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EFFECT OF FERROUS ION AND ASCORBATE-INDUCED LIPID PEROXIDATION ON LIPOSOMAL MEMBRANES

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Physical change of liposomal membranes due to peroxidation was examined by treating multilamellar liposomes of rat liver phosphatidylcholine with ferrous ion and ascorbate. Ferrous ion and ascorbate added exogenously induced peroxidation of the liposomal membranes. The peroxidation was dependent on the charge of liposomal membrane: neutral and negatively charged liposomes were sensitive to peroxidation, whereas positively charged liposomes were not. Ferrous ion induced breakdown of polyunsaturated fatty acid residues in phosphatidylcholine, the order of sensitivity of unsaturated fatty acids being as follows: docosahexaenoic acid, arachidonic acid, linoleic acid. Oleic acid was insensitive to peroxidation. Most of the water-soluble reaction products were released from liposomes immediately after they were formed, but phosphorus-containing products were not released from the liposomal membranes. One of the phosphorus-containing products remaining in the membrane had a similar R_F value to lysophosphatidylcholine on thin-layer chromatography. A permeability increase was always observed after a certain lag, indicating that accumulation of peroxidized products in membranes may cause perturbation leading to loss of barrier function. This permeability increase was not specific, since large molecules such as dextran (M_r 75 000) could be released in the same way as glucose. Decrease of vesicle size during peroxidation was observed by electron microscopy.

Introduction

Lipid peroxidation has been widely investigated because of its suspected role in a variety of pathological conditions [1–3]. The pathological consequencies of lipid peroxidation may be associated with alterations of membrane functions. Such possibility is supported by several lines of research. In red cells [4,5], mitochondria [6], microsome [7] and lysosome [8], loss of membrane integrity or inactivation of integral enzymes were observed during lipid peroxidation. The molecular mechanism of membrane damages induced by lipid peroxidation has not been, however, well understood.

It is generally accepted that peroxidation of unsaturated fatty acids in various cellular membranes leads to loss of double bonds or breaks in fatty acid side chains in the membranes. Based on previous reports that liposome premeability is sensitive to autooxidation of lipids, both before and after dispersion [9,10], it is certain that peroxidation causes perturbation of lipid bilayers. Very little is, however, known about the mechanism of peroxidation-induced perturbation leading to membrane damage.

We studied the effects of peroxidation induced by ferrous ions and ascorbate on the physical structure of liposomal membranes, and the events causing severe perturbation of membranes.

Materials and Methods

Lipids and chemicals. Phosphatidylcholine of rat liver and that of egg yolk were prepared by chromatography on Aluminium Oxide Neutral and Unicil. The fatty acid composition of rat liver phosphatidylcholine used throughout the present study was as follows: 25.1% palmitic acid, 22.7% stearic acid, 6.9% oleic acid, 13.3% linoleic acid, 25.5% arachidonic acid and 6.5% docosahexaenoic acid. Preparation and extraction of [^{32}P]phosphatidylcholine from rat liver were performed by the procedure of Inoue and Kinsky [11]. β,γ-Dipalmitoyl-D,L-α-glycerophosphocholine, dicetylphosphate, stearylamine and cholesterol were purchased from the Sigma Chemical Company, St. Louis, MO, U.S.A. All lipids gave single spots on silica gel thin-layer chromatography. Other chemicals and enzymes were purchased from the following companies: Oriental Yeast Company, Tokyo (hexokinase, glucose-6-phosphate dehydrogenase and NADP); Sigma Chemical Company, St. Louis, MO, U.S.A. (superoxide dismutase and catalase); Wako Pure Chemical Industries, Ltd., Osaka (2-thiobarbituric acid, ATP); Radiochemical Centre, Amersham, U.K. (D-[6-^{3}H]glucose, inorganic [^{32}P]-phosphate); New England Nuclear, Boston, MA, U.S.A. ([*carboxyl*-^{14}C]dextran).

Preparation of liposomes. Multilamellar liposomes were prepared as described previously [12]. The dried lipid film (3 μmol phosphatidylcholine) was swollen in 0.3 ml 0.3 M glucose. In some experiments, lipids were swollen in 0.3 M glucose containing trace amounts of [^{3}H]glucose (M_r 180) and [^{14}C]dextran (M_r 75 000). Untrapped glucose was removed by dialysing the preparations at room temperature against isotonic salt solution for more than 2 h. Untrapped dextran was removed from the liposome preparations by floating liposomes in 0.3 M sucrose [13].

Measurement of liposomal permeability. Glucose release from liposomes was assayed enzymatically with hexokinase and glucose-6-phosphate dehydrogenase following the method of Kinsky et al. [14] with a slight modification. The amount of NADPH generated was determined fluorometrically with a Recording Spectrofluorophotometer (Shimadzu Seisaku-sho RF 501 apparatus). Release of [^{3}H]glucose (M_r 180) and [^{14}C]dextran (M_r 75 000) was assayed in an ultrafiltration cell (Amicon Model 12, Amicon Corp., MA, U.S.A.), equipped with a Millipore membrane filter (type PH, pore size 0.3 μm, Japan Millipore Ltd., Tokyo, Japan) following the method of Oku et al. [13].

Peroxidation of liposomal lipids. Lipid peroxidation in liposomes (phosphatidylcholine 0.1 mM) was induced by the addition of 5 μM $FeSO_4$ and 0.1 mM ascorbic acid at room temperature in 10 mM Tris-buffered saline (pH 7.4) and was stopped by further addition of 5 mM hydroquinone. The degree of peroxidation (the amounts of thiobarbituric acid-reactive substances produced) was estimated by the 2-thiobarbituric acid method [15] and expressed as equivalents of malondialdehyde. A standard curve was made using tetraethoxypropane, a precursor of malondialdehyde.

Lipid analysis. The fatty acid compositions of intact and peroxidized phosphatidylcholine were determined by gas-liquid chromatography (Shimadzu Seisaku-sho, GC-OAP). Lipids were dissolved in 2 ml methanol in water (1 : 1) and hydrolyzed by incubation with 0.27 ml 85% KOH at 75°C for 1 h. Then the mixture was cooled, acidified with 6 M HCl, and extracted three times with diethyl ether. The extracts were combined, washed three times with concentrated aqueous KCl and evaporated. The residue was methylated with CH_2N_2 and the methyl esters were analyzed on a column (3 mm × 3 m) of 20% EGSS-X on chromosorb W AW (80–100 mesh) (Gasukuro Kyogyo Co.) at 180°C. The absolute amounts of each fatty acid in samples were obtained by comparison of the peaks with those of pentadecanoic acid, an internal standard [16]. The flow rate of nitrogen gas was 40 ml/min. Thin-layer chromatography was performed using precoated silica gel plates (20 × 5 cm, Merck).

Measurement of turbidity. Turbidity of liposome preparations was measured at 450 nm in a double beam spectrophotometer (Shimadzu Seisakusho).

Electron micrography. A drop of liposome solution (phosphatidylcholine of about 5 mM) was placed on a 180-mesh copper grid previously coated with carbon film. Excess sample was removed with filter paper and then 2% sodium phosphotungstate (pH 6.5) was applied to the grid. Excess staining solution

was removed with filter paper after 10–15 s and the grid was immediately examined under an electron microscope (Hitachi HU 11-B) at 75 kV.

Preparation of multilamellar liposomes deprived of trapped glucose in the outermost water space. Multilamellar liposomes prepared as described above were incubated in various hypotonic solutions at room temperature for 30 min. The tonicity was adjusted to 300 mosM by adding a suitable amount of sodium chloride, and the preparations were further dialyzed against 0.15 M sodium chloride for 2 h. The amounts of trapped glucose in liposomes preincubated in the medium of 210, 120 and 30 mosM, which were expressed relative to that in control liposomes without pretreatment (100), were 78, 52 and 31, respectively. With decrease in osmolarity of the medium, the amount of trapped glucose decreased, suggesting that liposomes pretreated hypotonically may lose glucose trapped in the outer water spaces.

Results

Increase of glucose permeability of liposomal membrane during lipid peroxidation

When liposomes consisting of rat liver phosphatidylcholine, dicetylphosphate and cholesterol were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for various times at room temperature, lipid peroxidation occurred almost linearly during the first 30 min, whereas increased permeability to glucose was observed after a lag of 10 min (Fig. 1). After incubation for 20 min, almost all the trapped glucose was released. On the other hand, when the liposomes consisted of egg yolk phosphatidylcholine, dicetylphosphate and cholesterol, only 50% of the trapped glucose was released, even after incubation for 70 min. During this period, no formation of thiobarbituric acid-reactive substances was observed. Liposomes consisting of dipalmitoylphosphatidylcholine, dicetylphosphate and cholesterol showed neither increased glucose release nor formation of thiobarbituric acid-reactive substances, indicating that ferrous ion and ascorbic acid had no direct effect on their permeability. It is noteworthy that the addition of superoxide dismutase (50 μg/ml) or catalase (10 μg/ml) did not have any appreciable influence on the reaction (data not shown).

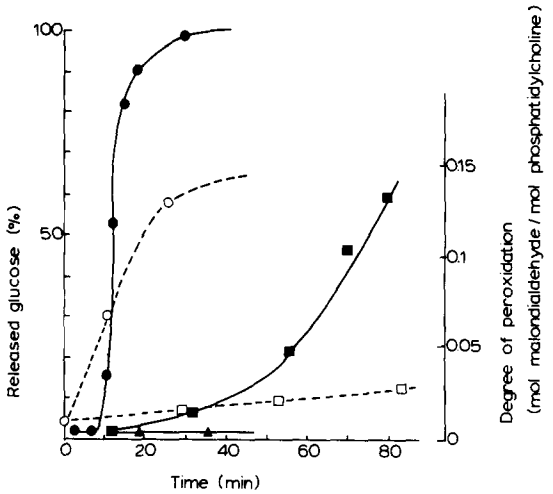


Fig. 1. Increase of glucose permeability of liposomal membrane during lipid peroxidation. Liposomes (0.1 mM phospholipid) consisting of phosphatidylcholine, dicetylphosphate and cholesterol (molar ratio 10 : 1 : 10) were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid at room temperature for various times. Glucose release from liposomes of rat liver phosphatidylcholine (●), egg yolk phosphatidylcholine (■) and dipalmitoylglycerophosphocholine (▲) was assayed. The amounts of thiobarbituric acid-reactive substances formed in liposomes of rat liver phosphatidylcholine (○) and of egg yolk phosphatidylcholine (□) were also determined.

Next, the effect of ferrous ion and ascorbic acid on liposomes pretreated hypotonically was examined (Fig. 2). Lipid peroxidation in liposomes pretreated hypotonically proceeded similarly to that in control liposomes, but release of trapped glucose was delayed: the time required for 50% of the maximum glucose release were 10.5 min in intact control liposomes, 13 min in liposomes preincubated in 210 mosM sodium chloride solution and 15 min in liposome preincubated in 120 mosM medium. These findings suggested that peroxidation proceeds stepwise in multilamellar liposomes; the outermost lamellae may be damaged first by peroxidation and then the damage may proceed sequentially toward the center of the liposomes. The delay in glucose release in hypotonically treated liposomes can be explained by the absence of glucose in the aqueous spaces between outermost and adjacent lamellae.

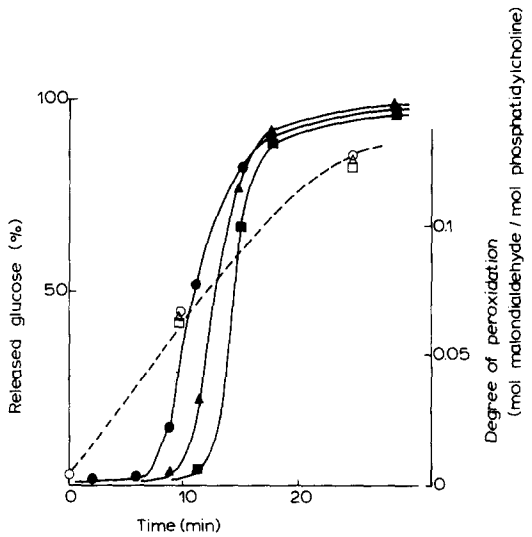


Fig. 2. Peroxidation-induced permeability increase in liposomes pretreated hypotonically. Rat liver phosphatidylcholine liposomes with no glucose trapped in the outermost water spaces were prepared as described in the Materials and Methods. These liposomes were treated with ferrous ions and ascorbate as described for Fig. 1. Glucose release from liposomes without pretreatment (●), and from those pretreated in 210 mosM (▲) and 120 mosM (■) sodium chloride, was assayed. At the same time, the amounts of thiobarbituric acid-reactive substances formed were assayed in control liposomes (○), and those pretreated in 210 mosM (△) and 120 mosM (□) sodium chloride solution.

Nonselective release of markers from liposomes upon peroxidation of lipid

Liposomes containing [^{3}H]glucose and [^{14}C]-dextran as trapped markers were incubated with $FeSO_4$ and ascorbic acid for various times at room temperature and then the amounts of markers released were assayed in an ultrafiltration cell equipped with a membrane filter [13]. Peroxidation of lipids induced nonspecific release of glucose and dextran from liposomes, since the rates of release of the two markers were similar (data not shown). Under the present experimental conditions, however, the osmotic mechanism should not work, since liposomes were prepared in physiological saline with a trace amount of radioactive markers and aqueous compartments of liposome contain almost the same solutes as medium [13]. These results indicate that lipid peroxidation causes initially disruption of membrane structures, resulting in the release of large molecules such as dextran. In the case of living cells or organelles, it is possible that the release of large molecules proceeds by a colloid-osmotic mechanism due to the formation of membrane lesions that are initially too small to permit the escape of large molecules but are sufficiently large to allow the exchange of ions.

Morphological change of liposomes

Morphological change of liposomes incubated with ferrous ion and ascorbate was determined by

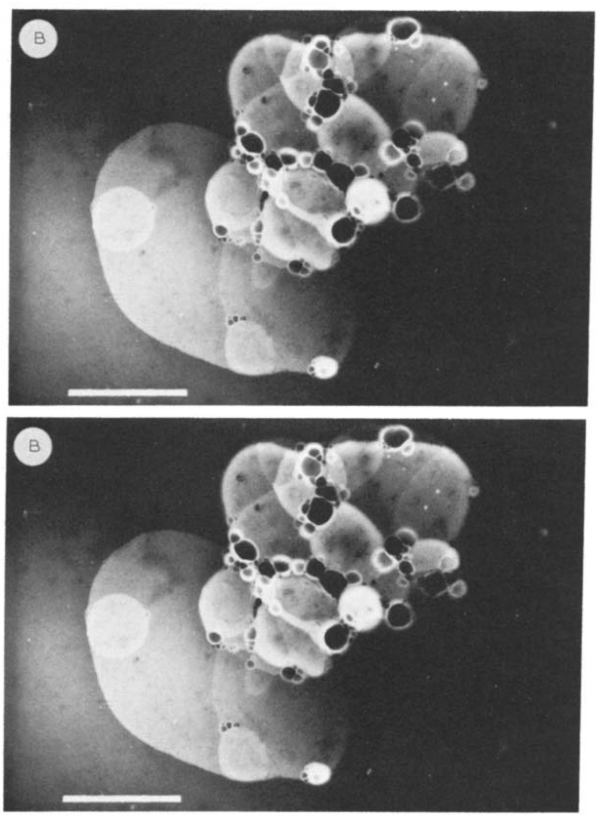


Fig. 3. Electron micrograph of peroxidized liposomes. (A) Peroxidized liposomes. Liposomes consisting of rat liver phosphatidylcholine, dicetylphosphate and cholesterol were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for 30 min. The reaction was stopped by further addition of 5 mM hydroquinone. The mixtures were centrifuged at 100 000 × g for 30 min to concentrate liposomes. The degree of peroxidation in the preparations was 0.05 mol malondialdehyde/mol phosphatidylcholine. Scale, 1 μm. (B) Control liposomes. The same liposomes as those in (A) were observed by electron microscopy without any treatment.

electron microscopy (Fig. 3). In a preparation of peroxidized liposomes forming 0.05 mol malonedialdehyde per mol phosphatidylcholine, the average diameters of liposomes (about 0.3 μm) were significantly smaller than those of untreated liposomes (about 1 μm) and the number of large liposomes decreased in some degree. The decrease in diameter of liposomes is consistent with the decrease in turbidity of the liposome preparations observed during lipid peroxidation. It is noteworthy that peroxidized preparations still consisted of closed multilamellar vesicles.

Changes of fatty acid composition of rat liver phosphatidylcholine during peroxidation

Peroxidation-induced change of the fatty acid composition of rat liver phosphatidylcholine in liposomal membranes was examined by gas-liquid chromatography (Fig. 4). Throughout the experiments, the ratio of palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) or oleic acid ($C_{18:1}$) to an internal standard, pentadecanoic acid was not changed significantly, indicating that contents of these fatty acids were not affected by peroxidation, in agreement with previous observations [7,15]. The contents of highly unsaturated fatty acids, such as docosahexaenoic acid ($C_{22:6}$) and arachidonic acid ($C_{20:4}$) decreased linearly as a function of lipid peroxidation. The amount of linoleic acid ($C_{18:2}$) also decreased significantly.

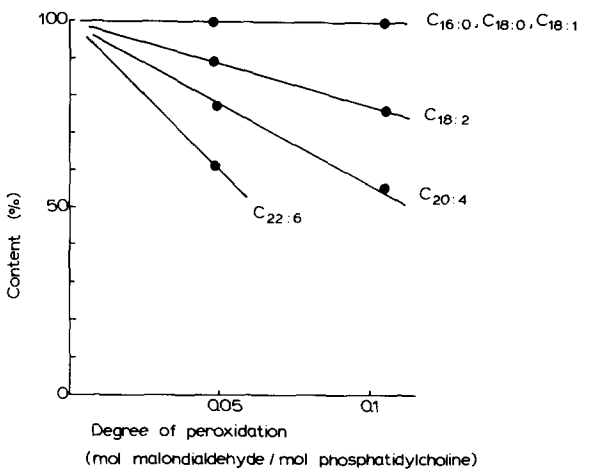


Fig. 4. Change of fatty acid composition during peroxidation. Rat liver phosphatidylcholine liposomes were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for various times and then lipids were extracted by chloroform/methanol (2 : 1) . The lipid extracts obtained were hydrolyzed and esterified as described in Materials and Methods. Changes of fatty acids are expressed as percentages of the original values for each fatty acid.

Chromatographic analysis of phosphate compounds

Chloroform/methanol extracts of peroxidized liposomes were analyzed by thin-layer chromatography. Autoradiograms of thin-layer plates

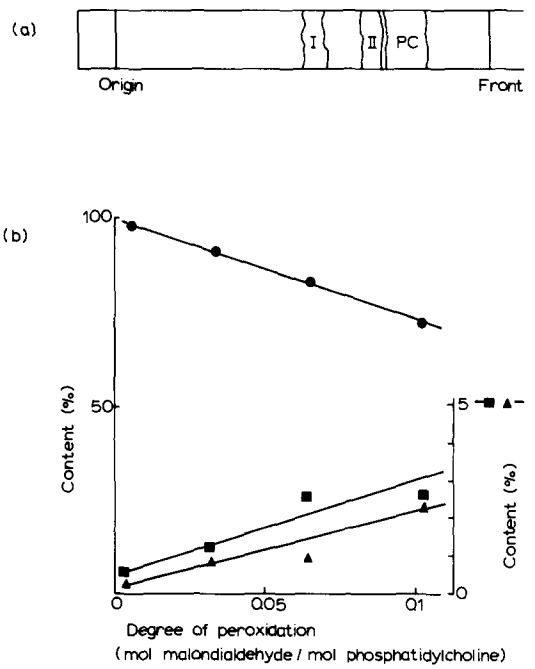


Fig. 5. Relative distribution of radioactivity after chromatography of chloroform/methanol extracts from reaction mixtures containing ^{32}P-labeled phosphatidylcholine liposomes. Liposomes consisting of ^{32}P-labeled rat liver phosphatidylcholine, dicetylphosphate and cholesterol (molar ratio 10 : 1 : 10) were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for various times at room temperature. Lipids were extracted with 6 vol. chloroform/methanol (2 : 1) and the extracts were subjected to chromatographic analysis in chloroform/methanol/water (65 : 35 : 8). Appropriate areas from the thin-layer plate shown in (a) were scraped off and counted. PC (●) corresponds to phosphatidylcholine and regions I (■) and II (▲) contain visible radioactive spots. Recovery of radioactivity in each spot was expressed as a percentage of the radioactivity recovered from the whole area between the solvent front and the origin.

revealed at least two new ^{32}P-labeled compounds as discrete spots after liposomes had been incubated with ferrous ion and ascorbate (Fig. 5a). Counting appropriate areas scraped from the plates showed that the radioactivity in regions corresponding to phosphatidylcholine decreased linearly as a function of formation of thiobarbituric acid-reactive substances (Fig. 5b), with concomitant increase in the radioactivities in the new spots I and II. However, the amount of radioactivity lost from phosphatidylcholine (about 25% under conditions where 0.1 mol malonedialdehyde per mol phosphatidylcholine was formed) was not recovered quantitatively in spots I and II (2.5 and 3%, respectively), but was mainly recovered from the area between the bottom of spot II and the origin. No discrete spots were detectable in this area; the radioactivity was spread diffusely over the area.

Release of thiobarbituric acid-reactive substances from liposomes

Rat liver phosphatidylcholine liposomes were treated with ferrous ion and ascorbate for various times and then the distribution of products was determined by filtering the mixtures through a millipore filter (Fig. 6). The amounts of thiobarbituric acid-reactive substances in the filtrates should indicate the amounts of these substances free from liposomes, since liposomes do not pass through a millipore filter [13]. The amount of thiobarbituric acid-reactive substances in the total reaction mixture was greater when hydroquinone was used to stop lipid peroxidation than when EDTA was used, though the amounts of thiobarbituric acid-reactive substances in the filtrates were similar. When EDTA was used to stop the reaction, the amount of thiobarbituric acid-reactive substances recovered in the filtrate was almost quantitative, since about 95% of the thiobarbituric acid-reactive substances in the total reaction mixture was recovered in the filtrate. But when hydroquinone was used, the recovery of thiobarbituric acid-reactive substances in the filtrate was about 80% of that in the total reaction mixture.

No appreciable damage of freshly added liposomes could be observed, after incubation, in the filtrates containing the highest amounts of thiobarbituric acid-reactive substances (data not shown).

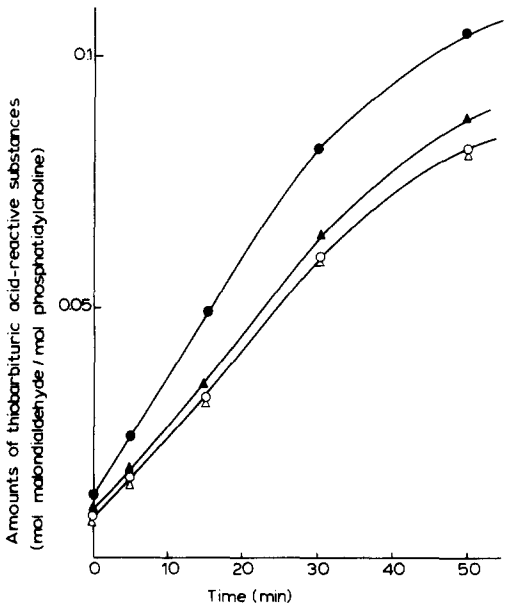


Fig. 6. Release of thiobarbituric acid-reactive substances from liposomes. Liposomes consisting of rat liver phosphatidylcholine, dicetylphosphate and cholesterol were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for various times at room temperature. The reaction was stopped by adding 5 mM hydroquinone (●, ○) or 2 mM EDTA (▲, △). An aliquot of the reaction mixture was applied to an ultrafiltration cell equipped with a Millipore membrane filter [13] and the filtrate was collected. Amounts of thiobarbituric acid-reactive substances in filtrates are shown as open symbols, and those in whole reaction mixtures as closed symbols.

Distribution of phosphate compounds after peroxidation of liposomes

Peroxidation of liposomes containing ^{32}P-labeled rat liver phosphatidylcholine did not affect the overall distribution of radioactivity; more than 99% of the isotope initially added to the reaction mixture was recovered in the chloroform extract regardless of whether or not the liposomes were treated with ferrous ion and ascorbate for 50 min (data not shown). When the reaction mixture was passed through a millipore filter, less than 0.5% of the radioactivity was recovered in the filtrate, though a significant amount of thiobarbituric acid-reactive substances was detected in the filtrate. These findings suggest that highly water-soluble phosphate

compounds were not produced when the liposomal membranes were peroxidized.

Effect of the liposomal composition on the sensitivity to peroxidation

Charge: The sensitivities of positively charged, negatively charged and neutral liposomes to peroxidation induced by ferrous ion and ascorbate were examined (Fig. 7). Liposomes with dicetylphosphate were most sensitive to peroxidation, followed by neutral liposomes. Positively charged liposomes were almost insensitive to peroxidation, since no appreciable formation of thiobarbituric acid-reactive substances was observed in liposomes with stearylamine. The sensitivity may depend on the electrostatic binding or accessibility of ferrous ions to the membrane surface. In fact, the amount of ferrous ions associated with negatively charged liposomes was much higher than that associated with positively charged liposomes (data not shown). When peroxidation was brought about by sonication, a different process from that induced by treatment with ferrous ion and ascorbate, peroxidation occurred independently of the charge of liposomes.

Cholesterol: Cholesterol did not have any significant influence on the sensitivity of liposomes to peroxidation induced by ferrous ion and ascorbate, since variation of the cholesterol content of liposomes from a molar ratio of 0.2 : 1 to phospholipid did not change the degree of peroxidation or increase glucose permeability.

Tocopherol: Glucose release and formation of thiobarbituric acid-reactive substances were measured after incubating liposomes containing α-tocopherol with ferrous ion and ascorbate (Fig. 8). Incorporation of 1 mol% α-tocopherol markedly inhibited the formation of thiobarbituric acid-reactive substances as well as glucose release from liposomes within 30 min. In the presence of 0.1 mol% α-tocopherol, a prolongation of the lag period for the formation of thiobarbituric acid-reactive substances and for

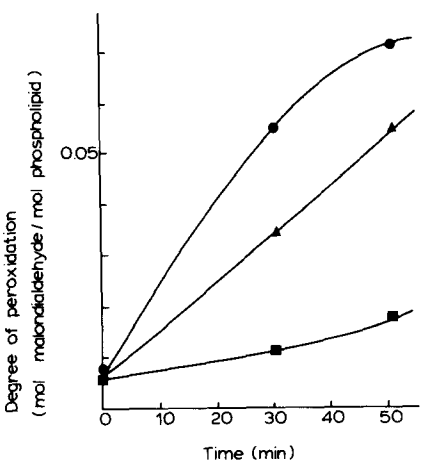


Fig. 7. Effect of surface charge on the susceptibility of liposomes to peroxidation. Liposomes consisting of rat liver phosphatidylcholine, dicetylphosphate and cholesterol (molar ratio 10 : 1 : 10) (●), rat liver phosphatidylcholine and cholesterol (molar ratio 1 : 1) (▲) and rat liver phosphatidylcholine, stearylamine and cholesterol (molar ratio 10 : 1 : 10) (■) were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for various times at room temperature.

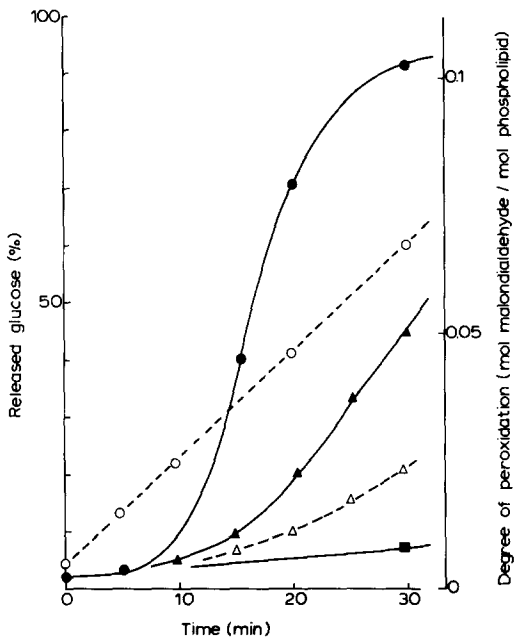


Fig. 8. Effect of α-tocopherol incorporated into liposomal membranes on peroxidation. Liposomes consisting of rat liver phosphatidylcholine, dicetylphosphate, cholesterol (molar ratio 10 : 1 : 10) and α-tocopherol were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid for various times at room temperature. Glucose release from liposomes without α-tocopherol (control) (●), those with 0.1 mol% of α-tocopherol (▲) and those with 1 mol% of α-tocopherol (■) was assayed enzymatically. Amounts of malondialdehyde formed were also determined in liposome preparations without α-tocopherol (○) and with 0.1 mol% α-tocopherol (△).

increase in glucose release was observed. It is generally accepted that the antioxidant activity of α-tocopherol may result from the scavenging radicals formed. Possibly, when α-tocopherol is consumed by reaction with radicals, the products such as tocopherol quinone do not show any inhibitory effect. In fact, the consumption of α-tocopherol during lipid peroxidation has been well demonstrated using ^{14}C-labeled tocopherol in the microsome system [17]. The prolonged lag period observed in the present system suggests that the added α-tocopherol is completely consumed within the period.

Effect of detergents on peroxidation of liposomal membranes

Peroxidation was determined in liposomes treated with Triton X-100, a nonionic detergent with critical micellar concentration of 0.24 mM or 0.015% (w/v), or with sodium dodecyl sulfate, an ionic detergent with critical micellar concentration of 8.3 mM or 0.22% (w/v). As shown in Fig. 9, Triton X-100 promoted lipid peroxidation at a final concentration of 0.04% (w/v) molar ratio of phosphatidylcholine to Triton X-100, 1 : 5), but inhibited lipid peroxidation at higher concentrations. Sodium dodecyl sulfate had no promoting effect on lipid peroxidation, but was inhibitory at higher concentrations.

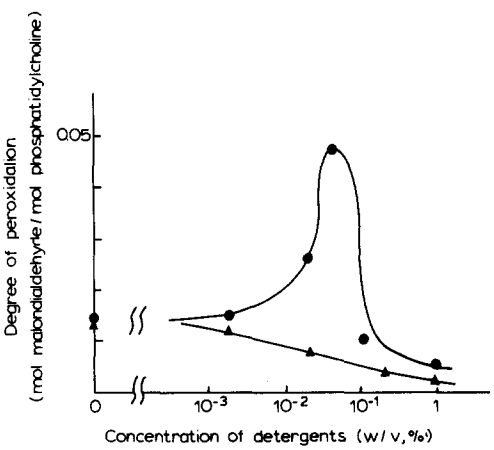


Fig. 9. Effects of detergents on the susceptibility of liposomes to lipid peroxidation. Liposomes consisting of rat liver phosphatidylcholine, dicetylphosphate and cholesterol (molar ratio 10 : 1 : 10) were incubated with 5 μM $FeSO_4$ and 100 μM ascorbic acid in the presence of various amounts of Triton X-100 (●) or sodium dodecyl sulfate (▲) for 5 min at room temperature and the amounts of thiobarbituric acid-reactive substances formed were determined.

Discussion

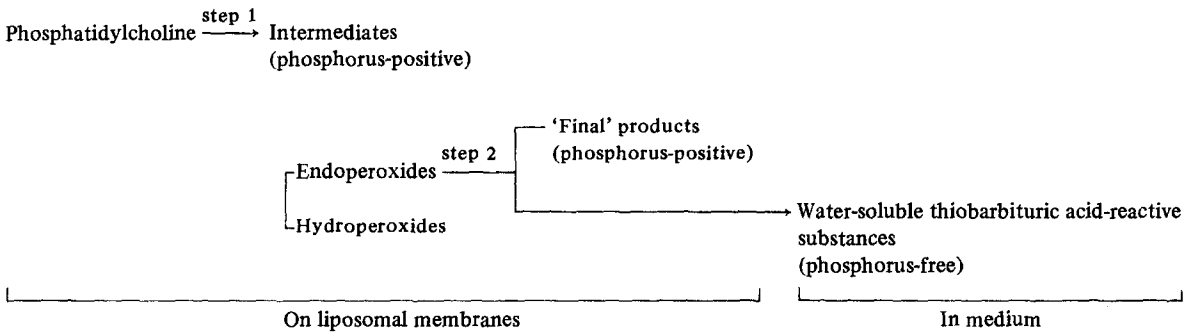
Ferrous ion and ascorbate, both of which were added exogenously, induced physical change of liposomal membranes consisting of rat liver phosphatidylcholine. Ferrous ions and ascorbate have often been used to induce lipid peroxidation because they are easy to handle. In this system, active oxygen species such as O_2^- and H_2O_2 do not play an important role as inducers of peroxidation, since neither superoxide dismutase nor catalase inhibited the reaction. The electrostatic association of ferrous ions with membranes seems to be essential for the peroxidation, because positively charged liposomes, which could not associate with ferrous ions, did not undergo appreciable peroxidation.

Ferrous ions in the presence of ascorbate caused decrease of intact phosphatidylcholine. The reaction seems to damage mainly molecular species having highly unsaturated fatty acids, since fatty acid analysis revealed a predominant decrease of highly unsaturated fatty acids, such as docosahexaenoic acid and arachidonic acid. During peroxidation, thiobarbituric acid-reactive substances were formed. The decrease of fatty acids, however, could not be quantitatively accounted for by the formation of thiobarbituric acid-reactive substances. Supposing that 1 mol thiobarbituric acid-reactive substance is formed from 1 mol polyunsaturated fatty acid, the amount of thiobarbituric acid-reactive substances generated corresponded to only 30% of the decrease of fatty acids.

As shown in Scheme I, it was proposed that thiobarbituric acid-reactive substances were generated via intermediates such as endoperoxides [18]. Hydroperoxides, the other intermediates, amy be semistable, not generating thiobarbituric acid-reactive substances under the present conditions [18,19]. Most of the thiobarbituric acid-reactive substances, probably malondialdehyde, were released from liposomes immediately after they were formed, whereas all the products containing phosphorus remained in the membranes.

It is noteworthy that the amount of thiobarbituric acid-reactive substances in the whole reaction mixture

Scheme I



depended on the compound used to stop the reaction: the amount with hydroquinone as the terminator was always higher than that with EDTA as the terminator. It seems likely that hydroquinone may interfere with step 1 in Scheme I, whereas EDTA may interfere with step 2 or both step 1 and 2, not only by chelating of ferrous ions but also by 'direct interaction' with peroxidized intermediates, since it interfered the formation of thiobarbituric acid-reactive substances even in the presence of 4 mM $MgCl_2$ (data not shown). The idea was also supported by our preliminary findings that peroxidation induced by sonication was inhibited by EDTA. Even in the presence of hydroquinone, step 2 may proceed during the procedure used for determining thiobarbituric acid-reactive substances. Since peroxidized intermediates were still associated with the membranes, the amount of thiobarbituric acid-reactive substances released from liposomes should be independent of the kind of terminator. In fact, the amount of thiobarbituric acid-reactive substances in the medium determined in the presence of EDTA was the same as that determined in the presence of hydroquinone. The reaction medium containing thiobarbituric acid-reactive substances did not cause appreciable perturbation of freshly added liposomes, indicating that the water-soluble thiobarbituric acid-reactive substances generated are not responsible for inducing perturbation. Thus, accumulation of intermediates and/or 'final' products in the membrane may cause perturbation.

The perturbation induced structural and functional changes in the liposomal membranes. Increase in permeability was observed after a certain lag, though production of thiobarbituric acid-reactive substances proceeded almost linearly, again indicating that sufficient perturbation to cause permeability change might result from accumulation of a certain amount of products in the membranes.

The permeability changes was nonspecific: large molecules such as dextran were released from liposomes as well as glucose even in the absence of an osmotic mechanism. Thus, peroxidation did not result in the formation of pores of limited size but disrupted the membrane structure severely. This disruption of the membrane structure was confirmed by electron microscopy, which showed that the average size of liposomes decreased during peroxidation. Disrupted membrane fragments may anneal, forming vesicles of smaller size.

Smolen and Shohet [10] studied the permeability properties of liposomes prepared from peroxidized human erythrocyte lipids. They postulated that the loss of the barrier function of the liposomal membrane might be associated with the production of surface-active lipid degradation products. They observed an increase in the proportion of lipid phosphorus recovered in lysophosphatidylcholine zones on thin-layer chromatograms. We also found an increase of a phosphorus-positive product with the same R_F value as lysophosphatidylcholine on thin-layer chromatograms. Previously, we observed that the incorporation of lyosphosphatidylcholine into liposomes have severe effects on the structural and functional properties of the membranes [20, 21]. Accumulation of lysophosphatidylcholine-like materials in membrane may be partly responsible for the observed perturbation. In this regard, it is interesting that oxidation of NADPH by rat liver microsomes resulted in the formation of products

more polar than normal phospholipids [16]. Accumulation of such products may result in perturbation of the bilayers, leading to loss of integral enzyme activities in microsome systems [7].

It should be noted that accumulation of materials not associated with the production of thiobarbituric acid-reactive substances also caused sufficient perturbation to induce permeability increase. In egg yolk phosphatidylcholine liposomes, ferrous ion induced appreciable permeability change with only slight production of thiobarbituric acid-reactive substances.

Perturbation of bilayers may stimulate the peroxidation process, since Triton X-100 showed stimulating activity towards ferrous ion-induced peroxidation. The inhibition observed in the presence of excess amounts of Triton X-100 may be due to the dilution of the substrate by detergents.

We could not observe any stimulation of peroxidation by sodium dodecyl sulfate. At this time, we cannot explain why the detergent is ineffective.

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References

1 Tappel, A.L. (1973) Fed. Proc. 32, 1870–1874
2 Slater, T.F. (1972) in Free Radical Mechanisms in Tissue Injury (Slater, T.F., ed.), pp. 118–166, Pion, London
3 Plaa, G.L. and Witscki, H. (1976) Annu. Rev. Pharmacol. 16, 125–141
4 Pfeifer, P.M. and McCay, P.B. (1971) J. Biol. Chem. 246, 6401–6408
5 Benedetti, A., Casini, A.F., Ferrali, M. and Comporti, M. (1979) Biochem. J. 180, 303–312
6 Hunter, F.E., Jr., Gebicki, J.M., Hoffstein, P.E., Weinstein, J. and Scott, A. (1963) J. Biochem. Chem. 238, 828–835
7 Högberg, J., Bergstrand, A. and Jakobsson, S.V. (1973) Eur. J. Biochem. 37, 51–59
8 Chen, Kuo-Lan and McCay, P.B. (1972) Biochem. Biophys. Res. Commun. 48, 1412–1418
9 Leibowitz, M.E. and Johnson, M.C. (1971) J. Lipid Res. 12, 662–670
10 Smolen, J.M. and Shohet, S.B. (1974) J. Lipid Res. 15, 273–280
11 Inoue, K. and Kinsky, S.C. (1974) Biochemistry 9, 4767–4776
12 Inoue, K. (1974) Biochim. Biophys. Acta 139, 390–402
13 Oku, N., Nojima, S. and Inoue, K. (1980) Biochim. Biophys. Acta 595, 277–290
14 Kinsky, S.C., Haxby, J.A., Zoph, D.A., Alving, C.R. and Kinsky, C.B. (1969) Biochemistry 8, 4149–4158
15 Bernheim, F., Bernheim, M.L.C. and Wilbur, K.M. (1948) J. Biol. Chem. 174, 257–264
16 May, H.E. and McCay, P.B. (1968) J. Biol. Chem. 243, 2288–2295
17 McCay, P.B., Poyer, J.L., Pfeifer, P.M., May, H.E. and Gillian, J.M. (1971) Lipids 6, 297–306
18 Pryor, W.A. and Stanley, J.R. (1975) J. Org. Chem. 40, 3615–3617
19 Frankel, E.N., Evans, C.D., McConnell, D.G., Selke, E. and Dutton, H.J. (1961) J. Org. Chem. 26, 4663–4669
20 Kitagawa, T., Inoue, K. and Nojima, S. (1976) J. Biochem. (Tokyo) 79, 1123–1133
21 Inoue, K., Suzuki, K. and Nojima, S. (1977) J. Biochem. (Tokyo) 81, 1097–1106