The mechanism of Fe^{2+}-initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical

Lixia TANG*, Yong ZHANG*, Zhongming QIAN† and Xun SHEN*[1]

*Institute of Biophysics, Chinese Academy of Sciences, Datun Road 15, Chaoyang District, Beijing 100101, People's Republic of China, and †Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

The mechanism of Fe^{2+}-initiated lipid peroxidation in a liposomal system was studied. It was found that a second addition of ferrous ions within the latent period lengthened the time lag before lipid peroxidation started. The apparent time lag depended on the total dose of Fe^{2+} whenever the second dose of Fe^{2+} was added, which indicates that Fe^{2+} has a dual function: to initiate lipid peroxidation on one hand and suppress the species responsible for the initiation of the peroxidation on the other. When the pre-existing lipid peroxides (LOOH) were removed by incorporating triphenylphosphine into liposomes, Fe^{2+} could no longer initiate lipid peroxidation and the acceleration of Fe^{2+} oxidation by the liposomes disappeared. However, when extra LOOH were introduced into liposomes, both enhancement of the lipid peroxidation and shortening of the latent period were observed. When the scavenger of lipid peroxyl radicals (LOO˙), N,N′-diphenyl-p-phenylene-diamine, was incorporated into liposomes, neither initiation of the lipid peroxidation nor acceleration of the Fe^{2+} oxidation could be detected. The results may suggest that both the pre-existing LOOH and LOO˙ are necessary for the initiation of lipid peroxidation. The latter comes initially from the decomposition of the pre-existing LOOH by Fe^{2+} and can be scavenged by its reaction with Fe^{2+}. Only when Fe^{2+} is oxidized to such a degree that LOO˙ is no longer effectively suppressed does lipid peroxidation start. It seems that by taking the reactions of Fe^{2+} with LOOH and LOO˙ into account, the basic chemistry in lipid peroxidation can explain fairly well the controversial phenomena observed in Fe^{2+}-initiated lipid peroxidation, such as the existence of a latent period, the critical ratio of Fe^{2+} to lipid and the required oxidation of Fe^{2+}.

Key words: chemiluminescence, initiation of lipid peroxidation, latent period, oxidation of ferrous ion.

INTRODUCTION

Lipid peroxidation in biological membranes has been considered as one of the major mechanisms of cell injury in aerobic organisms subjected to oxidation stress [1,2]. Hochstein et al. discovered the requirement for iron in initiating lipid peroxidation in the 1960s [3]. Since then, the mechanism involved in iron-dependent lipid peroxidation has been studied in many *in vitro* model systems, such as liposomes and microsomes [4,5]. However, the exact molecular mechanism has not yet been fully understood. Peroxidation in liposomes is usually studied after adding iron ions, e.g. Fe^{2+} (often as a chelated complex, e.g. ADP-Fe^{2+}), Fe^{3+} plus reducing agent (e.g. ascorbic acid) or chelated Fe^{3+} plus NADPH (in the case of microsomes). Since H_2O_2-degrading enzymes or scavengers of ˙OH rarely inhibit the iron-dependent peroxidation in either liposomal or microsomal systems [6–8], the ˙OH radical has been excluded as the possible initiator of ferrous- or ferric-ion-initiated lipid peroxidation. Whereas the mechanism is not completely understood, it is known that the redox chemistry of iron plays an important role in both the occurrence and the rate of lipid peroxidation. Many studies have shown that the iron-dependent lipid peroxidation in systems comprised initially of Fe^{2+} and liposomes requires some Fe^{2+} oxidation [9], but that in systems comprised initially of Fe^{3+} and liposomes it requires some Fe^{3+} reduction [10]. Some iron-oxygen species, such as the perferryl radical (with pentavalent iron), were suggested as the initiators of Fe^{2+}-initiated lipid peroxidation [3,11]. However, this speculation was proved wrong, since on thermodynamic grounds the perferryl ion is too poor to abstract the allyl hydrogen from methylene carbon [12]. Based mainly on the observation that partial but not complete oxidation would be required if starting with Fe^{2+}, while partial but not complete reduction would be required if starting with Fe^{3+}, Aust et al. hypothesized that a Fe^{2+}–Fe^{3+} complex may be the initiator for iron-dependent lipid peroxidation [13,14]. Unfortunately, this proposed complex was neither isolated nor observed. In fact, there was also some evidence against the requirement for the complex [15]. Besides looking for an iron complex as the initiator, the decomposition of the pre-existing lipid peroxides (LOOH) in liposomes induced by Fe^{2+} or Fe^{3+} was considered again as the mechanism involved in iron-dependent lipid peroxidation. In a typical study by Tadolini et al. [16], it was found that no peroxidation was observed with a variable $FeCl_2/FeCl_3$ ratio when phosphatidylcholine liposomes deprived of LOOH by triphenylphosphine (TPP) treatment were utilized. Nevertheless, many mechanisms have been proposed for the initiation of iron-dependent lipid peroxidation, but none of them can explain the diverse observations reported by different investigators.

Although the mechanism of iron-dependent lipid peroxidation is still a controversial and complicated issue, as a unique characteristic in Fe^{2+}-initiated lipid peroxidation, a short time lag after addition of Fe^{2+} in a liposomal system is frequently observed before lipid peroxidation starts [9,17]. It may provide an important clue to the truth behind this complicated problem.

Abbreviations used: LOOH, lipid peroxides; TPP, triphenylphosphine; DPPD, N,N′-diphenyl-p-phenylene-diamine; TBA, thiobarbituric acid; BHT, butylated hydroxytoluene; CL, chemiluminescence; TBARS, TBA-reactive substances; DTPA, diethylenetriaminepenta-acetic acid; LOO˙, lipid peroxyl radical.

[1] To whom correspondence should be addressed (e-mail shenxun@sun5.ibp.ac.cn).

Minotti and Aust [13] explained the time lag (or so-called latent period) as the time needed for accumulating Fe^{3+} to a certain extent by Fe^{2+} oxidation, and forming the Fe^{2+}–Fe^{3+} complex. Driomina et al. explained it as the time needed for reducing the membrane-bound Fe^{2+} by oxidation to such a degree that the ratio of Fe^{2+} concentration to lipid, $[Fe^{2+}]/[L]$, is below a critical ratio at which lipid peroxidation can start [18]. Whatever the mechanism involved in iron-dependent lipid peroxidation, the existence of a latent period implies that the species responsible for initiating lipid peroxidation is either formed or accumulated to such a degree that its reaction with lipid becomes dominant over all competitive reactions with others in the system consisting of liposomes, iron ions and chelator. It is reasonable to expect that any action that can change the length of the latent period by regulating the concentration of the species at the end of the latent period may provide some essential information on the initiator and actual mechanism involved in iron-dependent lipid peroxidation. In the present study, lipid peroxidation in a liposomal system was initiated by ADP-Fe^{2+}, and the effects of a second addition of Fe^{2+} at various moments during lipid peroxidation and of pre-existing LOOH on both lipid peroxidation and oxidation of ferrous ions were investigated. Particular attention was paid to the role of the pre-existing LOOH in liposomes and the dual functions of ferrous ions in either initiating or inhibiting lipid peroxidation.

MATERIALS AND METHODS

Chemicals

ADP, diethylenetriaminepenta-acetic acid (DTPA), $FeSO_4$, $FeCl_3$ and butylated hydroxytoluene (BHT) were purchased from Sigma. N,N'-Diphenyl-p-phenylene-diamine (DPPD) was from Acros. Trihydroxymethylaminomethane (as Tris), 1,10-phenanthroline, HCl, TPP, Methylene Blue and other chemicals were from Beijing Chemical Co. All chemicals were of analytical grade or better and used without further purification.

Preparation of liposomes

Phospholipids were obtained from egg yolk according to the method of Bligh and Dyer [19]. Liposomes were prepared by sonication of extracted phospholipids in 50 mM Tris/HCl buffer solution (pH 7.4) containing 0.1 M KCl or 50 mM acetate buffer (pH 5.0). The liposomes incorporated with TPP or DPPD were prepared by sonication of the phospholipids that had been mixed with either TPP or DPPD in chloroform and then dried under nitrogen. The indicated concentrations of TPP or DPPD in the text are expressed in terms of the concentration in the bulk liposome suspension.

Preparation of the liposomes containing excessive LOOH

Phospholipid hydroperoxides were prepared by the methods of Terao et al. [20]. A sample of 100 mg of egg phospholipids dissolved in 5 ml of methanol containing 0.1 mM Methylene Blue was illuminated with a 30-W tungsten projection lamp at room temperature for 12 h. The concentration of phospholipid hydroperoxides in the photodynamically treated phospholipid was determined by the iodometric method described below. The liposomes containing excessive LOOH were prepared by incorporating the desired amounts of the phospholipid hydroperoxide-rich phospholipid. The indicated concentrations of LOOH in the text are also for the bulk suspension.

Chemiluminescence (CL) measurement of lipid peroxidation

Liposome suspension (1.4 ml, 2 mg/ml lipid in 50 mM Tris/HCl buffer solution or 50 mM acetate buffer) containing ADP was placed in a quartz cuvette ($10 \times 10 \times 40$ mm). The cuvette was placed in a laboratory-made, computerized, high-sensitivity single-photon counter. After the cuvette had been kept in the counter in a dark room for 5 min, the CL of the liposomal system was monitored by EMI-9558B photomultiplier, which was cooled in order to minimize its background noise. Then, 200 s later, 0.6 ml of Fe^{2+} solution (pH 3.0) was injected rapidly into the cuvette and mixed with the liposome suspension, resulting in a chelated Fe^{2+}–liposome system containing initially 100 μM Fe^{2+} and the same concentration of ADP. The kinetic process of lipid peroxidation in the system was recorded as the profile of the lipid-peroxidation-concomitant CL [18,21].

Thiobarbituric acid (TBA) assay for lipid peroxidation

Lipid peroxidation was also assayed as TBA reactivity [22]. The liposome suspension containing iron chelator was exactly the same as that used in the CL assay (pH 7.4, 25 °C), and the same volume of suspension (1.4 ml) was placed in test tubes. At various times after addition of Fe^{2+}, the reaction suspension was mixed with an equal volume of cold trichloroacetic acid (30 %) to stop the peroxidation. TBA (1 ml, 1.5 % in distilled water) and 0.1 ml of BHT (2 % in ethanol) was then added in the stopped reaction mixture. Tubes were heated at 100 °C for 20 min, 2 ml of the final reaction mixture was transferred to Eppendorf tubes and centrifuged at 15 000 rev./min for 5 min. The absorbance of the supernatant was read at 532 nm as the production of TBA-reactive substances (TBARS).

Determination of LOOH

The content of LOOH in liposomes was determined spectrophotometrically as described by Buege and Aust [23] with minor modification. Liposome suspension (2 ml) was mixed thoroughly with 5.0 ml of chloroform/methanol mixture (2:1), followed by centrifugation at 1000 g for 5 min. Most of the upper layer was removed, and 3.0 ml of the lower layer was placed in a test tube and dried in a 45 °C water bath under a stream of nitrogen. While still under the nitrogen stream, 1.0 ml of acetic acid/chloroform (3:2), followed by 0.05 ml of potassium iodide, was quickly added, and the test tube was stoppered and shaken. The samples were placed in the dark at room temperature for exactly 5 min, followed by addition of 1.5 ml of cadmium acetate. The solution was mixed and centrifuged at 1000 g for 10 min. The absorbance of the upper phase was determined at 353 nm against a blank containing the complete assay mixture minus the liposomes. The absorption coefficient for I^-_2 was taken as 1.73×10^4 $M^{-1} \cdot cm^{-1}$.

Determination of the Fe^{2+} oxidation

The remaining Fe^{2+} after its oxidation was measured according to the method of Mahler and Elowe [24]. A single-dose or two split doses of Fe^{2+} were added to the liposomal system or buffer solution. At various times, 1,10-phenanthroline was added to stop the Fe^{2+} oxidation (the final concentration of 1,10-phenanthroline was 2.5 mM), and the absorbance at 515 nm was measured immediately using the reaction mixture minus Fe^{2+} as a blank.

RESULTS

Concomitant CL with the Fe^{2+}-initiated lipid peroxidation in liposomes containing various amounts of LOOH

Since CL is an overall index of radical reaction taking place in lipid peroxidation and can reflect the rate of peroxidation, the concomitant CL was used to monitor the kinetic process of lipid peroxidation. In order to verify the applicability of CL in monitoring the kinetics of lipid peroxidation, the kinetic curves of both CL and TBARS production during lipid peroxidation

initiated by 80 μM ADP-Fe^{2+} or DTPA-Fe^{2+} in the liposomes containing no additional LOOH were determined in parallel. The results are shown in Figure 1. It can be seen that the kinetics of the lipid peroxidation represented as the production of the TBARS are similar to those represented as the lipid-peroxidation-concomitant CL. The length of the latent period determined with the TBA method was close to that obtained from CL measurements, although the TBA assay measures the accumulation of TBARS but the CL measures the reaction rate. It seems that the kinetic process of the lipid-peroxidation-concomitant CL can be
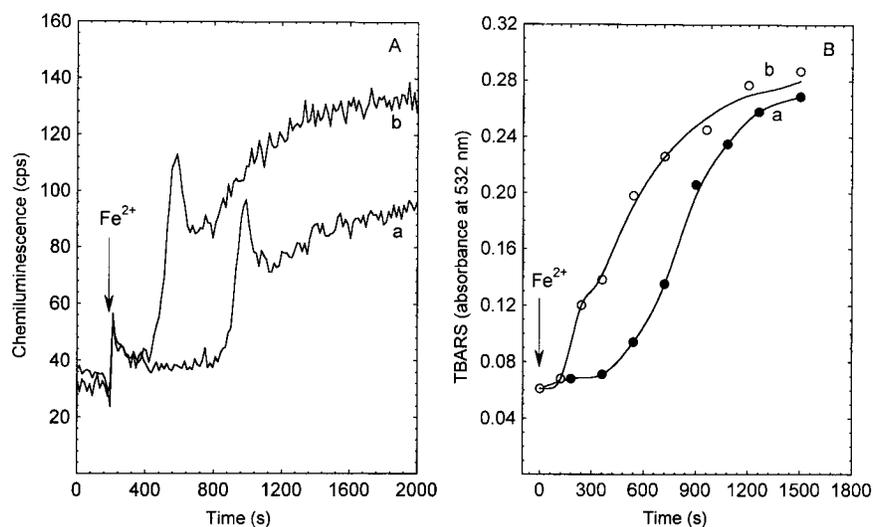
Figure 1   The kinetic curves of lipid peroxidation initiated by 80 μM (a) ADP-Fe^{2+} or (b) DTPA-Fe^{2+} in 1.4 mg/ml lecithin-formed liposomes (Tris/HCl buffer, pH 7.4)

(**A**) Detected by CL; (**B**) Detected by TBARS production.
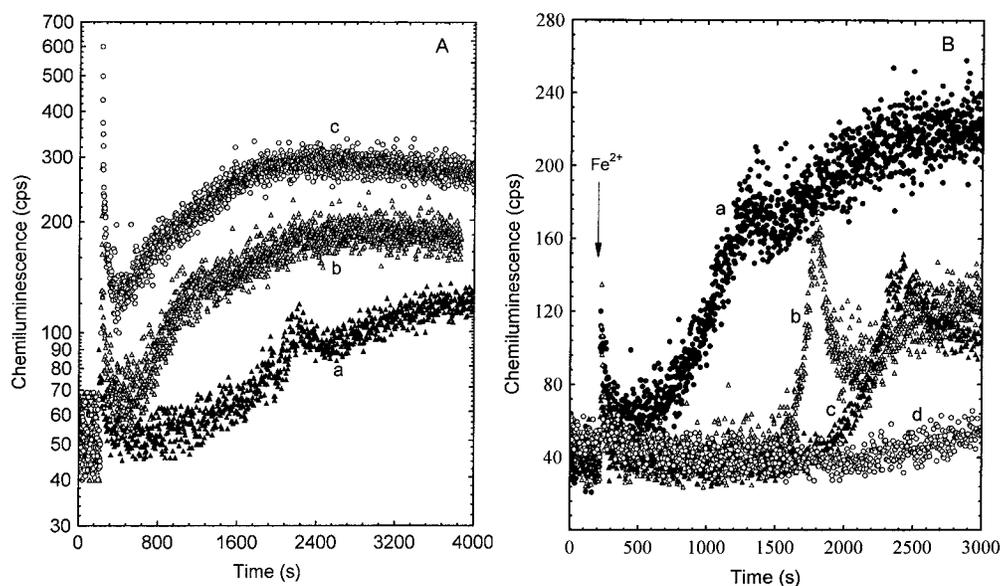
Figure 2   The effect of different concentrations of the pre-existing LOOH and of TPP incorporated in liposomes on the kinetics of lipid peroxidation initiated by 100 μM ADP-Fe^{2+} in 1.4 mg/ml lecithin-formed liposomes (Tris/HCl buffer, pH 7.4)

(**A**) Incorporated with (a) no extra LOOH, (b) 10.5 μM and (c) 17.5 μM extra LOOH. (**B**) Incorporated with (a) no TPP, (b) 3.5 μM, (c) 10.5 μM and (d) 17.5 μM TPP. The kinetic process was monitored by lipid-peroxidation-concomitant CL.
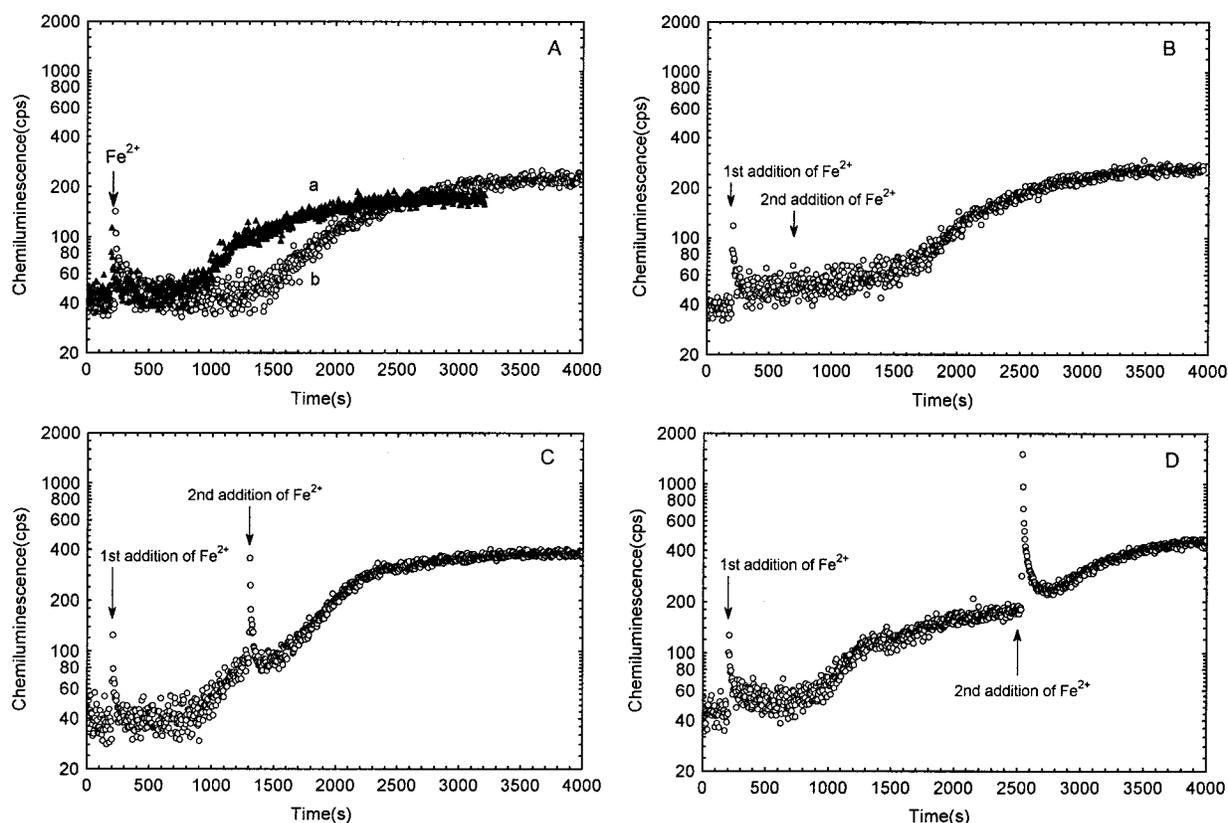
Figure 3   The effect of a second addition of 50 μM Fe^{2+} at different reaction stages on lipid peroxidation

(**A**) As the controls, lipid peroxidation initiated by 100 (a) or 150 μM Fe^{2+} (b) in the liposomal system (pH 7.4). (**B**–**D**) Lipid peroxidation was initiated by first addition of 100 μM Fe^{2+}-ADP, followed by a second addition of 50 μM Fe^{2+} 500, 1100 and 2300 s later, respectively.

divided into four stages as suggested by Driomina et al. [18]: (i) the first flash of CL appeared immediately after addition of Fe^{2+}, arising from decomposition of pre-existing LOOH in liposomes; (ii) a low-level stationary CL corresponding to the latent period; (iii) the rising phase of CL representing the start of lipid peroxidation; (iv) a high-level stationary CL reflecting the continuous chain reaction of lipid peroxidation in liposomes.

The CL following addition of 100 μM ADP-Fe^{2+} in the normally prepared liposomes and in the liposomes containing excessive LOOH was monitored. As shown in Figure 2(A), the latent period was shortened, the first CL flash was enhanced, and the rate of lipid peroxidation increased with the increase of the 'extra' LOOH content of the liposomes. In order to quantify the latent period, in the present study the latent period was defined as the time needed for the lipid-peroxidation-concomitant CL above baseline to reach 10 % of its maximum after addition of Fe^{2+}. In the studied liposomal systems, the latent period was reduced from 1400 s in normal liposomes to 450 s in the liposomes containing 10.5 μM extra LOOH. In the liposomes containing 17.5 μM extra LOOH, the latent period almost disappeared. Comparing the intensity at the high-level stationary CL stage, it was observed that lipid-peroxidation-concomitant CL increased by factors of 2 and 3 respectively in the liposomes containing 10.5 and 17.5 μM extra LOOH. The enhancement of the first flash by extra LOOH confirms that the flash arises from decomposition of pre-existing LOOH in liposomes.

To remove the pre-existing LOOH from liposomes, liposomes incorporated with different concentrations of TPP, which can

reduce LOOH to corresponding alcohol, were utilized and corresponding lipid peroxidation was measured. As shown in Figure 2(B), when TPP content in liposomes increased, the latent period in ADP-Fe^{2+}-initiated lipid peroxidation lengthened significantly, while the height of the first CL flash and the rate of lipid peroxidation decreased. When 17.5 μM TPP was incorporated into liposomes, the first CL flash almost disappeared and the peroxidation was hardly observed, which indicates that the pre-existing LOOH were almost completely eliminated. Further increase of TPP to 35 μM resulted in complete inhibition of lipid peroxidation (results not shown).

The effect of a second addition of Fe^{2+} introduced at various times on lipid peroxidation

As the controls, the CL concomitant with the lipid peroxidations initiated by 100 and 150 μM Fe^{2+} were recorded in the liposome suspension (pH 7.4) containing 100 μM ADP. As shown in Figure 3(A), it can be seen clearly that the addition of 150 μM Fe^{2+} resulted in a longer latent period and slightly higher lipid-peroxidation rate than the addition of 100 μM Fe^{2+}. However, what happens if 100 μM Fe^{2+} is added first and then 50 μM Fe^{2+} is added afterwards at various stages of the lipid peroxidation? Since the latent period in the 100 μM Fe^{2+}-initiated lipid peroxidation was about 600 s and the continuous chain reaction of lipid peroxidation started 1500 s later after the first addition of Fe^{2+} in the liposomal system studied, an experiment was carried out in which 50 μM Fe^{2+} was added 500, 1100 and 2300 s after

Table 1 The height of the second flash, resultant latent period and the final rate of lipid peroxidation (LPO) induced by the second addition of 50 μM Fe^{2+} at various times after the first addition of 100 μM in the liposomal system

1st addition of Fe^{2+} (μM)	2nd addition of Fe^{2+}		Latent period(s)	Height of 2nd flash(c.p.s.)	Final rate of LPO-CL(c.p.s.)
	Concentration (μM)	Time(s)			
150	0	–	1300	–	210
100	0	–	600	–	140
100	50	300	1300	≈ 0	210
100	50	500	1300	≈ 0	215
100	50	800	600	40	240
100	50	1100	600	230	340
100	50	1600	600	710	410
100	50	2400	600	1280	440

The lipid peroxidations were initiated in the liposome suspension (1.4 mg of lipid/ml, in Tris/HCl buffer, pH 7.4). The final rate of the lipid peroxidation was measured as the CL intensity in the high-level stationary stage. c.p.s., counts/s.
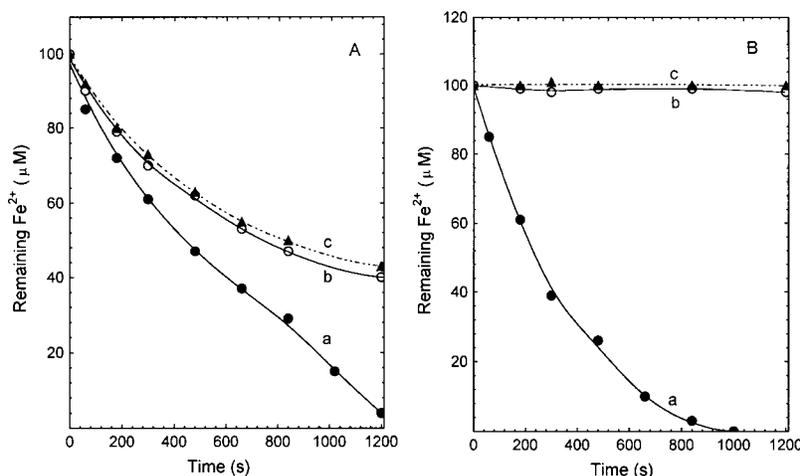
Figure 4   The oxidation of 100 μM ADP-Fe^{2+} in Tris/HCl buffer (pH 7.4) (A) and acetate buffer (pH 5.0) (B) in the absence or presence of liposomes

(a) 1.4 mg/ml liposomes (●); (b) buffer only (○) and (c) 2 ml of 1.4 mg/ml liposomes incorporated with 35 μM TPP (▲). The remaining Fe^{2+} was measured at various times after addition of Fe^{2+}.

the first addition. The results are shown in Figures 3(B), 3(C) and 3(D) respectively. It was found that: (i) when 50 μM Fe^{2+} was added within the latent period, the time lag was extended and the resultant time lag was just the same as that observed in 150 μM Fe^{2+}-initiated lipid peroxidation. In other words, two split doses of Fe^{2+} added to the liposomal system within the first-addition-dictated latent period are equivalent to the single dose of the total Fe^{2+} in terms of both the resultant apparent latent period and the final rate of lipid peroxidation. In addition to this unique observation, almost no flash was induced by the second addition of Fe^{2+}, indicating that the LOOH in liposomes during the latent period were so low that their decomposition by the newly added Fe^{2+} was negligible. (ii) When 50 μM Fe^{2+} was added after the end of the latent period either in the rising phase or in the high-level stationary CL phase, a significantly higher flash was observed immediately following the second addition of Fe^{2+}, indicating that more LOOH were accumulated and available for decomposition by newly added Fe^{2+}. Furthermore, the final rate of lipid peroxidation was obviously enhanced. The heights of the second flashes, the extension of the latent period and the final-intensities of the concomitant CL in the lipid peroxidation

caused by two split doses of Fe^{2+} are summarized in Table 1. From Table 1, it can be seen that the second dose of Fe^{2+} added at 300 and 500 s after the first addition of Fe^{2+}, still within the latent period, did not induce any observable flash and resulted in almost the same extension of the time lag (600 s was extended to 1300 s). When the second dose of Fe^{2+} was added after lipid peroxidation started, the later that Fe^{2+} was added, the higher the second flash and the rate of lipid peroxidation, indicating that the LOOH available for Fe^{2+}-catalysed decomposition began to accumulate after the latent period.

The oxidation of Fe^{2+} in the liposomal system

The oxidation of Fe^{2+} in Tris buffer (pH 7.4) and in acetate buffer (pH 5.0) was measured respectively in the absence or presence of liposomes. In the latter case, normal liposomes and the liposomes incorporated with TPP were used to see the effect of the pre-existing LOOH on the Fe^{2+} oxidation. The results are shown in Figure 4. It was found that Fe^{2+} oxidation became faster when liposomes were present. This effect was even more obvious in acidic buffer, where the Fe^{2+} almost did not undergo oxidation
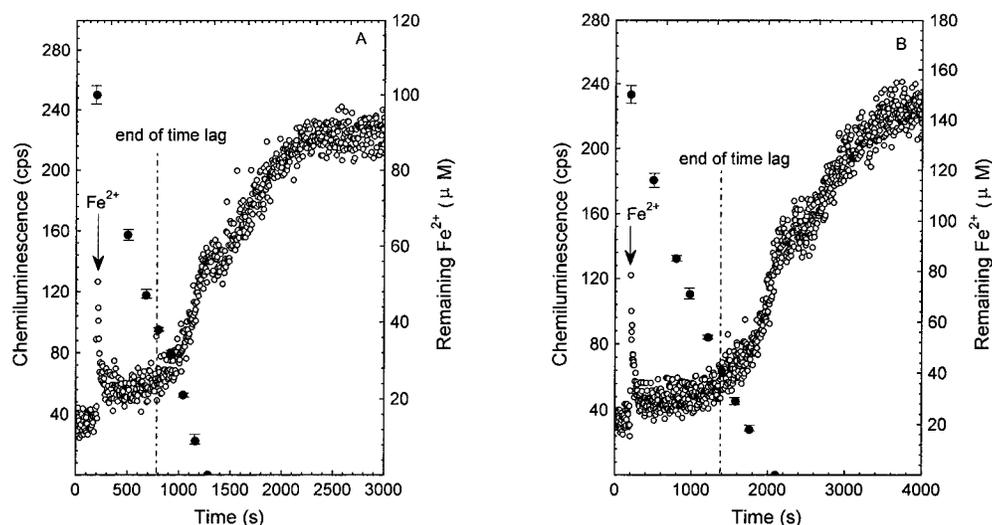
Figure 5 The kinetic profiles of the Fe^{2+}-initiated lipid peroxidation in a liposomal system (1.4 mg/ml lecithin-formed liposomes, 100 μM ADP in 50 mM Tris/HCl buffer) and the corresponding oxidation of Fe^{2+}

(**A**) 100 μM Fe^{2+} was added to the system; (**B**) 150 μM Fe^{2+} was added to the system. The kinetics of lipid peroxidation were monitored by concomitant CL. ●, Remaining Fe^{2+}.

in the absence of liposomes, but oxidized much faster in the presence of liposomes. The acceleration of Fe^{2+} oxidation in the presence of liposomes indicates that Fe^{2+} is oxidized not only by its autoxidation but also by liposomes. To know the effects of liposomes and pre-existing LOOH on the oxidation of Fe^{2+}, the kinetics of Fe^{2+} oxidation during lipid peroxidation initiated by different concentrations of Fe^{2+} or by two split doses of Fe^{2+} were determined. The oxidation of 100 and 150 μM Fe^{2+} in the liposomal system and the corresponding kinetic curves of their initiated lipid peroxidations were measured, and are shown in Figures 5(A) and 5(B) respectively. It can be seen that, in general, the lower the concentration of initially added Fe^{2+}, the shorter the latent period. However, it was interesting to note that although the time needed for the complete oxidation depends on the initial Fe^{2+} concentration in the system, the Fe^{2+} concentrations at the end of the latent period were almost the same regardless of the initial concentration of Fe^{2+}. This may suggest that the lipid peroxidation can really start only when the concentration of Fe^{2+} is reduced to a critical value.

To further verify this criterion, the concentration of Fe^{2+} at the end of the latent period in the lipid peroxidation of liposomes initiated by adding 150 μM Fe^{2+}, or by adding 100 μM Fe^{2+} initially and then 50 μM Fe^{2+} 300 or 480 s later, to the liposomal system was determined. The concentration changes of Fe^{2+} during its oxidation in the same liposomal system under the above three different conditions are shown in Figure 6. It was surprising to find that although Fe^{2+} was added in different time sequences, the corresponding latent periods were almost the same (≈ 1300 s), and the Fe^{2+} concentrations at the end of the periods were almost the same. The critical concentration of Fe^{2+} was about 40 μM under all three conditions. This value is even the same as the value determined when 100 μM Fe^{2+} was added to initiate lipid peroxidation in the same liposomal system. The results suggest that the oxidation of Fe^{2+} is also regulated by liposomes through lipid peroxidation.

When Fe^{2+} was added to the liposomal system, where the liposomes were incorporated with 35 μM TPP, the kinetics of the Fe^{2+} oxidation were the same as in liposome-free buffer, either
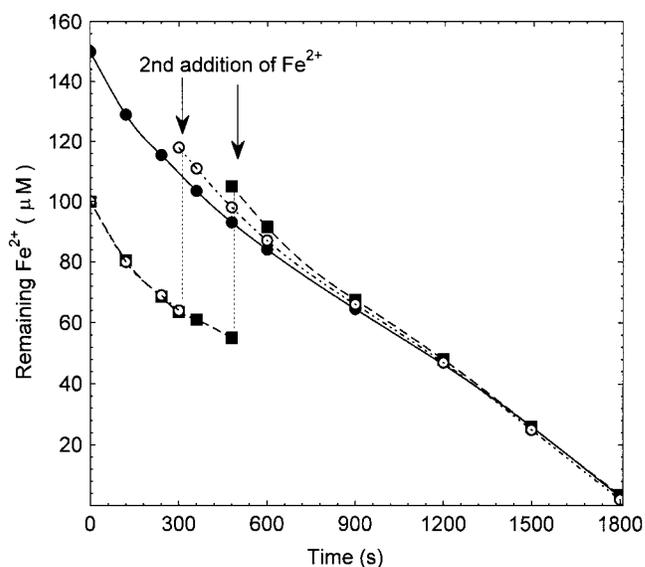
Figure 6 The oxidation kinetics of Fe^{2+} in the liposome suspension (1.4 mg/ml) containing 100 μM ADP

There were three different conditions: 150 μM Fe^{2+} added at the beginning as a single dose (●); 100 μM Fe^{2+} added at first, then 50 μM Fe^{2+} added 300 s later (○); and 100 μM Fe^{2+} added at first, then 50 μM Fe^{2+} added 480 s later (■).

at neutral or acidic pH (see curves c in Figures 4A and 4B). The disappearance of the acceleration of Fe^{2+} oxidation by removing LOOH from liposomes suggests that the pre-existing LOOH play an important role in accelerating the oxidation of Fe^{2+}.

The evolution of the LOOH during Fe^{2+}-initiated lipid peroxidation

In order to investigate the evolution of LOOH in liposomes during Fe^{2+}-initiated lipid peroxidation and to verify that
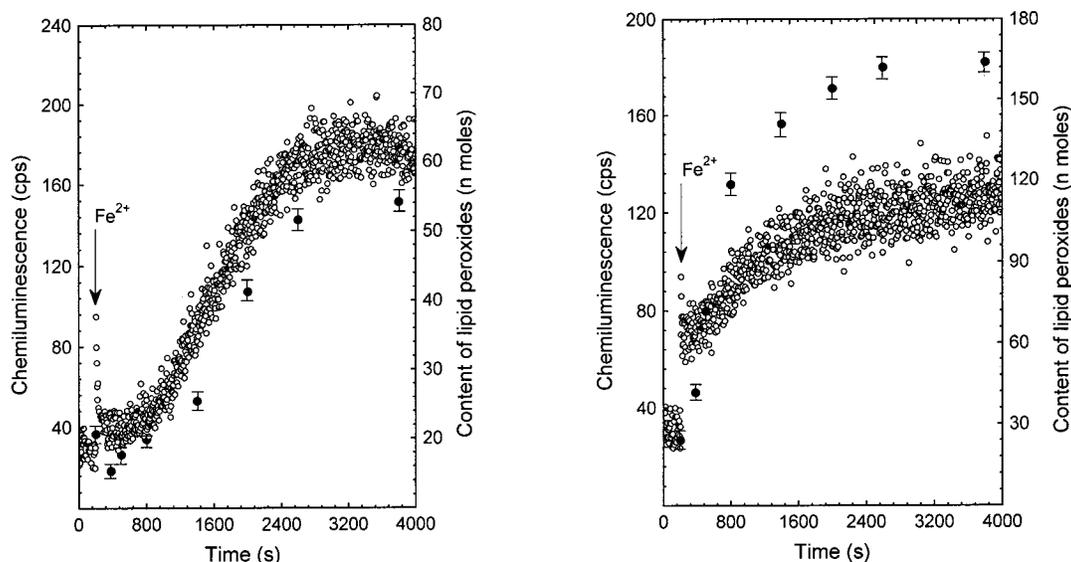
Figure 7   The content of lipid hydroperoxides during lipid peroxidation initiated by 100 μM ADP-Fe^{2+} in 2 ml of liposome suspension (1.4 mg/ml) prepared in Tris/HCl buffer (left-hand panel) and in acetate buffer (right-hand panel)

The lipid peroxidation took place at 25 °C and the content of lipid peroxide (●) was determined by iodometric assay (see text) in three independent measurements. The bars represent S.D.
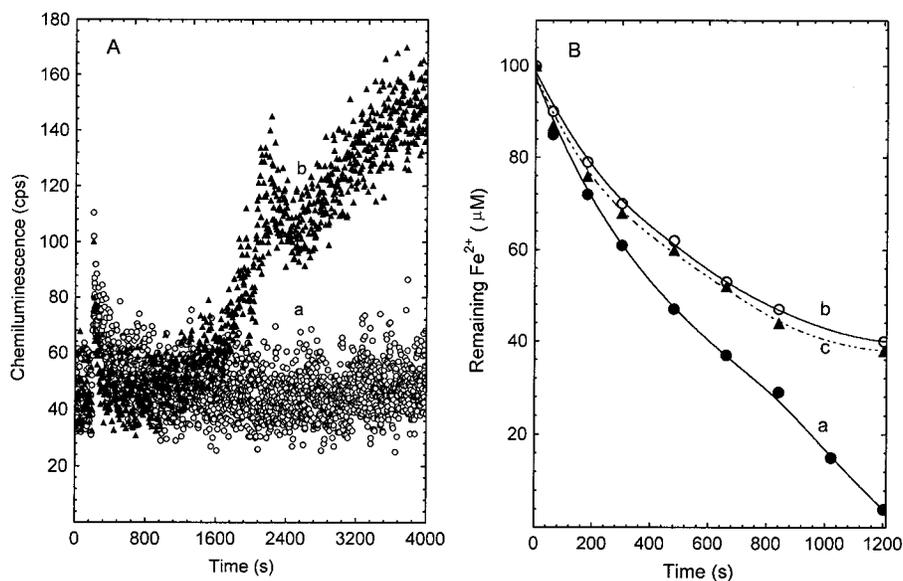
Figure 8   The lipid peroxidation initiated by 100 μM ADP-Fe^{2+} (A) and the corresponding oxidation of Fe^{2+} (B) in 1.4 mg/ml liposomes

Liposomes were incorporated with 35 μM DPPD (a) or without DPPD (b). Curve c in (**B**) shows the Fe^{2+} oxidation in liposome-free Tris/HCl buffer (pH 7.4).

the height of the flash induced by addition of Fe^{2+} reflects the content of LOOH existing or accumulated in liposomes, the content of LOOH in liposomes during lipid peroxidation was measured in neutral and acidic buffers. The results are shown in Figure 7, on which the kinetic curves of the concomitant CL are also plotted. It can be found that at neutral pH the LOOH content drops sharply upon addition of Fe^{2+}, and is then kept at a very low level with a slow-rising slope during the latent period. As lipid peroxidation is intensified, the LOOH content increases and finally reaches a higher stationary level when the continuous

chain reaction of lipid peroxidation takes place. However, when liposomes were suspended in acetate buffer, the LOOH content almost immediately began to increase without an observable drop upon the addition of Fe^{2+}. In accordance with that, almost no time lag could be observed in the Fe^{2+}-initiated lipid peroxidation under acidic conditions. Although the concomitant CL was lower, the content of LOOH during lipid peroxidation was higher in acidic buffer than at neutral pH. It is interesting to find that whatever the pH of the buffer, both the variation of LOOH content in liposomes and the lipid-peroxidation-con-

comitant CL had similar kinetic patterns. It should be pointed out that the measurement of LOOH content in liposomes during lipid peroxidation demonstrated that only when the content of LOOH rises significantly does the latent period end and the lipid peroxidation really start.

The effect of DPPD on lipid peroxidation and Fe²⁺ oxidation

In order to investigate the role of lipid peroxyl radical (LOO˙) in the initiation of the lipid peroxidation induced by Fe²⁺ and the regulation of the Fe²⁺ oxidation by liposomes, hydrophobic DPPD, an effective scavenger of LOO˙ [25], was incorporated into liposomes. The CL of the Fe²⁺-initiated lipid peroxidation in either DPPD-incorporated or DPPD-free liposomes was measured in Tris/HCl buffer (pH 7.4). The effect of liposomes incorporated with DPPD on the oxidation of Fe²⁺ in the same buffer was also determined. The results are shown in Figure 8. It was found that neither lipid peroxidation nor the acceleration of Fe²⁺ oxidation in the liposomes incorporated with 35 μM DPPD could be observed. The inability of Fe²⁺ to initiate lipid per-oxidation in the DPPD-incorporated liposomes implies that the LOO˙ may be the species that initiates lipid peroxidation. The failure of the DPPD-incorporated liposomes to accelerate the oxidation of Fe²⁺ suggests that the LOO˙ generated in liposomes during the lipid peroxidation might also be responsible for the extra oxidation of Fe²⁺.

DISCUSSION

The following points concerning the role of ferrous ions, the existing LOOH and the peroxyl radicals may be drawn from the observed facts in this study. (i) The existence of a latent period before lipid peroxidation starts is a unique characteristic in Fe²⁺-initiated lipid peroxidation in liposomal or microsomal systems. It implies that there is a process leading to formation of the species responsible for initiating the chain reaction of lipid peroxidation at a sufficient concentration after addition of Fe²⁺ in the system. If the species did not react with Fe²⁺, the second addition of Fe²⁺ within the latent period should not affect the formation of the species and could not cause any significant change in the length of the time lag. However, a lengthening of the latent period was observed, which indicates that the species responsible for initiation of the lipid peroxidation can be suppressed by newly added ferrous ions. In addition to that, the observed apparent latent period in the lipid peroxidation initiated by the single dose of 150 μM Fe²⁺, or 100 μM Fe²⁺ at first and 50 μM Fe²⁺ later, in the liposomal system remained the same, as long as the second dose of Fe²⁺ was added before peroxidation started (see Table 1). This indicates that the suppression of the initiators by Fe²⁺ depends essentially on the overall added Fe²⁺ rather than solely on the ions added second. Thus it seems that ferrous ions possess two functions: initiating lipid peroxidation by reaction with liposomes on one hand, and inhibiting per-oxidation by suppressing the species responsible for initiating the chain reaction of lipid peroxidation on the other. (ii) When TPP, an effective compound to reduce organic hydroperoxides through its biphilic insertion into and then cleavage of the O–O bond [26,27], was used to remove the pre-existing LOOH in liposomes, Fe²⁺ could no longer initiate the peroxidation. It suggests that the pre-existing lipid hydroperoxides are necessary for Fe²⁺-initiated lipid peroxidation. In contrast to that, introduction of extra LOOH in liposomes intensified the Fe²⁺-initiated per-oxidation and shortened the latent period. This effect may suggest that it is actually the reaction of Fe²⁺ with pre-existing LOOH in liposomes that is responsible for the initiation of lipid per-

oxidation. (iii) When DPPD, a well-established and effective scavenger of LOO˙ [25,28], was incorporated into liposomes, neither the initiation of peroxidation by Fe²⁺ nor the acceleration of Fe²⁺ oxidation was observed. This indicates that LOO˙ may be the real initiator of the Fe²⁺-initiated lipid peroxidation and that it might react with ferrous ions. (iv) It was observed that Fe²⁺ was oxidized faster in the liposomal system than in buffer solution at either neutral or acidic pH. However, the oxidation was accelerated only by the LOOH-containing, not the LOOH-deprived, liposomes, which indicates strongly that it is the reaction of ferrous ions with the pre-existing LOOH in liposomes and/or the species generated by this reaction responsible for the extra oxidation of Fe²⁺. Inability to both initiate peroxidation and accelerate oxidation of Fe²⁺ in the system containing the liposomes incorporated with sufficient DPPD suggests strongly that the species responsible for initiating peroxidation and the species responsible for extra oxidation of Fe²⁺ may be identical and originate from the decomposition of LOOH. The present study suggests that Fe²⁺ initiates peroxidation in liposomes by decomposing LOOH, which leads to the formation of LOO˙ and also inhibits the peroxidation by reacting with LOO˙.

Using basic lipid chemistry (e.g. [29,30]), the following reactions are proposed to be involved in Fe²⁺-initiated lipid per-oxidation:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\cdot} + OH^{-} \tag{1}$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\cdot} + H^{+} \tag{2}$$

$$LO^{\cdot} + LH \rightarrow L^{\cdot} + LOH \tag{3}$$

$$LO^{\cdot} + Fe^{2+} + H^{+} \rightarrow Fe^{3+} + LOH \tag{4}$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \tag{5}$$

$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH \tag{6}$$

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow LO^* + LOH + {}^1O_2 \tag{7}$$

$$Fe^{2+} + LOO^{\cdot} + H^{+} \rightarrow Fe^{3+} + LOOH \tag{8}$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-} \tag{9}$$
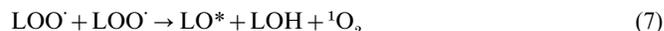
In the above equations, Fe²⁺ should be considered as chelated ferrous ion (i.e. ch-Fe²⁺), and LH stands for lipid with a number of allyl hydrogens that can be abstracted by LOO˙ and lipid alkoxyl radicals (LO˙). Reaction (1) is much faster than reaction (2). For example, the rate constant for the decomposition of t-butyl hydroperoxide by ATP-Fe²⁺ is 1.3×10^3 $M^{-1} \cdot s^{-1}$ [31], while the decomposition rate of linoleic acid hydroperoxide by AMP-Fe²⁺ was reported to be about 37.5-fold higher than that by AMP-Fe³⁺ [14]. Although the rate constant for reaction (3) has not been determined, the reaction is expected to be fast [29] and initiates lipid peroxidation. Reaction (4) is also fast and can suppress the formation of L˙ and LOO˙. For example, the rate constant of the reaction of t-butoxyl radical with Fe²⁺ was determined recently as 3.0×10^8 $M^{-1} \cdot s^{-1}$ [32]. Reactions (5) and (6) constitute a chain reaction leading to propagation of lipid peroxidation. The rate constant for reaction (5) is 10^9–10^{10} $M^{-1} \cdot s^{-1}$ if the oxygen partial pressure of the reaction mixture is 100 mmHg or greater [33]. The rate constant for reaction (6) was estimated as 3.0×10^5 $M^{-1} \cdot s^{-1}$ [29]. Reaction (7) is the main reaction to terminate the chain reaction of lipid peroxidation and is responsible for photon emission from the exited carboxyl (LO*) and the dimol reaction of singlet oxygen (¹O₂). Its rate constant was estimated as 2.2×10^3 $M^{-1} \cdot s^{-1}$ [29]. Reaction (8) has a rate constant estimated as 5×10^3 $M^{-1} \cdot s^{-1}$ at neutral pH [29] and may become another reaction to terminate the chain reaction of lipid peroxidation at higher concentrations of Fe²⁺. Reaction (9) stands for the autoxidation of ADP-Fe²⁺,

and its apparent rate constant, $k_9 \cdot [O_2]$, was determined as $5.7 \times 10^2 \, s^{-1}$ at neutral pH in this study. Based on the above chemistry, it seems we can quite reasonably explain the phenomena observed in this study. First of all, the latent period can be interpreted as the time needed to reduce the concentration of Fe^{2+} to such a degree that the peroxyl radicals, $LOO^{\cdot}$, can no longer be effectively suppressed by their reaction with the remaining Fe^{2+}, and to reach a substantially high level at which their attack on the fatty acid chain of the lipid becomes dominant. The reaction of Fe^{2+} with $LO^{\cdot}$ may also contribute to the formation of the latent period. It can also be expected from the chemistry that when the second dose of Fe^{2+} is added within the latent period, the latent period is lengthened because more ferrous ions are available to suppress $LOO^{\cdot}$ and $LO^{\cdot}$.

To gain insight into the role of the reaction of Fe^{2+} with $LOO^{\cdot}$ and the accumulation of LOOH in Fe^{2+}-initiated peroxidation, the evolution kinetics of the LOOH in liposomes were investigated. As shown in Figure 7 (left-hand panel), a rapid drop in LOOH, which is certainly due to reactions (1) and (2), was always observed immediately after addition of Fe^{2+} in the neutral liposomal system. Then, a low-level LOOH was followed during the latent period. During this period a quasi equilibrium between LOOH production and its decomposition exists. Besides reaction (5), some LOOH were generated as a result of the reaction of Fe^{2+} with $LOO^{\cdot}$. The newly produced LOOH can be decomposed again by Fe^{2+} to yield a new $LOO^{\cdot}$ through reactions (1–4). Thus a cycling of $LOO^{\cdot} \rightarrow LOOH \rightarrow LOO^{\cdot}$ is established at the expense of Fe^{2+}. As the first approximation, the following differential kinetic equation can be established:

$$\frac{d[LOOH]}{dt} = k_8[LOO^{\cdot}][Fe^{2+}] + k_6[LOO^{\cdot}][LH]$$

$$- k_1[LOOH][Fe^{2+}] - k_2[LOOH][Fe^{3+}] \quad (10)$$

where k_1, k_2, k_6 and k_8 are the rate constants for reactions (1), (2), (6) and (8) respectively. By applying the steady-state principle to equation (10) during the latent period, i.e. $d[LOOH]/dt = 0$, the following solution can be obtained:

$$[LOOH] = \frac{k_8 + k_6[LH]/[Fe^{2+}]}{k_1 + k_2([Fe^{3+}]/[Fe^{2+}])}[LOO^{\cdot}] \quad (11)$$

It seems that equation (11) can explain some important features observed in Fe^{2+}-initiated lipid peroxidation. (i) Since the rate constant k_8 for reaction (8) is much higher under acidic conditions than in neutral pH, the LOOH produced by this reaction increased so quickly that the initial drop, which was observed at neutral pH, disappeared, and a significantly higher level of LOOH was observed in the liposomes suspended in acetate buffer (pH 5.0; compare Figure 7, left and right panels). (ii) When the ratio of Fe^{2+} to lipid, $[Fe^{2+}]/[LH]$, decreases to a critical value, [LOOH] starts a rapid increase and the peroxidation starts. (iii) A low concentration of Fe^{2+} causes a short latent period in the initiated peroxidation. (iv) The ratio of Fe^{3+} to Fe^{2+}, $[Fe^{3+}]/[Fe^{2+}]$, does not significantly affect the latent period, since k_2 is at least one order of magnitude less than k_1.

In the early investigations by Aust and his co-workers [9,13,14], it was reported frequently that the ratio of Fe^{2+} to Fe^{3+} was a primary determining factor for the initiation of lipid peroxidation reactions and that the maximal initiation rates occurred at a ratio of 1:1. However, the work of Halliwell and co-workers on the stimulation of Fe^{2+}-dependent peroxidation by ascorbic acid, Al^{3+} or Pb^{2+} argued against the participation of an Fe^{2+}–Fe^{3+}–O_2 complex, or a critical 1:1 ratio of Fe^{2+} to Fe^{3+}, in the initiation of lipid peroxidation in liposomes and rat liver microsomes [15]. The present study showed that whether adding 100 or 150 μM

Fe^{2+} initially or adding 100 μM Fe^{2+} initially and then 50 μM Fe^{2+} later at various times during the latent period in the liposomal system, the concentration of the remaining Fe^{2+} at the end of the latent period was almost the same every time (about 40 μM). At least two different 'critical' ratios of Fe^{2+} to Fe^{3+} were observed by the end of the latent period: 1:2.5 for 100 μM Fe^{2+}-initiated and 1:3.75 for 150 μM Fe^{2+}-initiated peroxidation. It was also found that although the addition of a second dose of Fe^{2+} in the reaction system resulted in a different ratio of Fe^{2+} to Fe^{3+} during the latent period, either the time lag or the final peroxidation rate was not affected. Evidently, this study does not support the critical 1:1 ratio of Fe^{2+} to Fe^{3+} in the initiation of lipid peroxidation.

It seems that by including the reaction of ferrous ions with $LOO^{\cdot}$, which are generated initially by decomposition of the pre-existing LOOH and consequently formed through the Fe^{2+}-mediated cycling of $LOO^{\cdot} \rightarrow LOOH \rightarrow LOO^{\cdot}$ in the basic lipid chemistry, nearly all controversial phenomena observed in Fe^{2+}-initiated lipid peroxidation of liposomes, such as the existence of a latent period, the critical ratio of $[Fe^{2+}]$ to lipid, the required oxidation of Fe^{2+}, the acceleration of Fe^{2+} oxidation by liposomes, the effects of pH, iron chelators and the pre-existing LOOH on the lipid peroxidation, may be explained without conflicting each other. Physiologically, the dependence of Fe^{2+}-initiated peroxidation on pre-existing lipid hydroperoxides may imply that the iron-catalysed peroxidation, which is not Fenton-reaction-dependent, may not be serious *in vivo* since some endogenous enzymes, such as glutathione peroxidase and glutathione reductase, remove LOOH from the cell membrane and keep the peroxides at very low levels *in vivo*.

REFERENCES

1 Poli, G., Albano, E. and Dianzani, M. U. (1987) The role of lipid peroxidation in liver damage. Chem. Phys. Lipids **45**, 117–142

2 Sies, H. (1985) Oxidative Stress, Academic Press, London

3 Hochstein, P., Nordenbrand, K. and Ernster, L. (1964) Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and mitochondria. Biochem. Biophys. Res. Commun. **14**, 323–328

4 Benedetti, A., Comporti, M. and Esterbauer, H. (1980) Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. Biochim. Biophys. Acta. **620**, 281–296

5 Halliwell, B. (1976) An attempt to demonstrate a reaction between superoxide and hydrogen peroxide. FEBS Lett. **72**, 8–10

6 Gutteridge, J. M. C. (1984) Ferrous ion-EDTA-stimulated phospholipid peroxidation: a reaction changing from alkoxyl-radical-to hydroxyl-radical-dependent initiation. Biochem. J. **224**, 697–701

7 Morehouse, L. A., Tien, M., Bucher, J. R. and Aust, S. D. (1983) Effect of hydrogen peroxide on the initiation of microsomal lipid peroxidation. Biochem. Pharmacol. **32**, 123–127

8 Beloqui, O. and Cederbaum, A. I. (1986) Prevention of microsomal production of hydroxyl radicals, but not lipid peroxidation, by the glutathione peroxidase system. Biochem. Pharmacol. **35**, 2663–2669

9 Minotti, G. and Aust, S. D. (1987) The requirement for iron(III) in the initiation of lipid peroxidation by iron(II) and hydrogen peroxide. J. Biol. Chem. **262**, 1098–1104

10 Tien, M., Bucher, J. R. and Aust, S. D. (1982) Thiol-dependent lipid peroxidation. Biochem. Biophys. Res. Commun. **107**, 279–285

11 Goddard, J. G. and Sweeney, G. D. (1987) Delayed, ferrous iron-dependent peroxidation of rat microsomes. Arch. Biochem. Biophys. **259**, 372–381

12 Koppenol, W. H. (1983) Thermodynamics of the Fenton-driven Haber-Weiss and related reaction. In Oxygen Radicals and Their Scavenger Systems. Vol. 1. Molecular Aspects (Cohen, G. and Greenwald, R. A., eds.), pp. 84–88, Elsevier Science, New York

13 Minotti, G. and Aust, S. D. (1987) The role of iron in the initiation of lipid peroxidation. Chem. Phys. Lipids **44**, 191–208

14 Bucher, J. R., Tien, M. and Aust, S. D. (1983) The requirement for ferric in the initiation of lipid peroxidation by chelated ferrous iron. Biochem. Biophys. Res. Commun. **111**, 777–784

15 Aruoma, O. I., Halliwell, B., Laughton, M. J., Quinlan, G. J. and Gutteridge, J. M. C. (1989) The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)-iron(III) complex. Biochem. J. **258**, 617–620

16 Tadolini, B., Cabrini, L., Menna, C., Pinna, G. G. and Hakim, G. (1997) Iron(III) stimulation of lipid hydroperoxide-dependent lipid peroxidation. Free Radicals Res. **27**, 563–576

17 Aust, S. D., Bucher, J. R. and Tien, M. (1984) Evidence for the initiation of lipid peroxidation by a ferrous-dioxygen-ferric chelate complex. In Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M. and Tait, D. J., eds.), pp. 147–152, Walter de Gruyter Co., Berlin

18 Driomina, E. S., Sharov, V. S. and Vladimirov, Y. A. (1993) Fe^{2+}-induced lipid peroxidation kinetics in liposomes: the role of surface Fe^{2+} concentration in switching the reaction from acceleration to decay. Free Radicals Biol. Med. **15**, 239–247

19 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917

20 Terao, J., Asano, I. and Matsushita, S. (1985) Preparation of hydroperoxy and hydroxy derivatives of rat liver phosphatidylcholine and phosphatidylethanolamine. Lipids **20**, 312–317

21 Shen, X., Tian, J., Zhu, Z. and Li, X. (1991) Chemiluminescence study on the peroxidation of linoleic acid initiated by the reaction of ferrous iron with hydrogen peroxide. Biophys. Chem. **40**, 161–167

22 Braughler, J. M., Duncan, L. A. and Chase, R. L. (1986) The involvement of iron in lipid peroxidation: importance of ferric to ferrous ratios in initiation. J. Biol. Chem. **261**, 10282–10289

23 Buege, J. and Aust, S. D. (1987) Microsomal lipid peroxidation. Methods Enzymol. **52**, 302–318

24 Mahler, H. R. and Elowe, D. G. (1954) Studies on methalloflavoproteins. II. The role of iron in diphosphopyridine nucleotide cytochrome c reductase. J. Biol. Chem. **210**, 165–179

25 Noguchi, N., Yamashita, H., Gotoh, N., Yamamoto, Y., Numano, R. and Niki, E. (1998) 2,2′-Azobis (4-methoxy-2,4-dimethylvaleronitrile), a new lipid-soluble azo inhibitor: application to oxidations of lipid and low-density lipoprotein in solution and in aqueous dispersions. Free Radicals Biol. Med. **24**, 259–268

26 Adam, W. and Weinkotz, S. (1995) Triphenylphosphine reduction of thiophene endoperoxides: nucleophilic attack on sulfur versus biphilic insertion into the peroxide bond. Tetrahedron Lett. **36**, 7431–7432

27 Balci, M. (1981) Bicyclic endoperoxides and synthetic applications. Chem. Rev. **81**, 91–108

28 Shi, H., Noguchi, N. and Niki, E. (1999) Comparative study on dynamics of antioxidative action of alpha-tocopheryl hydroquinone, ubiquinol, and alpha-tocopherol against lipid peroxidation. Free Radicals Biol. Med. **27**, 334–346

29 Vladimirov, Y. A. (1986) Free radical lipid peroxidation in biomembrane: mechanism, regulation and biological consequences. In Free Radicals, Aging and Degenerative Diseases (Johnson, J. E., Walford, R., Harman, D. and Miquel, J., eds.), pp. 14–195, Allan R. Liss, New York

30 Borg, D. C. and Schaich, K. M. (1988) Iron and hydroxyl radicals in lipid oxidation: Fenton reactions in lipid and nucleic acids co-oxidized with lipid. In Oxy-radicals in Molecular Biology and Pathology (Cerutti, P. A., Fridovich, I. and McCord, J. M., eds.), pp. 427–441, Alan R. Liss, New York

31 Rush, J. D. and Koppenol, W. H. (1990) Reactions of Fe(II)-ATP and Fe(II)-citrate complexes with t-butyl hydroperoxide and cumyl hydroperoxide. FEBS Lett. **275**, 114–116

32 Mihaljevic, B., Medved-Rogina, B. and Razem, D. (1999) Rate constant for the reaction of the t-butoxyl radical with Fe(II) ion. Radiat. Phys. Chem. **55**, 593–597

33 Pryor, W. A. (1973) Free radical reactions and their importance in biochemical systems. Fed. Proc. **2**, 1862–1869