Autoxidation of Lecithin Liposomes in the Presence of Lysozyme

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The effects of the three-dimensional arrangement of the constituents on the interactions of the peroxidizing lipids with proteins were studied by using large oligolamellar vesicles prepared by the reverse-phase technique. Egg lecithin liposomes with hen-egg lysozyme localized either inside or outside the vesicles were oxidized in the presence of ferric iron. Protein dimerization, TBA values, and permeation of [^{14}C]glucose and of protein as well as vesicle structure were monitored during oxidation. Lysozyme polymerization, measured by protein dimer appearance, and lipid oxidation (TBA) are not greatly affected by initial protein location. The presence of protein outside the vesicles exerts a slight retardation of oxidation. α-Tocopherol inhibits lecithin oxidation and lysozyme polymerization. Changes in the vesicle structure as shown by electron micrographs and significant protein leakage are caused by vesicle oxidation. The presence of cholesterol increases TBA values without affecting protein polymerization.

Proteins exposed to peroxidizing lipids cross-link and become insoluble and undergo other changes. This has been shown in studies in dehydrated model systems (Kanner and Karel, 1976; Schaich and Karel, 1976), in emulsions (Roubal and Tappel, 1966; Desai and Tappel, 1963; Funes et al., 1982), and also in certain conditions in vivo and in food products (Logani and Davies, 1980; McCay, 1981).

Free radical reactions involving proteins are often initiated in the lipid phase by autoxidation and/or irradiation. The free radical chain transfer from lipids to proteins can occur by lipid hydroperoxides or by fatty acid fragments which can be volatile and/or water soluble. However, subsequent reactions of protein do not necessarily include incorporation of lipid fragments (Funes and Karel, 1981). Organization of the protein–lipid system can affect free radical transfer reactions (Strahm et al., 1981). Despite containing active catalysts of lipid oxidation, intact biological membranes show substantial resistance to autoxidation (Vladimirov et al., 1980). It is not understood whether the organization within the membranes or specific membrane components causes the protective effects.

Model systems approaching the situation in membranes consist mostly of liposomes or vesicles whose bilayered or multilayered structures simulate the organization of a natural membrane. Many studies have been reported on oxidative changes in lecithin liposomes and possible protective effects of various antioxidants (Kunimoto et al., 1981; Morita and Tsushimi, 1981; Muller-Runkel et al., 1981; Fukuzawa et al., 1981; Nakano et al., 1980). Recently, Barclay and Ingold (1980, 1981) reported that the formation of lecithin liposomes in aqueous dispersion as compared to the same lipid compound in homogeneous solution inhibits oxidation as shown by kinetic data. These studies did not investigate changes in other membrane components such as protein.

Strahm et al. (1981) have studied protein–peroxidizing lipid interaction in a lecithin liposome system containing one of three different proteins, including lysozyme. In this work, lysozyme was partially inside the phospholipid vesicles and partially outside the liposomes, and no evidence could be obtained on which fraction was more relevant in that interaction.

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In the present study we investigate the lipid–protein interactions using as the model system egg yolk lecithin (LEC) liposomes with hen egg lysozyme (LYS) localized either inside or outside the vesicles. Large unilamellar or oligolamellar vesicles were prepared by the reverse-phase technique (Szoka and Papahadjopoulos, 1978). The effects of α-tocopherol and of cholesterol upon incorporation into the vesicles were also studied. Electron microscopy and permeation studies were conducted to characterize the vesicles.

MATERIALS AND METHODS

Experimental Design. The experiments were designed to study protein–peroxidizing lipid interactions in organized model systems consisting of LEC liposomes with LYS localized either inside or outside the vesicles. For controls, we prepared two other systems: (1) vesicles without LYS and (2) LYS–LEC suspensions forming no vesicles because they contained the surface active agent Triton X-100.

Materials. LEC (phosphatidylcholine) and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification; LYS (3× crystallized) was from ICN Nutritional Biochemicals (Cleveland, OH) and α-tocopherol from Eastman Kodak Co. (Rochester, NY). Aquasol and uniformly labeled [^{14}C(U)]glucose were obtained from New England Nuclear (Boston, MA).

Preparation of the Vesicles. Protein-containing lipid vesicles have been prepared by the reverse-phase technique in accordance with Szoka and Papahadjopoulos (1978). During the whole procedure all flasks were flushed thoroughly with nitrogen and all operations were carried out in nitrogen atmosphere; all preparations were stored under nitrogen until the start of the experiment.

Our preparations consisted of 1.0 g of LEC solubilized in 130 mL of chloroform and 130 mL of peroxide-free isopropyl ether. Saline solution (39 mL; 137 mM NaCl–2.6 mM KCl) containing the protein (10 mg/mL LYS) was added at this point. The suspension was sonicated for 5 min at 45 °C in a bath-type sonicator. The resulting "inverted micelles" were kept at room temperature for 30 min to allow stabilization. The solvents were removed under slight vacuum at 35 °C. With further addition of small portions of saline solution the organic solvents were completely evaporated. The untrapped protein was removed by centrifugation (3 times, 10 min at 10000 rpm), followed by removal of the supernatant and resuspension of the sedimental vesicles in fresh saline solution. A very small residue of protein persisted with repeated centrifugation, probably due to disrupting some of the vesicles. This residue was removed by allowing the vesicles to settle

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overnight at 5 °C and removing the supernatant. The suspension was then adjusted to 100 mL with the saline solution and used for the oxidation studies.

When α-tocopherol or cholesterol was incorporated into the system, they were solubilized in the organic phase with the LEC. [^{14}C(U)]Glucose was added to the saline solution and incorporated into the system.

Vesicles with the LYS outside were prepared in a similar way with an LYS-free solution. Protein was added after vesicles were formed to a final concentration of 2.0 mg of LYS/mL of saline solution.

Controls are as follows: (1) vesicles without LYS were prepared in a way similar to the above but with an LYS-free saline solution; (2) a vesicle-free preparation was obtained by adding 2.5 mL of Triton X-100 aqueous solution (25% v/v) to 100 mL of saline solution containing 1 g of LEC and 200 mg of LYS.

Oxidation Experiments. All oxidations were initiated by addition of ferric ion to a final concentration of 0.22 mM $FeCl_3$ as the catalyst. Oxidation was carried out in air at 37 °C with constant gentle agitation to ensure homogeneous oxygen distribution. Samples taken periodically were analyzed as follows: TBA values were determined in 0.1 mL of the preparation by a modification of the method of Asakawa and Matsushita (1980). To 0.1 mL of the sample were added 0.1 mL of a 10 millimolar solution of EDTA, 1.5 mol of 0.2 M glycine–HCl buffer (pH 3.6), and 1.5 mL of TBA reagent solution (0.5 g of TBA plus 0.3 g of $NaDodSO_4$ in 100 mL of H_2O). The mixture was vortexed for 1 min and heated in a boiling water bath for 15 min. It was then cooled in an ice bath. One milliliter of glacial acetic acid and 2 mL of CH_3Cl were added. The mixture was shaken and centrifuged at 10 000 rpm for 15 min. The optical density of the supernatant was determined at 532 nm by using a 1-cm cuvette.

Ten milliliters of preparation was extracted 3 times with chloroform–methanol (2:1) (Bligh and Dyer, 1959) to separate lipids from proteins. The aqueous phase was freeze-dried and then dissolved in aliquots of distilled water. LYS determination was performed in accordance with Lowry et al. (1951) by using pure LYS as the standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by using the method of Weber and Osborn (1969) as modified by Pozzio and Pearson (1976). Sample preparation was described by Funes et al. (1980). Destained gels were scanned colorimetrically at 550 nm in a Hitachi Perkin-Elmer UV–visual gel scanner with a 339A Hewlett-Packard integrator.

Nonoxidizing conditions were achieved by maintaining the vesicles under nitrogen and in the absence of iron at 37 °C.

[^{14}C]Glucose Permeation Study. LYS-free liposomes were prepared as above with 300 mg of LEC and 11.7 mL of saline solution containing 120 mg of uniformly labeled [^{14}C(U)]glucose (6.25×10^{-2} μCi/mg glucose). The untrapped glucose was removed by centrifugation, and then the solution volume was adjusted to 30.0 mL with saline solution. Half of this preparation was oxidized and half was maintained in nonoxidizing conditions. Samples (1.0 mL) were removed periodically and centrifuged (10 min; 10 000 rpm) to give a vesicle-free supernatant. A portion of this supernatant (0.1 mL) was diluted with 10.0 mL of Aquasol in a 20-mL glass scintillation vial. The vials were counted in a Beckman LS-7000 scintillation counter. ^{14}C was counted with 78–80% efficiency.

Lysozyme Permeation Study. LYS-containing liposomes were incubated in oxidizing and nonoxidizing conditions. Periodically, samples (1.0 mL) were removed and filtered through a Millex H-A 0.45-μm filter (Millipore Corp., Bedford, MA); then protein was determined in the filtrate.

Table I. Experimental and Calculated LEC Vesicle Parameters

diameter (electron microscopy), nm	236
encapsulation ratio (^{14}C glucose)	6.04
glucose entrapment, %	16.0
total surface area (Pidgeon and Hunt, 1981), nm^2	1.78×10^{20}
calculated total surface area (unilamellar), nm^2	2.72×10^{20}
calculated total surface area (bilamellar), nm^2	1.50×10^{20}
lysozyme entrapment, %	3.9–5.1
lysozyme:lecithin ratio	0.035–0.050
lysozyme concentration inside vesicles (original solution 10.0 mg/mL), mg/mL	2.5–3.3
nonencapsulated lysozyme, % total protein	0.5–0.7

Enzyme activity was determined by using the Worthing LYS reagent set as described by Funes et al. (1980) with the modification of Sessa and Weissmann (1970) to determine "latent" LYS activity.

Electron Microscopy. The liposome preparation was negatively stained with 2% uranyl acetate in water, and pictures were taken with a JEOL 10B transmission electron microscope.

Lecithin Fatty Acid Composition. The fatty acid composition of LEC was determined by gas chromatography using a Perkin-Elmer Sigma 3 gas chromatographer and a 10% DEGS on 80–100 Chromosorb WAW column (Supelco, Inc.). Methyl esters were prepared in accordance with Morrison (1964) by using boron fluoride–methanol (14%). The determined fatty acid composition was 16:0 (35.0%), 18:0 (13.2%), 18:1 (32.4%), 18:2 (19.4%), and 18:3 (less than 0.1%).

RESULTS

Vesicle Characterization. Table I summarizes experimental and calculated parameters of LEC vesicles prepared by the reverse-phase technique with LYS or labeled [^{14}C]glucose entrapped into their internal aqueous phase.

Vesicle size was determined directly from electron micrographs (at different degrees of magnification) of two different preparations of LYS-containing liposomes, by using *Tobacco mosaic* virus and T_4 virus as standards, and in accordance with Szoka et al. (1980). A mean size (diameter) of 236 nm (s = 105 nm) was determined. Protein-containing vesicles appear to be oligolamellar and unilamellar with a large internal aqueous phase.

Encapsulation ratio was determined by entrapment of labeled [^{14}C]glucose. Vesicles were prepared as described above for LYS-free vesicles but by using a saline solution containing 4 mg of glucose/mL. The amount of entrapped glucose was determined by counting the ^{14}C label, and the amount of entrapped water was calculated by assuming that the glucose-encapsulated concentration is the same as in the original solution. The amount of lipid was determined by extraction with chloroform–methanol as described above. The encapsulation ratio was defined as the ratio of entrapped water to lipid (v/v) and was found equal to 6.04. The observed glucose encapsulation was 16.0%, for an original solution of 4 mg of glucose/mL of saline solution.

By use of the diameter determined by electron microscopy, the encapsulation ratio, and the method of Pidgeon and Hunt (1981), the total surface area was calculated as 1.79×10^{20} nm^2 and the number of liposomes in a vesicle

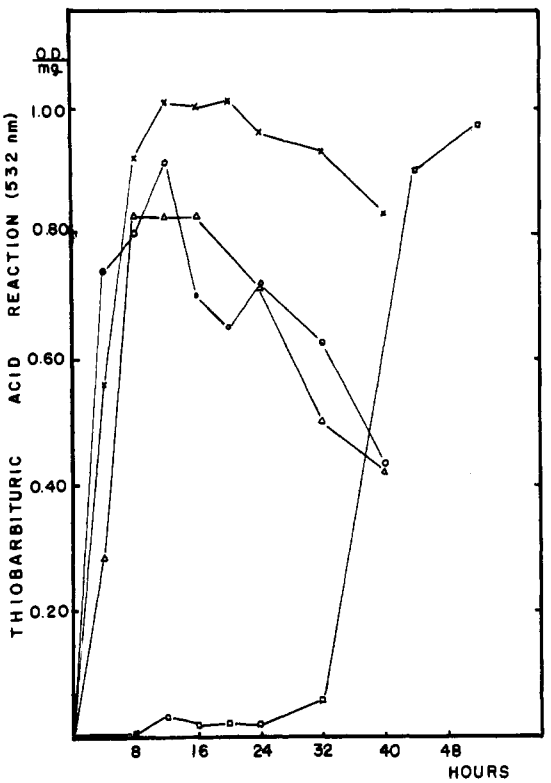

Figure 1. Peroxidation of lecithin vesicles, as measured by TBA, as a function of incubation time. (□) Lysozyme inside; (Δ) lysozyme outside; (×) no lysozyme; (○) lysozyme-containing vesicles incubated in nonoxidizing conditions.

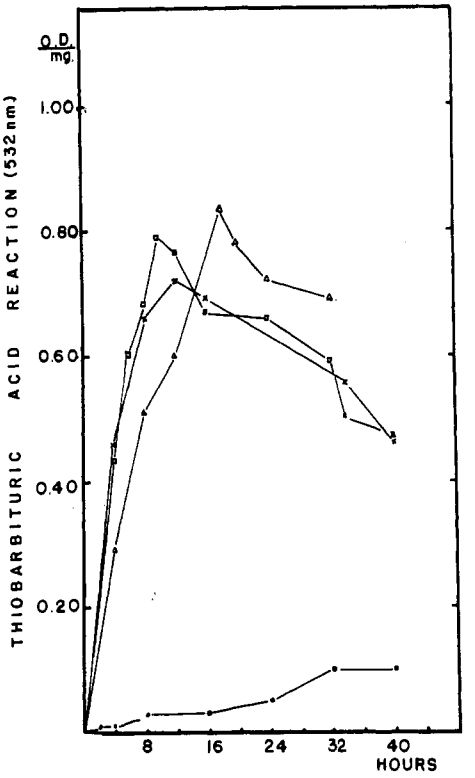

Figure 2. Peroxidation of lysozyme-containing lecithin vesicles, as measured by TBA, as a function of incubation time. (□) Vesicles with 3.0% mol of α-tocopherol; (○) vesicles with 0.3% mol of α-tocopherol; (Δ) system with Triton X-100; (×) vesicles with 25% cholesterol.

preparation as 1.02×10^{15}, both quantities per gram of lipid.

The total surface area of unilamellar and bilamellar spherical homogeneous liposomes for 1 g of LEC (average hydrated molecular weight 800 g/mol) was calculated by assuming a value of 0.70 nm^2/molecule of phospholipid and a bilayer thickness of 4.0 nm (Mimms et al., 1981). In the bilamellar population and a water layer thickness of 7.0 nm was assumed (Pidgeon and Hunt, 1981). The proximity of the observed and theoretical surface areas suggests that these vesicles are oligolamellar and unilamellar.

In protein-containing vesicles, LYS encapsulation was determined by two methods: (1) determination of the biological activity of an aliquot of solubilized unoxidized liposomes with Triton X-100 (1%) (Sessa and Weissmann, 1970) using pure LYS as the standard and (2) determination of the protein content (Lowry method after lipid extraction with chloroform–methanol, 2:1). Both methods gave comparable results, in the range of 3.9–5.1% for different preparations. The protein:lipid ratio was in the range 0.015–0.020 (w/w). The concentration of LYS inside the vesicle was calculated by using the encapsulation ratio and the measured LYS encapsulation percentage. Values were 2.5–3.3 mg of LYS/mL of aqueous phase (the original LYS concentration was 10 mg of LYS/mL of saline solution).

Nonencapsulated LYS in unoxidized LYS-containing vesicles was determined in the supernatant of sedimented liposomes (after 3–5 h in the refrigerator, under nitrogen). Biological activity determinations gave values of 0.5–0.7% of the total LYS content in the preparation. Measurement of protein concentration by the Lowry method was not sensitive enough to detect this concentration.

Vesicle Oxidation. LEC liposome systems with different initial LYS locations were oxidized and the TBA values were measured. The following systems were assayed: (1) liposomes with entrapped LYS (1 g of LEC–0.02 g of LYS–100 mL of saline solution); (2) vesicles with lysozyme outside (1 g of LEC–0.2 g of LYS–100 mL of saline); (3) vesicles with no LYS.

Figure 1 shows the TBA value course for those systems. Protein within vesicles has apparently no effect on lipid oxidation rate. Protein outside protein-free vesicles exerts only a slight retardation of oxidation. A slight oxidation is observed in the control caused by the impossibility of complete elimination of oxygen.

The lipid oxidation course was also determined in a vesicle-free preparation obtained by addition of Triton X-100 [2.5 mL of 25% (v/v) solution] to a saline solution (100 mL) containing LEC (1 g) and LYS (0.2 g). Figure 2 shows the occurrence of TBA-reactive products with time in such system; LEC is oxidized at the same rate as in the liposome system and is independent of LYS concentration. The occurrence of a maximum in the TBA values is typical of the autoxidation process and is due to buildup and subsequent decomposition of primary products.

Figure 2 also shows the effects of α-tocopherol incorporation into the LYS-containing vesicle system. Incorporation of 0.3% mol of α-tocopherol/mol of LEC fails to give an observable induction period. However, it is possible that a short induction period (less than 4 h) was induced but could not be detected. The incorporation of 3.0% mol of α-tocopherol/mol of LEC is able to protect the lipids for as long as 32 h of incubation with oxygen and ferric iron.

The incorporation of 25% (w/w) of cholesterol into the lipid phase resulted in slight acceleration of lipid oxidation as measured by TBA reaction (Figure 2).

Protein Dimerization. LYS dimerization was monitored by the disappearance of the LYS monomer and formation of the LYS dimer. The relative concentrations of these fractions were estimated by scanning gels after

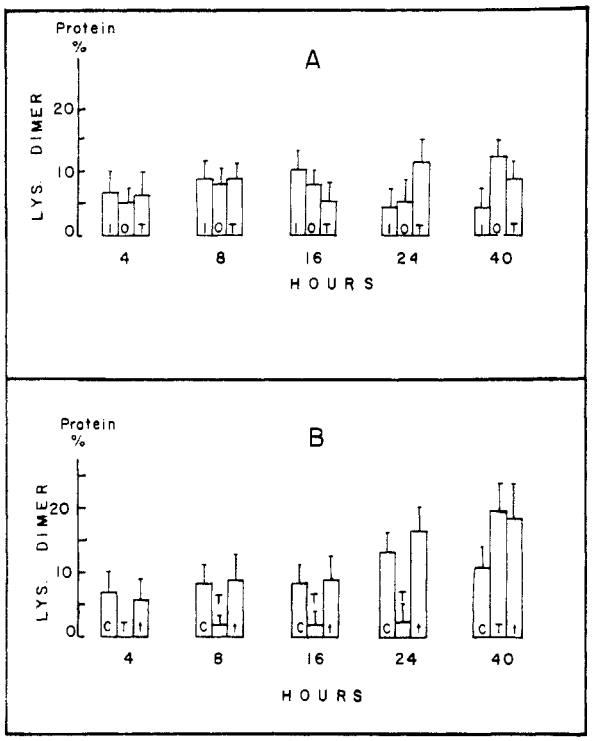

Figure 3. Lysozyme dimerization, expressed as a percentage of total protein, as a function of incubation time. Graph A: I, vesicles with lysozyme inside; O, vesicles with lysozyme outside; T, system with Triton X-100. Graph B: C, vesicles with 25% cholesterol; T, vesicles with 3.0% mol of α-tocopherol; t, vesicles with 0.3% α-tocopherol.

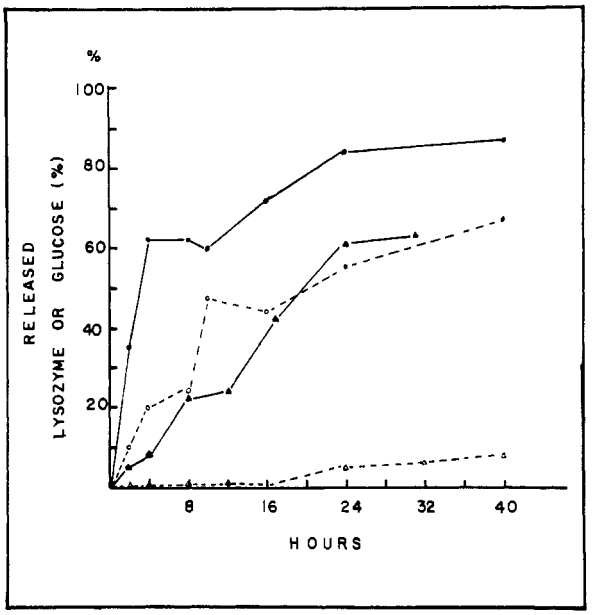

Figure 4. Lecithin vesicle permeation in nonoxidizing (open figures) and oxidizing (closed figures) conditions. (△, ▲) [^{14}C]-Glucose release; (○, ●) lysozyme release.

electrophoretic separation and by determining the total soluble protein after lipid extraction. In calculating the percentages, we assume that the optical density of each soluble fraction is equal to a constant multiplied by its weight.

Figure 3A shows the percentage of LYS dimer in oxidized protein-containing vesicles compared to oxidation with protein outside the vesicles and to similar oxidized systems solubilized with Triton X-100. Since some LYS dimer (2–5%) was detected at the beginning of some experiments (immediately after lipid extraction and before incubation), we only report the dimer formed during the incubation. The standard deviation of this determination was 3–5%.

Formation of the dimer started early with 6% of the newly formed dimer detected after 4 h of oxidation. After 16 h the amount of dimer reached a plateau, probably due to formation of higher polymers.

We could not detect significant differences in the amount of LYS dimer formed upon incubation due to the presence of protein inside or outside the vesicles or in the vesicle-free system.

Figure 3B shows the LYS dimerization when α-tocopherol and cholesterol are added to the lipid phase. The addition of 3.0% mol of α-tocopherol/mol of LEC prevents LYS dimerization, coinciding with its effect in preventing LEC oxidation. A total of 0.3% mol of α-tocopherol/mol of LEC does not affect either oxidation (Figure 2) or LYS polymerization. Incorporation of 25.0% (w/w) of cholesterol into the lipid phase does not lead to an increase in protein polymerization, even though it does accelerate oxidation slightly.

Permeation Studies. Figure 4 shows release of LYS or of [^{14}C]glucose from LEC liposomes upon incubation at 37 °C under oxidizing conditions (oxygen and 0.22 mM Fe^{3+}) and under nonoxidizing conditions (no Fe^{3+}, nitrogen). These results clearly show glucose release from these large unilamellar vesicles even when there is no lipid oxidation. Singer (1981) has reported glucose and Na^+ permeability in liposomes formed from saturated phosphatidylcholines which display a permeability maximum in the region of the lipid transition temperature. Also, Pick (1981) has reported a significant permeability of sorbitol in large soybean liposomes prepared by the freezing and thawing technique. Permeability to LYS under nonoxidizing conditions is negligible during the first 24 h of oxidation. After 24 h, when some lipid oxidation has taken place due to the impossibility of complete elimination of oxygen, some leakage occurs. In any case, protein loss is never higher than 9.0% of initial encapsulated protein.

Under oxidizing conditions [^{14}C]glucose efflux becomes very rapid, giving a loss of 60.0% of the originally encapsulated glucose during the initial 4 h of oxidation. Kunimoto et al. (1981) have reported similar glucose and dextran efflux for oxidizing multilamellar liposomes of egg LEC in the presence of ferrous iron and ascorbate. LYS release from peroxidizing vesicles occurs almost linearly during the first 24 h of oxidation, reaching a value of 63.0% after 40 h of incubation.

These results show that glucose permeation can take place in unoxidized LEC vesicles. Blok et al. (1975) have postulated that near the phase transition temperature, permeability of small solutes in vesicles of saturated phosphatidylcholines is due to permeation through statistical "pores". Our experiments were performed at 37 °C—far above the broad phase transition of LEC (−5 °C) (Mason and Huang, 1978)—but such porosity may exist under our conditions. Before oxidation, these LEC vesicles are impermeable to LYS, but oxidation causes significant LYS leakage due to the bilayer damage which is also demonstrable by electron microscopy.

Electron Microscopy. Figure 5 shows electron micrographs of peroxidizing LEC vesicles after 0, 16, and 40 h of incubation. In this case, samples were taken from the same vesicle preparation and from the same oxidation study. Lipid oxidation causes deleterious changes in the bilayer structure of these vesicles. Initially it is possible to observe the lipid bilayer structure and relatively spherical vesicles. After 16 h, an important diminution

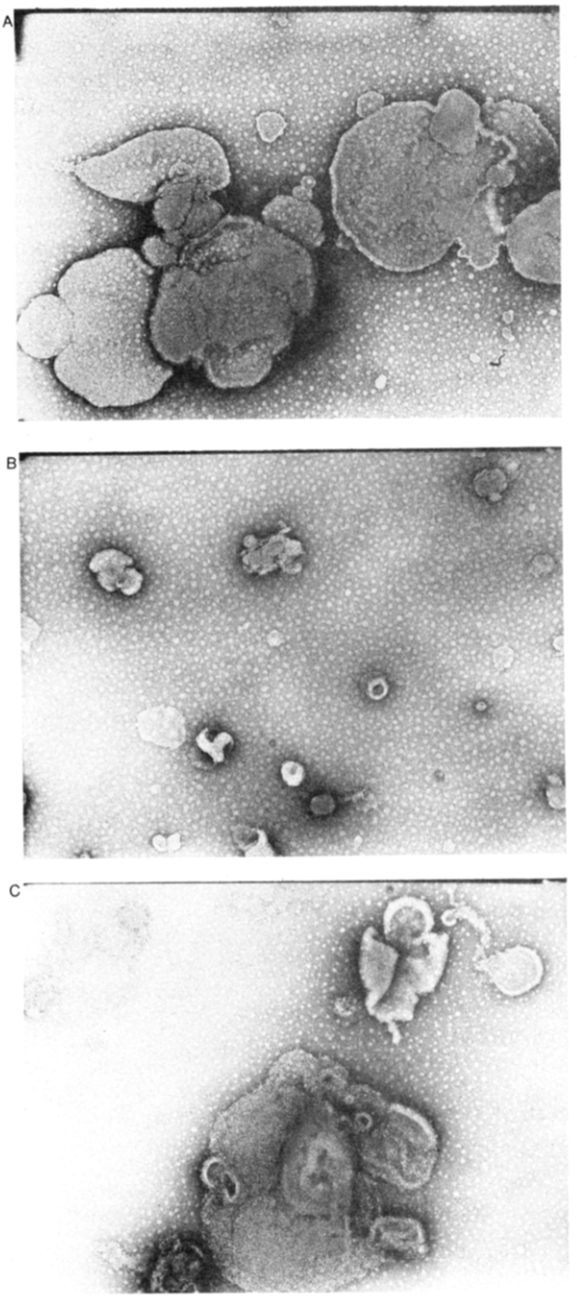

Figure 5. Electron micrographs of peroxidizing LEC vesicles after 0 (A), 16 (B), and 40 (C) h of incubation.

in vesicle size has taken place although bilayer structures are still visible. At the end of our study (40 h) only traces of the bilayer remain and very chaotic structures are observed.

DISCUSSION

Peroxidation of lipids is a free-radical chain process affected by various environments and compositional factors (Labuza, 1971; Logani and Davies, 1980). The presence of protein in biological materials introduces an important component in this oxidative process affecting the lipid oxidation course and the quality of the protein fraction (Karel, 1977, 1980). Protein–peroxidizing lipid interactions under different environmental conditions and different degrees of protein–lipid contact have been studied (Kanner and Karel, 1976; Funes et al., 1980; Leake and Karel, 1982; Funes et al., 1982). Mechanisms established in those systems may differ from those in more complex, organized, and dynamic systems such as membranes in cells, subcellular organelles, and tissues. The present study continues previous work (Strahm et al., 1981) on the role of organization in the protein–peroxidizing interaction.

We understand organization as the molecular arrangements of protein, lipids and other biological components in membrane of cells, subcellular organelles and tissues, whether "in vivo", post-mortem (e.g., in foods), or in "synthetic" systems (e.g., liposomes). In this study we used large oligolamellar LEC liposomes with LYS localized either inside or outside the vesicles. This system, compared to the nonlocalized protein multilayer vesicle system used before (Strahm et al., 1981), provides the organization factor with a closer similarity to real membrane cells which consist of a lipid bilayer associated with localized proteins. Although LYS is a soluble nonmembrane protein, it is reported to be a good model for peripheral activity, partially penetrating membrane protein (Kimelberg and Papahadjopoulos, 1971).

Recently, Barclay and Ingold (1980, 1981) have studied the kinetics of autoxidation of LEC in homogeneous solution in chlorobenzene and as a bilayer dispersion in aqueous solution. They have demonstrated that the physical structure of LEC bilayers makes them more resistant than would be expected on the basis of their chemical composition. They attributed the low efficiency of the free radical initiator to the higher microviscosity of lecithin in bilayers, compared to that in an organic solvent. Thus, the initial radicals formed are prevented from diffusing apart. They suggest the lower oxidizability of the vesicles is due to a low chain propagation rate due to expulsion of the polar peroxyl radicals from the autoxidizable region of the bilayer.

In the localized protein and LEC vesicle systems used in this study, we are introducing LYS as another component which, in accordance with previous work (Karel et al., 1975; Schaich and Karel, 1976; Kanner and Karel, 1976), can react with peroxidizing lipids, originating protein free radicals among other products. We have proposed the mechanism

initiation

$$\mathrm{LOOH + PH \rightarrow [LOOH\cdots HP] \rightarrow LO\cdot + H_2O + P\cdot} \quad (1a)$$

$$\rightarrow \mathrm{LO\cdot + \cdot OH + PH} \quad (1b)$$

$$\mathrm{PH + \cdot OH \rightarrow P\cdot + H_2O} \quad (1c)$$

propagation

$$\mathrm{LOO\cdot + PH \rightarrow LOOH + P\cdot} \quad (2)$$

$$\mathrm{LO\cdot + PH \rightarrow LOH + P\cdot} \quad (3)$$

termination

$$\mathrm{2P\cdot \rightarrow P\text{–}P} \quad (4)$$

Protein dimerization has been taken as a measure of this protein–peroxidizing lipid interaction.

In the present study we were able to observe lysozyme dimerization within 4 h after lipid oxidation was initiated, contrasting with the longer periods observed in other lipid–LYS systems (Funes et al., 1980; Leake and Karel, 1982; Funes et al., 1982).

We suggest that lipid peroxy radicals migrate to the polar region of the bilayer (as Barclay and Ingold proposed) where they react with the aqueous-soluble LYS originating protein free radicals. Protein location, inside or outside the vesicle, would not affect this process, explaining our inability to observe differences in LYS dimerization due to protein location. This would be valid as long as the damage to the bilayer is not important; as the lipid oxidation progresses, regions of very different composition and polarity (due to the secondary lipid oxidation break-

down products) would coexist, making any prediction very difficult.

Our results show that LEC oxidation is affected neither by the presence of LYS nor by its location. A slight retardation effect is noted when LYS is located outside the vesicles at a concentration of 2 mg/mL. Strahm et al. (1981) observed a protein-induced reduction in the rate of oxygen uptake during the autoxidation of LEC multilamellar vesicles at a protein concentration of 10 mg/mL. Addition of Triton X-100 to an LYS–LEC aqueous system affects neither lipid oxidation nor LYS polymerization, although vesicle structure is destroyed. Probably the detergent concentration assayed (10.6 mM, above the Triton X-100 critical micellar concentration) results in mixed micelles of detergent and LEC; lipid free radicals would still be a hydrophobic lipid media presenting a similar behavior as the vesicle lipid bilayer.

Lipid oxidation is inhibited in our system to a large extent by addition of 3.0% mol of α-tocopherol/lipid. Fukuzawa et al. (1981) reported similar inhibition although their LEC liposome was monitored for only 60 min. We have found that α-tocopherol inhibits lipid oxidation as long as 32 h. After this period, lipid oxidation starts at the usual rate, causing immediate protein dimerization. Depletion of α-tocopherol as a radical quencher would cause the sharp lipid oxidation increment observed in our experiments.

Location of α-tocopherol inside the lipid bilayer and its higher reactivity toward lipid free radicals compared to that toward protein would explain its inhibitory effect in lipid oxidation and protein polymerization. Low α-tocopherol concentration (0.3% mol/lipid) does not show these inhibitory effects, at least not for periods of longer than 4 h.

Cholesterol reduces the permeability of membranes (Papahadjopoulos, 1974). We observed a significant increase in TBA values when cholesterol is present in the vesicle lipid bilayer, but this is not reflected on the LYS dimerization rate.

Our electron microscopy and permeation studies show that as the LEC oxidation progresses, important lipid bilayer damage takes place, causing destruction of vesicle organization. The photomicrographs taken after 40 h of oxidation show large "collapsed" bilayer structures, possibly indicating aggregation of LEC from several vesicles. The mechanism of this aggregation is not known. Fukuzawa et al. (1981) have studied multilamellar lecithin vesicles and have also observed drastic structural changes due to oxidation.

Our results indicate that as long as lipid oxidation is prevented (e.g., addition of antioxidants, absence of lipid oxidation catalysts, or absence of oxygen) the large vesicles maintain their structure and impermeability to macromolecules.

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Registry No. LYS, 9001-63-2; α-tocopherol, 59-02-9; cholesterol, 57-88-5; glucose, 50-99-7.

LITERATURE CITED

Asakawa, T.; Matsushita, S. *Lipids* **1980**, *15*, 137.
Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1980**, *102*, 7794.
Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6478.
Bligh, E. G.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911.
Blok, M. C.; van der Neut-Kok, E. C. M.; van Deenen, L. L. M.; de Gier, J. *Biochim. Biophys. Acta* **1975**, *406*, 187.
Desai, I. D.; Tappel, A. L. *J. Lipid Res.* **1963**, *4*, 204.
Funes, J.; Karel, M. *Lipids* **1981**, *16*, 347.
Funes, J.; Weiss, U.; Karel, M. *J. Agric. Food Chem.* **1982**, *30*, 1204.
Funes, J.; Yong, S.; Karel, M. *J. Agric. Food Chem.* **1980**, *28*, 794.
Fukuzawa, K.; Chida, H.; Tokumura, A.; Tsukatani, H. *Arch. Biochem. Biophys.* **1981**, *206*, 173.
Kanner, J.; Karel, M. *J. Agric. Food Chem.* **1976**, *24*, 468.
Karel, M. In "Biotechnological Applications of Proteins and Enzymes"; Bohak, Z.; Sharon, N., Eds.; Academic Press: New York, 1977; Chapter 19, p 317.
Karel, M. (1980) In "Autoxidation in Food and Biological Systems"; Simic, M.; Karel, M.: Eds.; Plenum Press: New York, 1980.
Karel, M.; Schaich, K.; Roy, R. B. *J. Agric. Food Chem.* **1975**, *23*, 159.
Kimelberg, H.; Papahadjopoulos, K. *J. Biol. Chem.* **1971**, *246*, 1142.
Kunimoto, M.; Inoue, K.; Nojima, S. *Biochim. Biophys. Acta* **1981**, *646*, 169.
Labuza, T. P. *CRC Crit. Rev. Food Technol.* **1971**, *2*, 355.
Leake, L.; Karel, M. *J. Food Sci.* **1982**, *47*, 737.
Logani, M. K.; Davies, R. E. *Lipids* **1980**, *15*, 485.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
Mason, J. T.; Huang, C. *Ann. N.Y. Acad. Sci.* **1978**, *308*, 29.
McCay, P. B. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1981**, *40*, 173.
Mimms, L. T.; Zampighi, G.; Nozaki, Y.; Tanford, C.; Reynolds, J. A. *Biochemistry* **1981**, *20*, 833.
Morita, M.; Tsushimi, N. *Agric. Biol. Chem.* **1981**, *45*, 1413.
Morrison, R.; Smith, L. H. *J. Lipid Res.* **1964**, *5*, 600.
Muller-Runkel, R.; Blais, J.; Grossweiner, L. I. *Photochem. Photobiol.* **1981**, *33*, 683.
Nakano, M.; Sugioka, K.; Nakamura, T.; Oki, T. *Biochim. Biophys. Acta* **1980**, *619*, 274.
Papahadjopoulos, D. *J. Theor. Biol.* **1974**, *43*, 329.
Pick, U. *Biochem. Biophys. Res. Commun.* **1981**, *212*, 186.
Pidgeon, C.; Hunt, A. C. *J. Pharm Sci.* **1981**, *70*, 173.
Pozzio, M. A.; Pearson, A. M. *Biochim. Biophys. Acta* **1976**, *490*, 27.
Roubal, W. T.; Tappel, A. L. *Arch. Biochem. Biophys.* **1966**, *113*, 5.
Schaich, K. M.; Karel, M. *Lipids* **1976**, *11*, 392.
Sessa, G.; Weissmann, G. *J. Biol. Chem.* **1970**, *245*, 3295.
Singer, M. *Chem. Phys. Lipids* **1981**, *28*, 253.
Strahm, A.; Sorensen, J.; Karel, M. *Lebensm.-Wiss. Technol.* **1981**, *14*, 192.
Szoka, F., Jr.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4194.
Szoka, F. C.; Olson, F.; Heath, T.; Vail, J.; Mayhew, E.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1980**, *601*, 559.
Vladimirov, Y. A.; Olemev, V. I.; Suslou, T. B.; Cheremisina, Z. P. *Adv. Lipid Res.* **1980**, *17*, 173.
Weber, K.; Osborn, M. *J. Biol. Chem.* **1969**, *244*, 4406.

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