Relation of lipid peroxidation to loss of cations trapped in liposomes

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ABSTRACT Lipid peroxidation and alterations in cation loss have been induced in liposomes by ferrous ion, ascorbic acid, reduced and oxidized glutathione, and gamma radiation. Modifications of these effects by tocopherol and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were studied when these antioxidants were either incorporated in the membrane or were added to already formed liposomes prior to the addition of the chemical agent or to irradiation.

Lipid peroxidation, as indicated by the thiobarbituric acid test for malonic dialdehyde, did not correlate with alterations in cation loss. The largest amounts of lipid peroxidation induced by ascorbic acid and glutathione were associated with decreased cation loss. Inhibition of Fe^{2+}- and radiation-induced lipid peroxidation by antioxidants did not inhibit the associated increase in cation loss. Tocopherol was a more effective antioxidant than BHT when it was incorporated in the membrane, whereas BHT was more effective when it was added to the liposomes after formation.

SUPPLEMENTARY KEY WORDS ascorbic acid · ferrous ion · glutathione · radiation · membrane antioxidants

THE CLOSE ASSOCIATION between lipid peroxidation and increased permeability both in red blood cells (1–3) and in subcellular organelles such as mitochondria (4–6), microsomes (7), and lysosomes (8, 9) suggests a possible causal relationship. However, the precise way in which the lipid peroxidation process might alter permeability is not clear. Peroxidation of unsaturated fatty acids in the various cellular membranes could lead to loss of double bonds or breaks in fatty acid side chains in the region of double bonds, thus producing physical changes in the membrane. Alternatively, there may be little or no local effect at the site of lipid peroxidation. Rather, secondary effects of lipid peroxidation products on proteins (10–12) and enzymes (13), and inhibition of metabolic pathways (14), may lead to increased permeability.

The present work was done to establish whether local alterations in membrane lipids, which must occur with lipid peroxidation, alter membrane permeability. Use is made of liposomes, phospholipid spherules which exhibit ion discrimination, osmotic swelling, and response to a variety of agents which accelerate or retard loss of ions or molecules from the spherules in a way that qualitatively mimics their action on natural membrane-bounded structures (15). Changes in loss of ions from the spherules induced by various agents have been attributed to alterations in permeability (16–17). These structures are used to eliminate the possible effect of lipid peroxidation on components of the cell other than membrane lipids, and to establish whether lipid peroxidation in a pure lipid membrane leads to increased permeability of the membrane.

MATERIALS AND METHODS

Materials

Lecithin, phosphatidylethanolamine, and phosphatidylinositol (all labeled peroxide-free) were obtained from General Biochemicals, Chagrin Falls, Ohio. These lipids were chromatographically pure as judged by thin-layer chromatography, using chloroform–methanol–glacial acetic acid–water 100:50:13:3 on Adsorbosil-5 Prekotes (Applied Science Laboratories, Inc., State College, Pa). Dicetyl phosphate was obtained from K and K Labora-

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Abbreviations: BHT, 2,6-di-*tert*-butyl-4-methylphenol; TBA, 2-thiobarbituric acid.

tories, Inc., Plainview, N.Y. Cholesterol and oxidized and reduced glutathione were from Mann Research Laboratories, Orangeburg, N.Y. D-α-Tocopherol (vitamin E), L-ascorbic acid, Triton X-100, and ferrous chloride were from Fisher Scientific Co., Silver Spring, Md. 2-Thiobarbituric acid was purchased from Eastman Organic Chemicals, Rochester, N.Y. BHT was a gift of Shell Chemical Co. as Ionol C.P. Dialysis tubing (0.25-inch diameter) was obtained from Arthur H. Thomas Co., Philadelphia, Pa. Sephadex G-50 (coarse) was from Pharmacia Fine Chemicals, Piscataway, N.J., and choline chloride and spectroquality *n*-butanol were from Matheson Scientific Co., Beltsville, Md.

^{24}Na ($t_{1/2}$, 15 hr), ^{22}Na ($t_{1/2}$, 2.6 yr), and glucose-1-^{14}C (Amersham/Searle Corp., Des Plaines, Ill.) were used as radioactive tracers in these experiments. ^{24}Na was made in the Walter Reed Research Reactor from crystalline NaCl, using a thermal neutron flux of 10^{12} neutron/sec/cm^2. ^{38}Cl was allowed to decay before use. ^{22}Na was obtained from Abbott Laboratories, Baltimore, Md., as $^{22}NaCl$. Scintanalyzed PPO (2,5-diphenyloxazole), Scintanalyzed toluene, and ethylene glycol monoethyl ether (Cellosolve) were obtained from Fisher Scientific Co.

Glassware was washed in sulfuric acid–dichromate cleaning solution. Deionized water was used in all experiments.

Preparation of Liposomes

Liposomes were made using a modification of the method of Bangham, Standish, and Watkins (16). Chloroform solutions containing lecithin, cholesterol, dicetyl phosphate, phosphatidylethanolamine, and phosphatidylinositol (mole % composition: 57.4, 8, 10, 14.8, and 9.8, respectively) were pipetted into a 100-ml round-bottom flask. In some experiments, an antioxidant (D-α-tocopherol or BHT in chloroform solution) was added to the lipid to give a final concentration of 0.1 or 1 mole %. The chloroform was evaporated to dryness under a stream of nitrogen with the flask suspended in a water bath at 30–40°C. Enough 0.145 M NaCl, water if necessary, and 2.5–10 μCi of $^{24}NaCl$ or $^{22}NaCl$ were added to the dried lipid so that the final concentration of total lipid was 16.6 μmoles/ml, usually in a total volume of 6 ml of 0.145 M NaCl. The flask was shaken gently until all lipid was removed from the wall of the flask and the resultant milky suspension was sonicated for 1 min in a Branson water bath type sonicator. The liposomes were allowed to swell (17) at room temperature for 2–3 hr. They were then placed on a 1.5 × 30 cm column packed with coarse Sephadex G-50 swollen in 0.145 M choline chloride and eluted from the column with 0.145 M choline chloride. The liposomes came through the column first and emerged in a fraction about one-third as concentrated as the original sample, followed by the untrapped electrolyte (Fig. 1). Usually, the liposomes were collected in a volume of 15–16 ml.

EXPERIMENTAL PROCEDURE

Ascorbic Acid, Reduced and Oxidized Glutathione, and Ferrous Chloride

The material being studied was dissolved in 0.145 M choline chloride with water added if necessary to adjust total osmolarity to 0.145 M. Solutions were made fresh for each experiment. The pH of the 0.145 M choline chloride was 5.0. With the ascorbic acid and glutathione solutions, this pH was obtained by addition of small amounts of concentrated KOH. The pH remained unchanged throughout the experiments.

Initial solution and serial dilutions of each agent were made so that 0.2 ml of the test solution added to a 1-ml aliquot of the liposomes gave the desired concentration.

Radiation Studies

In these experiments, 3.2-ml aliquots of liposomes were pipetted into 8 × 74 mm Pyrex test tubes and radiated with a ^{60}Co source in a gamma cell. A cork was placed in each tube almost touching the liquid to minimize the free air space and, consequently, ozone production. The temperature during radiation was controlled with a water bath at 22 ± 1.5°C. Dose rate was calibrated with ferrous sulfate dosimeters. Samples were removed as the desired dose was achieved up to a maximum of 300 K-rad.

Antioxidants

In some experiments, BHT or D-α-tocopherol was added to the liposomes after their passage through the column and prior to addition of the chemical agent or to irradiation. The antioxidant was dissolved in absolute ethanol so that 3 or 8 μl added to 4.4 or 6.7 ml of liposomes achieved the desired final concentration.

Cation Loss

1-ml aliquots of liposomes were pipetted into 0.25-inch diameter dialysis bags; 0.2 ml of test solution or 0.2 ml of 0.145 M choline chloride was then added. The bags were tied and placed in 1.1 × 9.9 cm glass test tubes containing 5 ml of 0.145 M choline chloride. The samples were placed, at 1-min intervals, into a 37°C water bath shaker (Eberbach Corp., Ann Arbor, Mich.), shaking at 88 oscillations/min. Each sample was removed after 30 min. Duplicate or triplicate aliquots of all samples were incubated. To one aliquot of liposomes in each experiment, 0.2 ml of 1.2% Triton X-100 in 0.145 M choline chloride, which physically disrupts the spherules, was added, and the solution was incubated in the same fashion

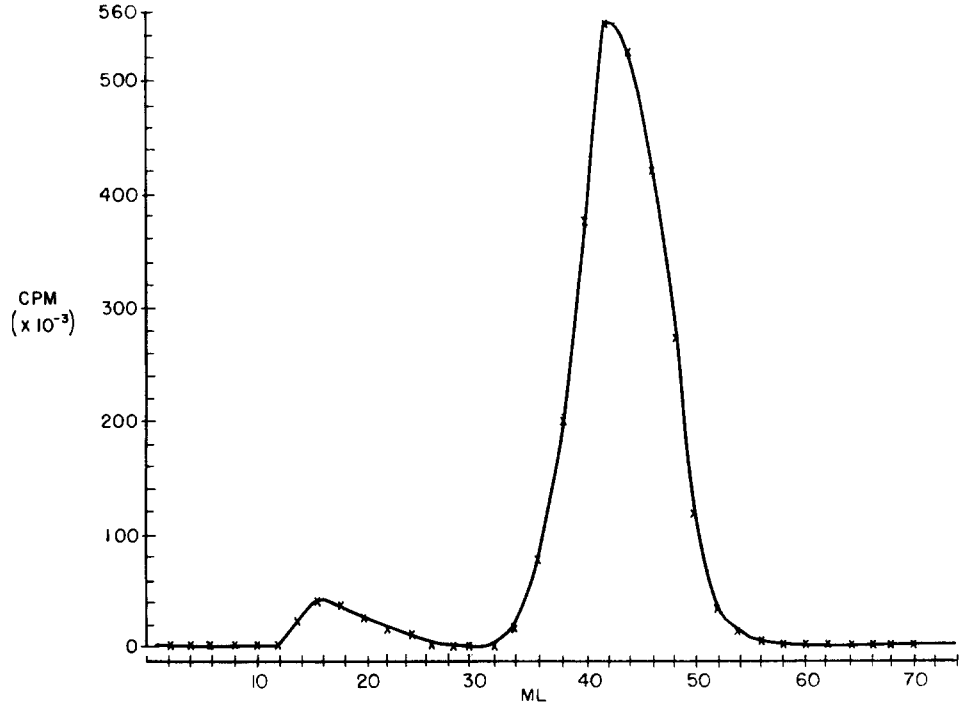

FIG. 1. Appearance first of ^{22}Na trapped in liposomes (5.3%) and then untrapped ^{22}Na (94.7%) after 6 ml of liposomes dispersed in 0.145 M NaCl + 3 μCi ^{22}Na were applied to a Sephadex G-50 (coarse) column equilibrated with 0.145 M choline chloride. Ordinate is cpm $\times 10^{-3}$ and abscissa is cumulative volume of eluate.

in order to measure the maximum rate of leakage possible under the experimental conditions. After incubation, radioactivity in 3-ml aliquots of each sample was determined in a Nuclear-Chicago automatic gamma spectrometer. Samples were counted long enough to attain an error of less than 2%. The dialysis bags were opened and the TBA test was done immediately on the residual liposomes.

TBA Test

Malonic dialdehyde was measured using a modification of the TBA reaction (1, 18). Malonic dialdehyde is a breakdown product formed during lipid peroxidation, but its formation is considered to parallel the extent of lipid peroxidation (19, 20). Residual liposomes from duplicate or triplicate samples for each concentration of the chemical agent studied or for each irradiation dose were mixed, and 2-ml aliquots were added to 1.3 ml of 10% trichloroacetic acid. After mixing, each sample was filtered through S & S qualitative filter paper (no. 595). 0.5 ml of 0.67% thiobarbituric acid in water was added to 2.5 ml of filtrate, and the samples were placed in a boiling water bath for 15 min. The pink pigment was extracted with 3 ml of spectroquality *n*-butanol to eliminate slight residual opalescence, and after centrifugation the absorbance was measured at 530 nm using a Beckman DB spectrophotometer. To assure that determination of TBA values of dialyzed liposomes did not give a false picture of relative amounts of malonic dialdehyde formed, in several experiments TBA tests were done on both dialyzed and nondialyzed liposomes kept at 37°C for 30 min.

Lipid Quantitation

In some experiments, lipid concentration was measured in the liposome suspension after passage through the Sephadex column. The lipids were extracted from 0.2-ml aliquots of liposomes by the method of Folch, Lees, and Sloane Stanley (21). The washed lower phase was dried under a stream of nitrogen, and the total lipid was quantitated using the method of Amenta (22). 7.0 ml of a solution of 2.5 g of $K_2Cr_2O_7$ dissolved in 1 liter of 36 N H_2SO_4 was added to the dried lower phase, to a blank, and to a standard. The standard consisted of a dried chloroform solution of lipids in the same mole ratio as present in the liposomes. After heating in a boiling water bath for 45 min, the change in absorbance at 350 nm was measured.

Glucose Loss

To ensure that the patterns of cation loss observed in these studies represented a nonspecific phenomenon, in some experiments 2.1 μCi of glucose-1-^{14}C was added to unlabeled NaCl solution to give a liposome suspension in

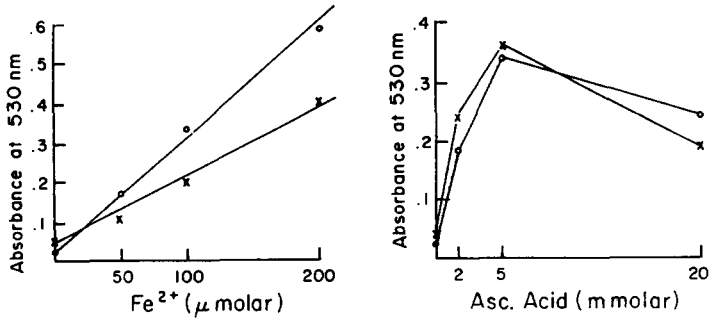

FIG. 2. Malonic dialdehyde formation in dialyzed (-x-x-) and nondialyzed (-o-o-) liposomes incubated at 37°C for 30 min. Ferrous ion or ascorbic acid was added in 0.2 ml of 0.145 M choline chloride to 1 ml of liposomes to achieve final concentrations.

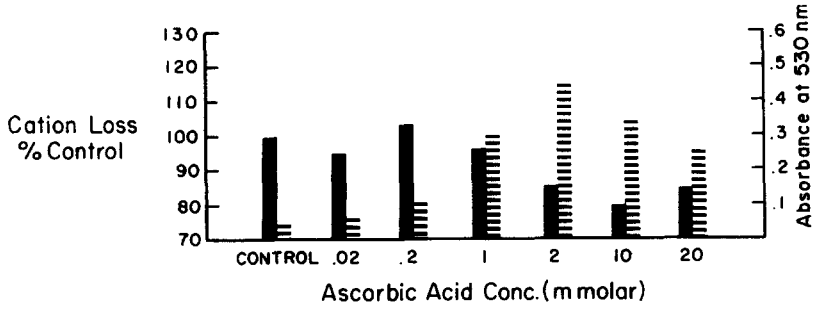

FIG. 3. Cation loss and malonic dialdehyde formation induced by ascorbic acid. Solutions of increasing concentrations of ascorbic acid in 0.2 ml of 0.145 M choline chloride were added to 1 ml of liposomes to give desired final concentration. Solid blocks represent cation loss as percent of control, and striped blocks represent malonic dialdehyde formation as measured by absorbance at 530 nm.

6.0 ml of 0.01 M glucose and 0.140 M NaCl. The liposomes were prepared as described previously. Glucose loss was measured by adding 2.0 ml of dialysate to a mixture of 6.8 ml of 0.6% 2,5-diphenyloxazole in toluene and 8.2 ml of Cellosolve. Radioactivity was determined using a Tri-Carb liquid scintillation spectrometer, model 3003 (Packard Instrument Co., Inc., Downers Grove, Ill.). Experiments were done with added ascorbic acid and ferrous chloride, and in both cases glucose loss and lipid peroxidation followed patterns similar to those found when NaCl was used alone.

RESULTS

In all experiments, "leakage" of sodium ions from control samples ranged from 0.05 to 0.15 μeq, corresponding to a net count rate of 300–1000 cpm. Samples treated with Triton X-100 released 17–25 times as many sodium ions as did control samples. Lipid concentration of liposomes after passage through the Sephadex column was 4.3 ± 0.3 mg/ml.

Lipid Peroxidation in Dialyzed and Nondialyzed Liposomes

When liposomes were irradiated or exposed to ascorbic acid, ferrous ion, or reduced and oxidized glutathione, the relative amounts of pink pigment formed in the TBA test were the same, whether samples were dialyzed at 37°C for 30 min or were simply kept at 37°C for 30 min in a test tube. Similarly, BHT inhibited lipid peroxidation induced by ferrous ion and ascorbic acid in dialyzed and nondialyzed samples. Fig. 2 illustrates the data obtained with ascorbic acid and ferrous ion. We used TBA values in residual phospholipid spherules after dialysis as an indication of the degree to which lipid peroxidation had progressed.

Ascorbic Acid

Increasing concentrations of ascorbic acid (actually a mixture of ascorbic acid and ascorbate) resulted first in an increase in lipid peroxidation, with a subsequent decrease at higher concentrations. Conversely, cation loss was close to control values at low concentrations of ascorbic acid, and dropped well below control values at higher concentrations (Fig. 3).

Ferrous Ion

As shown in Fig. 4, higher concentrations of ferrous ion were associated with both increased cation loss and increased lipid peroxidation. However, addition of BHT (20 μmoles/liter) to liposomes before addition of ferrous ion inhibited peroxidation approximately 50%, without affecting the increased cation loss caused by ferrous ion (Table 1, A).

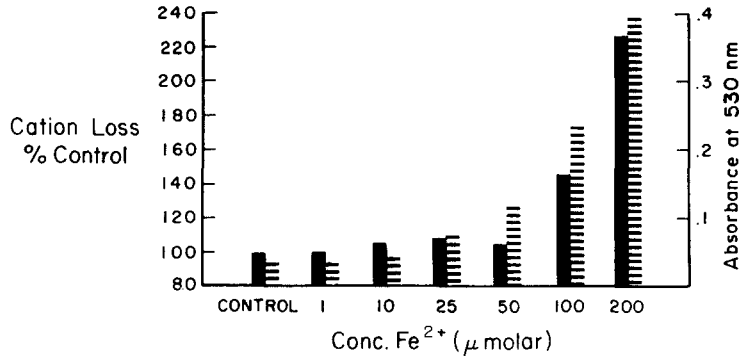

FIG. 4. Cation loss and malonic dialdehyde formation induced by ferrous ion. Increasing concentrations of ferrous ion in 0.2 ml of 0.145 M choline chloride were added to 1 ml of liposomes to give desired final concentration. Solid blocks represent cation loss as percent of control, and striped blocks represent malonic dialdehyde formation as measured by absorbance at 530 nm.

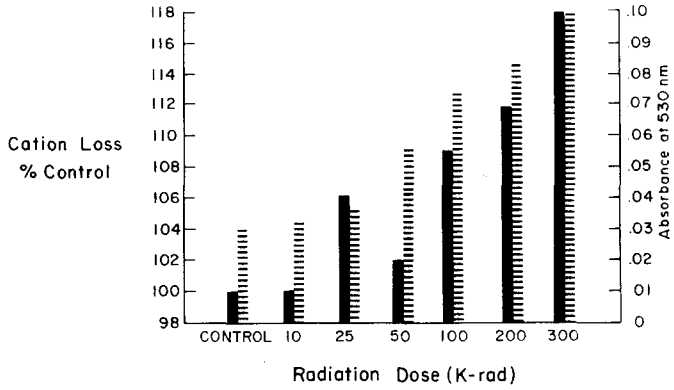

FIG. 5. Cation loss and malonic dialdehyde formation induced by radiation. 3.2-ml aliquots of liposomes were irradiated with increasing doses of gamma radiation. Solid blocks represent cation loss as percent of control, and striped blocks represent malonic dialdehyde formation as measured by absorbance at 530 nm.

Radiation

When liposomes were irradiated over the dose range 10–300 K-rad, cation loss increased slightly with increasing dosage, corresponding to relatively small amounts of lipid peroxidation (Fig. 5). However, whereas BHT produced some inhibition of peroxidation, there was no effect on the increased cation loss produced by irradiation (Table 1, B).

Glutathione

Reduced glutathione induced a progressively larger increase in cation loss with increasing concentration, although there was little change in TBA values compared with controls. Oxidized glutathione and mixtures of oxidized and reduced glutathione induced small amounts of lipid peroxidation with a decrease in cation loss (Fig. 6).

BHT Incorporation

When BHT in a mole ratio of 1% was incorporated into liposomes during their preparation, there was marked inhibition of lipid peroxidation compared with control liposomes prepared without BHT. When the mole ratio of BHT was reduced to 0.1%, there was only minimal inhibition of peroxidation. At both concentrations, there was no apparent effect of inhibition of peroxidation on the changes in cation loss induced either by ferrous ion or ascorbate (Table 2, A and B).

Preincorporation of Vitamin E

1% vitamin E was very effective in inhibiting lipid peroxidation. 0.1% vitamin E was also an effective inhibitor, but did not inhibit as completely as at the higher concentration. Again, inhibition of peroxidation did not affect the changes in cation loss induced by ferrous ion or ascorbic acid (Table 2, C and D).

Vitamin E and BHT

When added to already formed liposomes at similar concentrations, BHT was a much more effective inhibitor of ascorbate- and ferrous ion-induced lipid peroxidation than was vitamin E (Table 1, A, and Table 3).

TABLE 1 EFFECT OF BHT ON FERROUS ION- AND RADIATION-INDUCED CHANGES IN CATION LOSS AND MALONIC DIALDEHYDE FORMATION

	No Antioxidant		BHT 20 μmoles/liter	
	Na^+ Release	Absorbance	Na^+ Release	Absorbance
A.				
Control	100	0.030 (0.025–0.034)	100 (88–116)	0.021 (0.016–0.024)
Fe^{2+} 100 μmoles/liter*	202 (153–260)	0.242 (0.210–0.262)	199 (119–277)	0.106 (0.075–0.140)
Fe^{2+} 200 μmoles/liter†	339 (284–393)	0.319 (0.306–0.332)	355 (261–440)	0.154 (0.137–0.171)
B.				
Control	100	0.032 (0.030–0.033)	102 (98–106)	0.030 (0.028–0.032)
300 K-rad†	128 (125–131)	0.096 (0.095–0.096)	130 (129–130)	0.069 (0.067–0.070)

BHT in 8 μl of absolute ethanol was added to 6.7 ml of liposomes just prior to addition of ferrous ion, or in 3 μl of absolute ethanol to 4.4 ml of liposomes prior to irradiation. Ferrous ion in 0.2 ml of 0.145 M choline chloride solution, or choline chloride solution alone as control, was added to 1 ml of liposomes. Na^+ release expressed as percent of control, and malonic dialdehyde formation as absorbance at 530 nm.

* The ranges of values from five experiments are in parentheses.

† The ranges of values from two experiments are in parentheses.

TABLE 2 LIPID PEROXIDATION AND CATION RELEASE IN LIPOSOMES PREPARED WITH BHT OR VITAMIN E

	No Antioxidant		1% BHT	
	Na^+ Release	Absorbance	Na^+ Release	Absorbance
A*				
Control	100	0.027 (0.023–0.030)	100	0.018 (0.014–0.021)
Ascorbic acid 2 mmoles/liter	88 (82–93)	0.467 (0.422–0.511)	99 (85–113)	0.069 (0.068–0.069)
Fe^{2+} 100 μmoles/liter	179 (122–236)	0.185 (0.179–0.191)	203 (176–230)	0.055 (0.054–0.055)
			0.1% BHT	
B†				
Control	100	0.028 (0.023–0.031)	100	0.024 (0.023–0.025)
Ascorbic acid 2 mmoles/liter	92 (71–108)	0.380 (0.225–0.677)	90 (84–97)	0.351 (0.209–0.567)
Fe^{2+} 100 μmoles/liter	182 (125–280)	0.157 (0.102–0.252)	164 (126–218)	0.114 (0.091–0.153)
			1% Vitamin E	
C*				
Control	100	0.032 (0.025–0.039)	100	0.018 (0.017–0.019)
Ascorbic acid 2 mmoles/liter	66 (52–80)	0.424 (0.227–0.620)	55 (48–62)	0.034 (0.029–0.039)
Fe^{2+} 100 μmoles/liter	209 (200–217)	0.141 (0.084–0.198)	245 (230–260)	0.035 (0.032–0.037)
			0.1% Vitamin E	
D†				
Control	100	0.025 (0.019–0.031)	100	0.023 (0.021–0.024)
Ascorbic acid 2 mmoles/liter	90 (71–108)	0.247 (0.225–0.279)	80 (70–90)	0.085 (0.061–0.106)
Fe^{2+} 100 μmoles/liter	202 (125–280)	0.117 (0.102–0.132)	205 (178–250)	0.066 (0.053–0.075)

Liposomes made with 1 mole % or 0.1 mole % BHT or vitamin E incorporated at the time of preparation were compared with simultaneously prepared liposomes made without antioxidant. Ferrous ion and ascorbic acid in 0.2 ml of 0.145 M choline chloride solution, or 0.2 ml 0.145 M choline chloride alone, were added to 1 ml of liposomes. Na^+ release expressed as percent of control, and malonic dialdehyde formation as absorbance at 530 nm.

* Ranges of values from two experiments are shown in parentheses.

† Ranges of values from three experiments are shown in parentheses.

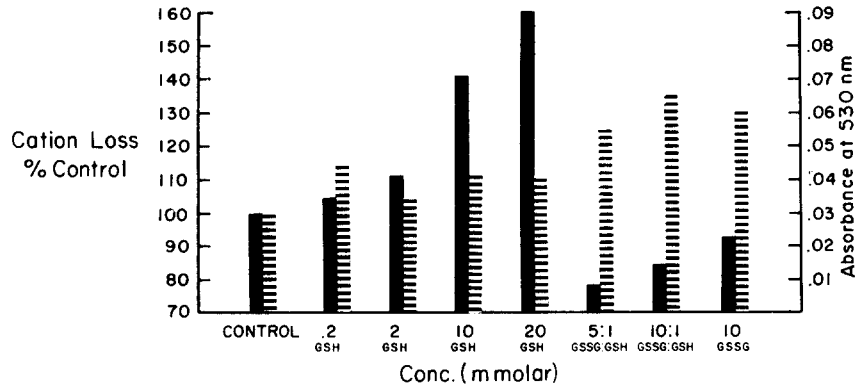

FIG. 6. Cation loss and malonic dialdehyde formation induced by reduced and oxidized glutathione. Solutions of increasing concentrations of reduced glutathione (*GSH*), mixtures of oxidized and reduced glutathione (*GSSG:GSH*), and oxidized glutathione (*GSSG*) dissolved in 0.2 ml of 0.145 M choline chloride solution were added to 1 ml of liposomes to give desired final concentration. Solid blocks represent cation loss as percent of control, and striped blocks represent malonic dialdehyde formation as measured by absorbance at 530 nm.

DISCUSSION

The exact relation of lipid peroxidation to cellular and subcellular injury produced by various agents is not known. Robinson (7) induced turbidity changes in rat brain microsomes with mixtures of ascorbate, iron, cysteine, and cystine, and concluded that lipid peroxidation resulted in increased membrane permeability. Hunter and his colleagues (4–6) studied the effect of ascorbate, ferrous ion, and oxidized and reduced glutathione on isolated rat liver mitochondria. They found a good correlation, both temporally and in order of magnitude, between swelling and lysis of the mitochondria and lipid peroxidation. They concluded that lipid peroxidation may be the cause of the permeability increase via disruption of double bonds in fatty acids. Wills and Wilkinson (8) arrived at a similar conclusion in studies with irradiated lysosomes. They showed that enzyme release increased with lipid peroxidation. In a subsequent paper (23), however, these authors presented evidence that oxidation of thiol groups to disulfide groups by free radicals formed during irradiation may be the primary biochemical damage, with lipid peroxidation merely occurring secondarily to configurational changes. In addition to injury to subcellular organelles, lipid peroxidation has been correlated with H_2O_2-induced hemolysis in vitro in red cells from humans with paroxysmal nocturnal hemoglobinuria (1), from tocopherol-deficient patients with abetalipoproteinemia (3), and from tocopherol-deficient mice exposed to hyperbaric oxygen in vivo (2).

TABLE 3 EFFECT OF VITAMIN E AND BHT ON ASCORBATE- AND FERROUS ION-INDUCED LIPID PEROXIDATION

	No Antioxidant	Vitamin E 25 μmoles/liter
	Absorbance	Absorbance
Control*	0.028 (0.027–0.029)†	0.028 (0.028–0.028)
Ascorbic acid 2 mmoles/liter	0.428 (0.339–0.516)	0.344 (0.291–0.397)
Fe^{2+} 100 μmoles/liter	0.138 (0.129–0.146)	0.100 (0.095–0.104)
		BHT 20 μmoles/liter
Control	0.029 (0.028–0.029)	0.029 (0.027–0.030)
Ascorbic acid 2 mmoles/liter	0.365 (0.339–0.390)	0.168 (0.129–0.206)

Vitamin E and BHT in 8 μl of absolute ethanol were added to 6.7 ml of liposomes prior to addition of ascorbic acid and ferrous ion. The latter were added to 1 ml of liposomes in 0.2 ml of 0.145 M choline chloride solution. Malonic dialdehyde formation is expressed as absorbance at 530 nm.

* Permeability studies were not done in these experiments.

† The values in parentheses represent the ranges of two experiments.

Thus, many workers have shown that lipid peroxidation is often closely associated with, and may well be the cause of, increased membrane permeability. In the present work, using a phospholipid spherule system, lipid peroxidation as indicated by the TBA test for malonic dialdehyde did not correlate with alterations in cation loss. Thus, cation loss and lipid peroxidation both increase with increasing doses of ferrous ion and radiation, but lipid peroxidation is inhibited by BHT in both cases with no reduction in the increased cation loss. Also notable is the lack of correlation between the changes in cation loss induced by reduced and oxidized glutathione and the relatively small amount of lipid peroxidation. Reduced glutathione alone causes a marked increase in cation loss, but oxidized glutathione alone and the reduced and oxidized forms together decrease cation loss although associated with larger amounts of lipid peroxidation. Most striking, however, is the induction of large amounts of lipid peroxidation by some concentrations of ascorbic acid, with a definite decrease in cation loss noted

over this concentration range. The results do not clarify the question of whether the products of lipid peroxidation are themselves important mediators of cellular injury in vivo, but they do suggest that any injurious effects they might have are mediated indirectly, possibly by disrupting structural proteins or enzyme systems. Several investigators have reported such effects. Wills (13) and Lewis and Wills (10) have demonstrated damage to sulfhydryl proteins and enzymes induced by lipid peroxidation, and Tappel (11) and Desai and Tappel (12) have demonstrated damage to proteins. Zirkle et al. (24) and O'Malley et al. (25) have demonstrated that decreased erythrocyte acetylcholinesterase levels in dogs exposed to hyperbaric oxygen are an effect of lipid peroxidation.

Further evidence that lipid peroxidation in fatty acid side chains does not itself increase cation loss is provided when antioxidants are incorporated in the liposomes. Both BHT and vitamin E at a mole ratio of 1% inhibit lipid peroxidation resulting from addition of ferrous ion and ascorbic acid, without affecting the cation loss. When the mole ratio is reduced to 0.1%, vitamin E is still quite effective in inhibiting peroxidation, while BHT has very little effect.

Of interest is the fact that BHT is a more effective antioxidant than vitamin E when added to the liposomes after their formation, whereas vitamin E is more effective when preincorporated into the membrane. Vitamin E is the main naturally occurring antioxidant. Our findings suggest that very low levels of vitamin E in natural membranes, possibly undetectable by present methods, may be an important factor in determining relative susceptibility of various membrane-bounded structures to lipid peroxidation. Wills and Wilkinson (26) found that more lipid peroxidation occurred in microsomes in response to irradiation than in other subcellular fractions studied, a fact unexplained by the relative content of polyunsaturated fatty acids. Red blood cell tocopherol is believed to be localized in the membrane, and it is in dynamic equilibrium with plasma tocopherol (27). However, it is not known whether the protection against lipid peroxidation shown in several studies is afforded by the plasma or cell-bound fraction. The present findings suggest that the membrane-bound fraction is protective, and they explain why BHT, which is less effective in the membrane, cannot completely replace vitamin E in animal diets (28).

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