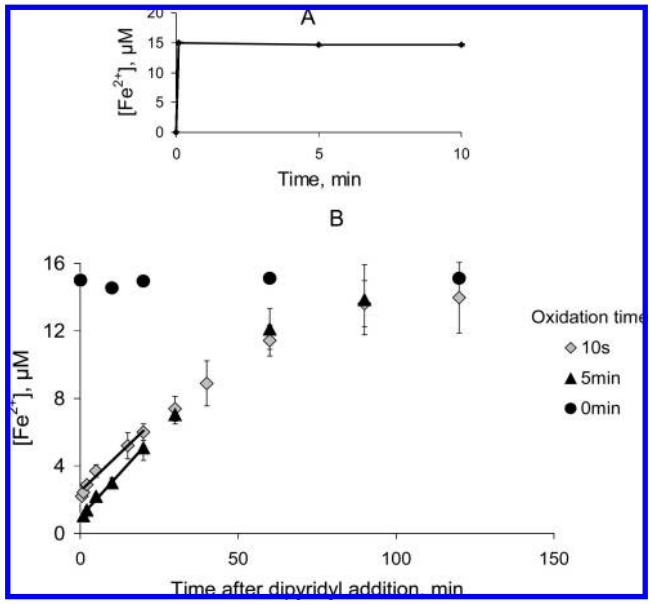

Figure 7. Changes in Fe^{2+} concentration in liposomes (6 mg/mL) after addition of 2,2-dipyridyl (**A**). 2,2-Dipyridyl was added to the liposome solution before addition of Fe^{2+}. (**B**) 2,2-Dipyridyl was added to the liposome solution after different oxidation periods (0, 10 s, and 5 min). The oxidation was induced by 15 μM Fe^{2+}. The increase in Fe^{2+} concentration during the first 20 min after addition of 2,2-dipyridyl was used for calculation of the rate of Fe^{3+} reduction.

Table 2. Concentration of Fe^{2+} and Rate (r_2) of Production of Fe^{2+} from Reduction of Fe^{3+} at 20 and 30 °C Measured at Different Times of Oxidation of Fatty Acids in Liposomes[a]

	$T = 20$ °C			$T = 30$ °C		
time (t), s	$[Fe^{2+}]_t$, μM	r_2, μM/min	k_2, min^{-1}	$[Fe^{2+}]_t$, μM	r_2, μM/min	k_2, min^{-1}
10	2.504	0.178	0.014	1.932	0.254	0.019
30	1.555	0.198	0.015	1.805	0.268	0.020
60	1.053	0.227	0.016	1.634	0.256	0.019
300	0.968	0.207	0.015	1.368	0.295	0.022

[a] Fe^{2+} was measured as the 2,2-dipyridyl−Fe^{2+} complex. The rate constant was determined from the equation $k_2 = r_2/[Fe^{3+}]$. The concentration of Fe^{3+} is given by total added Fe minus Fe^{2+}. The lipid concentration in the liposomes was 6 mg/mL.

low activation energy for this process indicates that this might be a transport-limited process (*31*).

Fe^{3+} is the rate-limiting factor for the redox cycling between Fe^{2+} and Fe^{3+} during the constant oxygen uptake. At equilibrium, the rate of peroxide decomposition by Fe^{2+} (reaction 3) and Fe^{3+} (reaction 1) can be described by the equation:

$$[Fe^{2+}][LOOH]k_1 = [Fe^{3+}][LOOH]k_2$$

where $[Fe^{2+}]$ and $[Fe^{3+}]$ are the iron concentrations, [LOOH] is the peroxide concentration, and k_1 and k_2 are reaction constants for reactions 3 and 1, respectively.

At equilibrium, the concentration of Fe^{2+} was found to be less than one-tenth of the Fe^{3+} concentration. The ratio between the rate constants for those reactions was calculated to be $k_2/k_1 \approx 1/10$ as the peroxide concentration is the same for both reactions.

DPPD and Lipid Oxidation. The radical chain reaction is supposed to involve peroxy radicals $LOO^{\bullet}$. Addition of the peroxy radical scavenger *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) (*28*) should therefore inhibit the lipid oxidation and

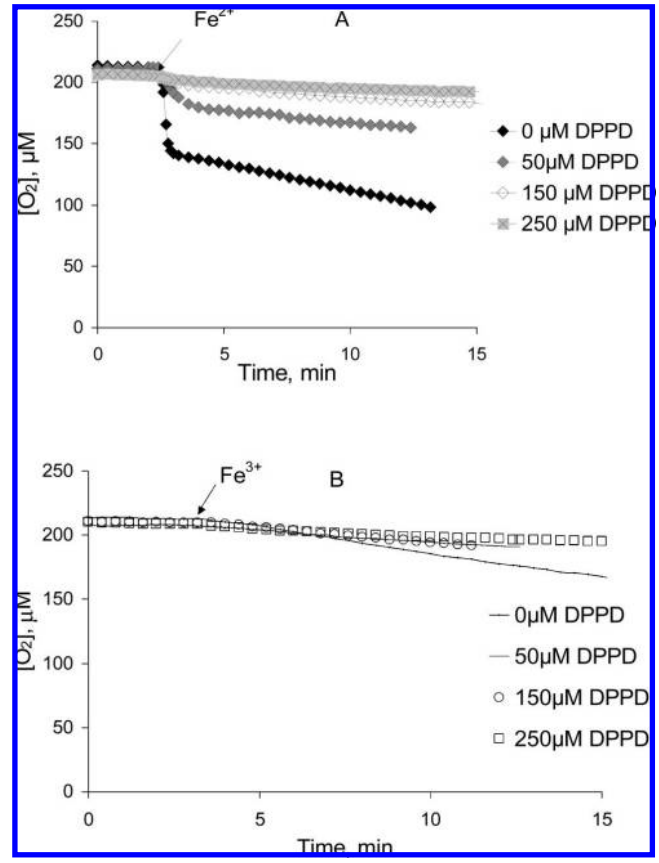

Figure 8. Changes in concentration of dissolved oxygen in liposome solutions (6 mg/mL) with different concentrations (0–250 μM or 0–42 μmol/g PL) of *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD). 15 μM Fe^{2+} or Fe^{3+} was used to catalyze the oxidation.

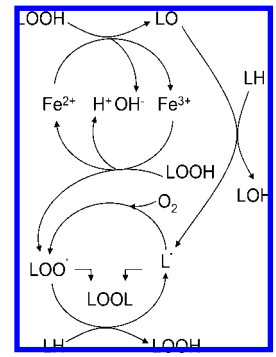

Figure 9. Proposed mechanism for oxidation of unsaturated fatty acids.

thereby reduce the oxygen uptake. Addition of DPPD to the liposomes reduced both the initial drop in dissolved oxygen induced by oxidation of Fe^{2+} and the constant oxygen uptake induced by both Fe^{2+} and Fe^{3+} (**Figure 8**). The inhibition increased with increasing concentrations of DPPD, and at a concentration of 250 μM DPPD oxygen consumption was completely inhibited. These results indicate that peroxy radicals are involved in the iron-mediated oxidation of unsaturated fatty acids.

Lipid Oxidation in Liposomes. From the observations described above we propose that Fe^{2+} induced a rapid oxidation in liposomes, which was observed as an initial drop in dissolved oxygen. At a lipid concentration of 6 mg/mL and 15 μM Fe^{2+} the rate was >258 μM O_2/min. As Fe^{2+} was oxidized to Fe^{3+}, the rate of oxidation reached a constant rate of 4–6 μM oxygen/min proportional to the concentration of iron added (*17*).

Table 3. Rate of Linear Oxygen Uptake (OUR) after Two Repetitive Additions of Iron (7.5 μM)[a]

	Fe^{2+}			Fe^{3+}		
[Fe], μM	7.5[b]	15[b]	15[c]	7.5[b]	15[b]	15[c]
OUR, μM/min	2.5 ± 0.3	5.2 ± 0.4	2.8 ± 0.2	2.2 ± 0.2	3.9 ± 0.2	0.4 ± 0.1
OUR/[Fe]	0.33 ± 0.05	0.35 ± 0.03	0.19 ± 0.01	0.30 ± 0.04	0.26 ± 0.01	0.03 ± 0.01

[a] Oxygen uptake rate per mole of iron (OUR/[Fe]) is means ± SE (n = 4). [b] Iron working solution at pH ≈2. [c] Iron working solution at pH ≈3.5.

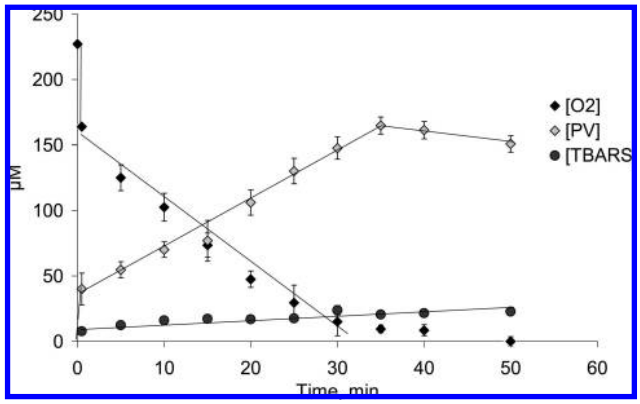

Figure 10. Changes in dissolved oxygen concentration [O_2], peroxide [PV], and thiobarbituric reactive substances [TBARS] as a function of time. The phospholipid concentration in liposomes was 9 mg/mL, and 7.5 μM Fe^{2+} was used to catalyze the oxidation.

A decrease in oxidation rate when Fe^{2+} was oxidized to Fe^{3+} was also observed by Yoshida and Niki (*32*), but in their study the decrease was not quantified.

When Fe^{2+} was added to the solution of liposomes, the initial oxygen uptake was due to radical production via breakdown of peroxides by Fe^{2+} (**Figure 9**). As the oxidation rate of Fe^{2+} via peroxide breakdown was observed to be 79 μM/min, the alkoxy radical formation rate was also assumed to be 79 μM/min. One alkoxy radical further propagated lipid peroxidation by chain branching, leading to production of several peroxides accompanied by oxygen consumption (lower cycle in **Figure 9**) as a rate of 258 μM O_2/min was measured during the initial drop in oxygen. When equilibrium between Fe^{2+} and Fe^{3+} was achieved after the initial fast oxygen uptake, a constant oxygen uptake rate was observed.

Only the linear oxygen uptake was observed after addition of Fe^{3+} to the liposome solution. Reaction of Fe^{3+} with peroxides was slower (~0.25 μM Fe^{3+}/min) than the oxidation of Fe^{2+} by peroxides measured during the initial drop. Due to this, reduction of Fe^{3+} is the limiting factor for circulation between Fe^{2+} and Fe^{3+} in the *radical generating cycle* (**Figure 9** upper cycle) when equilibrium was reached. On the basis of equal oxidation and reduction rates of the two iron species, the rate of radical production in the radical generating cycle can be calculated to 0.5 μM/min. Both peroxy and alkoxy radicals formed in the radical production cycle are proposed to subtract bisallylic hydrogen from fatty acids (*2*). The generated lipid radical ($L^{\bullet}$) takes part in the *peroxide production cycle*, leading to formation of several peroxides as shown in the lower cycle in **Figure 9**. The oxygen consumption at constant oxygen uptake was observed to be 4–6 μM O_2 /min. Constant oxygen uptake rate has been shown to be independent of the concentration of dissolved oxygen from 100% saturation to at least 5% (*13*). To achieve a constant oxygen uptake rate, the concentration of radicals, in specific lipid radicals ($L^{\bullet}$), must be constant. Their concentrations are regulated by the production in the radical generating cycle and removal by the termination reactions. As the radical concentrations are constant when the oxidation rate is constant, the production rate and the termination rate should be equal. On the basis of these assumptions it is possible to calculate that the peroxy and lipid radicals in average would react approximately 10 times in the peroxide producing cycle before they are removed by termination reactions.

According to previous observations, the linear oxygen uptake rate should be the same when adding Fe^{2+} or Fe^{3+}. Repetitive addition of Fe^{2+} and Fe^{3+} was used to catalyze oxidation in liposomes (**Figure 2**). After the first addition of iron, the rate of linear oxygen uptake (OUR) per mole of iron was similar both for Fe^{2+} and for Fe^{3+} (**Table 3**). However, the second addition gave a slightly lower OUR per mole of Fe^{3+} than for Fe^{2+}; this could be due to the higher sensitivity of Fe^{3+} to changes in pH. To investigate this, Fe^{2+} and Fe^{3+} working solutions were made at pH 2 and 3.5 and oxidation experiments performed with 15 μM iron. The oxidation rates were lower when iron solutions with higher pH were used. The increase in working solution pH led to approximately 47% reduced OUR for Fe^{2+} and 90% for Fe^{3+}, which reflects the solubility product constants 5×10^{-17} and 3×10^{-39} for the corresponding hydroxides (*33*). This indicates that iron hydroxides (*20*) play a role in determining the concentration of active iron on the surface of the liposomes. Repetitive additions of Fe^{3+} probably increased the fraction of iron hydroxide more than addition of Fe^{2+}, leading to lower oxidation rates for Fe^{3+} at higher pH.

After addition of Fe^{2+} to liposomes, an increase in PV and TBARS values followed the oxygen consumption (**Figure 10**). When all of the dissolved oxygen was depleted, decomposition of peroxides by iron could occur through the radical production cycle, resulting in consumption of peroxides. Due to this reaction a decrease in PV values was observed after depletion of dissolved oxygen in the liposome solution.

In conclusion, the initial drop in dissolved oxygen, after adding Fe^{2+} to liposomes, is due to oxidation of Fe^{2+} to Fe^{3+} (rate > 79 μM Fe^{2+}/min at 0.6% lipid concentration and 15 μM Fe^{2+} added), resulting in formation of alkoxy radicals. Fe^{3+} is reduced to Fe^{2+} by peroxides at a slow rate (0.25 μM Fe^{3+}/min at 30 °C) in a pseudo-first-order reaction with respect to the Fe^{3+} concentration. When equilibrium between Fe^{2+} and Fe^{3+} is reached, the linear oxygen uptake is observed. Both alkoxy and peroxy radicals formed from breakdown of peroxides by Fe^{2+} and Fe^{3+} react with fatty acids, giving a lipid radical capable of reacting with oxygen. The net result is production of lipid peroxides accompanied by oxygen consumption.

ABBREVIATIONS USED

CumOOH, cumene hydroperoxide; DPPD, *N*,*N*′-diphenyl-*p*-phenylenediamine; PUFA, polyunsaturated fatty acid; EDTA, ethylenediaminetetraacetic acid; LOOH, lipid peroxide; $L^{\bullet}$, lipid radical; $LOO^{\bullet}$, peroxy radical; $LO^{\bullet}$, alkoxy radical; MES, 2-morpholinoethanesulfonic acid; PL, phospholipids; OUR, oxygen uptake rate; PV, peroxide value; TBARS, thiobarbituric reactive substances; TPP, triphenylphosphine.

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