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Some Studies on Lipid Peroxidation in Monomolecular and Bimolecular Lipid Films

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Summary. Hydrogen peroxide generated from dissolved oxygen through the alloxan-dialuric acid cycle affected both the permeability and the stability of lipid bilayer membranes. The permeability of the artificial membranes varied directly with the hydrogen peroxide concentration. Membrane stability varied inversely with the hydrogen peroxide concentration. Bilayers formed from solutions containing both phospholipid and the antioxidant vitamin E were less permeable and more stable in the presence of hydrogen peroxide than bilayers generated from solutions containing phospholipid alone. Peroxidation of phospholipid monolayers caused first an expansion of the films presumably through the introduction of peroxide groups. Further oxidation of phospholipid monolayers led to contraction of the films presumably through the formation of water-soluble products. The results of the monolayer studies and a consideration of the possible kinetics for the peroxidation reaction sequence have been used to explain the changes in the permeability and the stability of lipid bilayer membranes. Our data suggest that oxidation of lipid in biological membranes may first increase membrane permeability and then decrease membrane stability.

Many studies have provided evidence that the initial phases in the peroxidation of unsaturated lipids result in the formation of hydroperoxides. Subsequently, hydroperoxides decompose by a complex series of reactions (for reviews, *see* refs. [2] and [14]). Because of the complexity of the reactions involved, our present knowledge of the reaction sequences in lipid peroxidation is mainly based on studies with pure lipids. Information regarding the peroxidation of complex lipid mixtures in tissues and tissue extracts is generally inferred from the identification of an end-product such as malonaldehyde.

It is widely accepted now that lipid peroxides and/or their decomposition products damage membrane structure. Erythrocytes from vitamin E-deficient animals are lysed by dialuric acid [3, 4, 10, 11, 13, 16, 18], a hydroquinone which reacts with dissolved molecular oxygen forming alloxan and hydrogen

peroxide [6], and possibly the superoxide radical [7]. Horwitt, Harvey, Duncan and Wilson [11], Bunyan, Green, Edwin and Diplock [4] and Tsen and Collier [18] have demonstrated a correlation between dialuric acid-induced hemolysis and lipid peroxidation which was identified by the reaction of malonaldehyde with thiobarbituric acid. Nevertheless, it has been suggested that lipid peroxidation is not involved specifically in control of membrane permeability [17].

We have studied the effect of peroxidation on bimolecular and monomolecular lipid films. We have used alloxan and the reducing agent ascorbic acid as a system for the controlled generation of very small amounts of hydrogen peroxide. Our results suggest that the initial oxidation reactions expand a lipid bilayer film, thus increasing its permeability. Subsequent oxidation reactions cleave lipid components, thus decreasing bilayer stability.

Materials and Methods

Egg-yolk lecithin was isolated and purified according to established procedures [15]. Dialuric acid and alloxan were purchased from K & K Laboratories, Plainview, N.Y., catalase and DL-α-tocopherol (vitamin E) from Sigma Chemical Co., St. Louis, Mo. Ascorbic acid was obtained from Merck & Co., Inc., Rahway, N. J., decane from Eastman Kodak Co., Rochester, N.Y., and castor oil from E. R. Squibb & Sons, New York. Commercial *n*-hexane was purified as described by Heikkila, Kwong and Cornwell [9]. All salts used were analytical grade.

Reagents were dissolved in 0.1 M NaCl. Oxygen uptake in these aqueous solutions was measured with an oxygen electrode as described by Deamer, Heikkila, Panganamala, Cohen and Cornwell [6]. All measurements were made at the ambient temperature, 24 to 26 °C.

Techniques for the formation of lipid bilayer membranes and the measurement of their electrical resistances were previously reported [19, 20]. The films were prepared in 0.1 M NaCl from decane solutions of the following composition: (a) 1 % egg-yolk lecithin, (b) 1 % egg-yolk lecithin with various weight percentages of vitamin E (based on lecithin + vitamin E). Reagents were introduced into one of the compartments of the bilayer cell. Ascorbic acid was added after alloxan or dialuric acid.

Monolayer studies were performed at the air-water interface on a $2 \times 50 \times 1$ cm Teflon trough using a constant pressure-variable area technique previously described [5]. The trough was filled with 0.02 M NaCl or 0.02 M NaCl containing alloxan and ascorbic acid. Alloxan and ascorbic acid were first dissolved separately in 0.02 M NaCl at appropriate concentrations. The two solutions were mixed just prior to the start of an experiment.

A known amount of egg-yolk lecithin dissolved in *n*-hexane-methanol (49:1, v/v) was spread on the aqueous subphase. The amount of lecithin was chosen so that each molecule occupied an area of approximately 300 $Å^2$ before the film was compressed. The monolayers were compressed using castor oil, which generates 17 ± 0.7 dynes/cm [5], as a piston oil. Surface area A was measured as a function of time. The initial A value was determined by applying the piston oil immediately after a film was formed. Other A data were obtained by applying the piston oil at stated time intervals after a film was formed.

Results and Discussion

Fig. 1 shows oxygen uptake in 0.1 M NaCl solutions containing alloxan and ascorbic acid. Addition of alloxan at a concentration of 7.1×10^{-5} M and ascorbic acid at a concentration of 7.1×10^{-4} M caused the consumption of 60% of the dissolved molecular oxygen within 20 min. An identical curve was obtained upon addition of dialuric acid at a concentration of 7.1×10^{-5} M and ascorbic acid at a concentration of 7.1×10^{-4} M. When both the alloxan and the ascorbic acid concentrations in the solution were de-

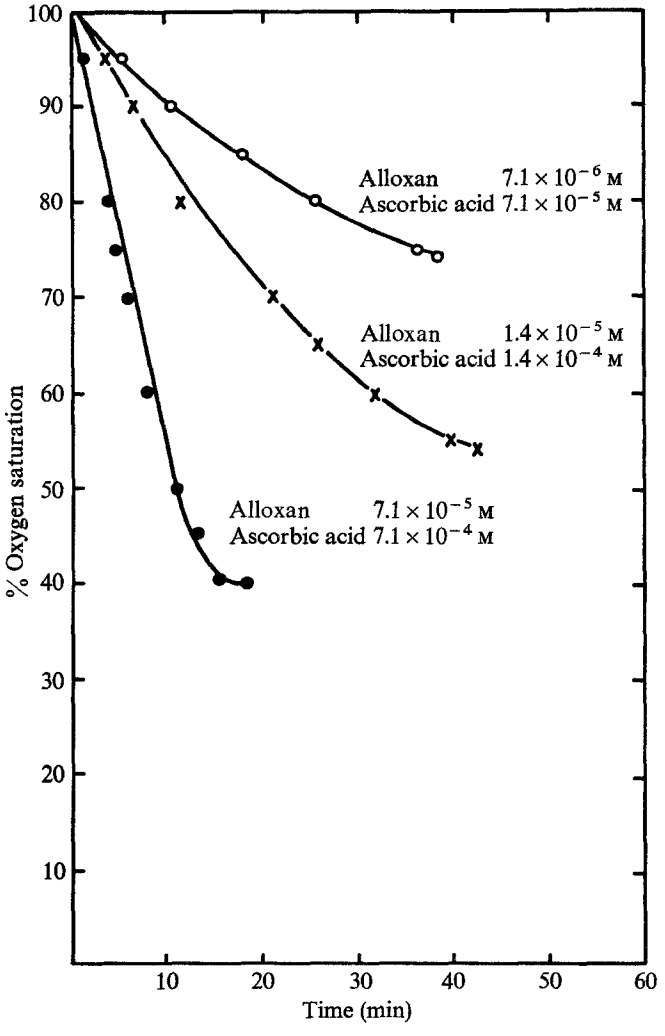

Fig. 1. Oxygen uptake measured with a Clark electrode. The aqueous solutions contained alloxan and ascorbic acid at the concentration indicated in the figure. Temperature was 24 to 26 °C

creased, oxygen consumption occurred at a slower rate and the amount of oxygen consumed was smaller. These data, which are in agreement with data reported earlier by Deamer *et al.* [6], show that one can control the amount of hydrogen peroxide formed by changing the concentrations of alloxan and ascorbic acid in the aqueous solution. At the same time one can control the rate at which hydrogen peroxide is generated.

Fig. 2 demonstrates the effects of the controlled generation of hydrogen peroxide by dialuric acid-ascorbic acid or alloxan-ascorbic acid on the d-c resistance and stability of lecithin bilayers formed in 0.1 M NaCl. Addition of 7.1×10^{-5} M dialuric acid and 7.1×10^{-4} M ascorbic acid to one side of the films decreased their resistances by a factor of 80 within 20 min. At this point the gradual decrease in resistance was interrupted by rupture of the membranes. A similar result was obtained when 7.1×10^{-5} M alloxan and 7.1×10^{-4} M ascorbic acid were added to one side of the bilayers. The change in bilayer resistance and the rupture of the films depended on the concentration of the reagents. A decrease in the concentrations of alloxan and ascorbic acid resulted in a slower decrease in film resistance, rupture of the films at a stage of higher resistance, and an increase in film duration before rupture. Addition of alloxan (at a concentration of 7.1×10^{-5} M) or ascorbic acid (at a concentration of 7.1×10^{-4} M) alone was very similar to the control (*see* No Addition, Fig. 2).

The effects of hydrogen peroxide on film permeability and stability were difficult to control by the direct addition of this reagent. When 10^{-4} to 10^{-6} M hydrogen peroxide was added directly to one side of the lecithin bilayers, the films ruptured immediately and resistance measurements were virtually impossible. Films were stable for 40 to 50 min and showed only small changes in d-c resistance when hydrogen peroxide was added in the 10^{-7} to 10^{-8} M range.

The data summarized above suggested that hydrogen peroxide first increased the permeability and subsequently decreased the stability of lecithin bilayers. This suggestion was supported by experiments with catalase and vitamin E. Lecithin bilayers were unstable in solutions containing more than 0.01 mg/ml catalase. When 7.1×10^{-5} M alloxan and 7.1×10^{-4} M ascorbic acid were added to solutions containing 0.005 mg/ml catalase, films showed an average resistance of 1.5×10^{6} Ω cm^2 at 15 min. The resistance did not change significantly for the duration of the films which averaged 30 min. When the catalase concentration was doubled, the average film resistance was 3.4×10^{6} Ω cm^2 at 15 min and film stability increased to 40 to 50 min. Thus, catalase inhibited the action of alloxan-ascorbic acid on bilayer resistance and bilayer stability just as catalase inhibited dialuric acid-induced hemolysis [7].

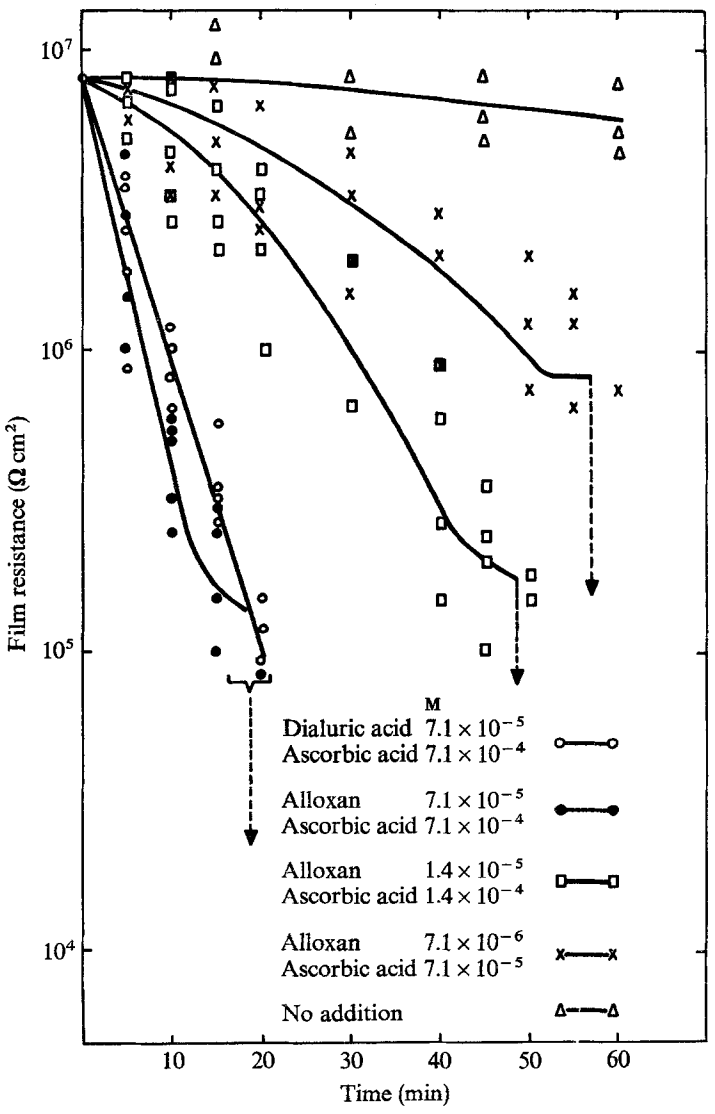

Fig. 2. The effects of various concentrations of dialuric acid and ascorbic acid and alloxan and ascorbic acid on the d-c resistance and stability of lipid bilayer membranes. The membranes were formed in 0.1 M NaCl from solutions containing egg-yolk lecithin. The reagents were added to one chamber at the concentrations indicated in the figure. Temperature was 24 to 26° C. The dotted line indicates the point of film rupture

The antioxidant vitamin E had a marked protective effect on lecithin bilayers. Fig. 3 shows the effects of 7.1×10^{-5} M alloxan and 7.1×10^{-4} M ascorbic acid on the d-c resistance and stability of bilayer membranes which had been formed from lecithin solutions containing increasing amounts of

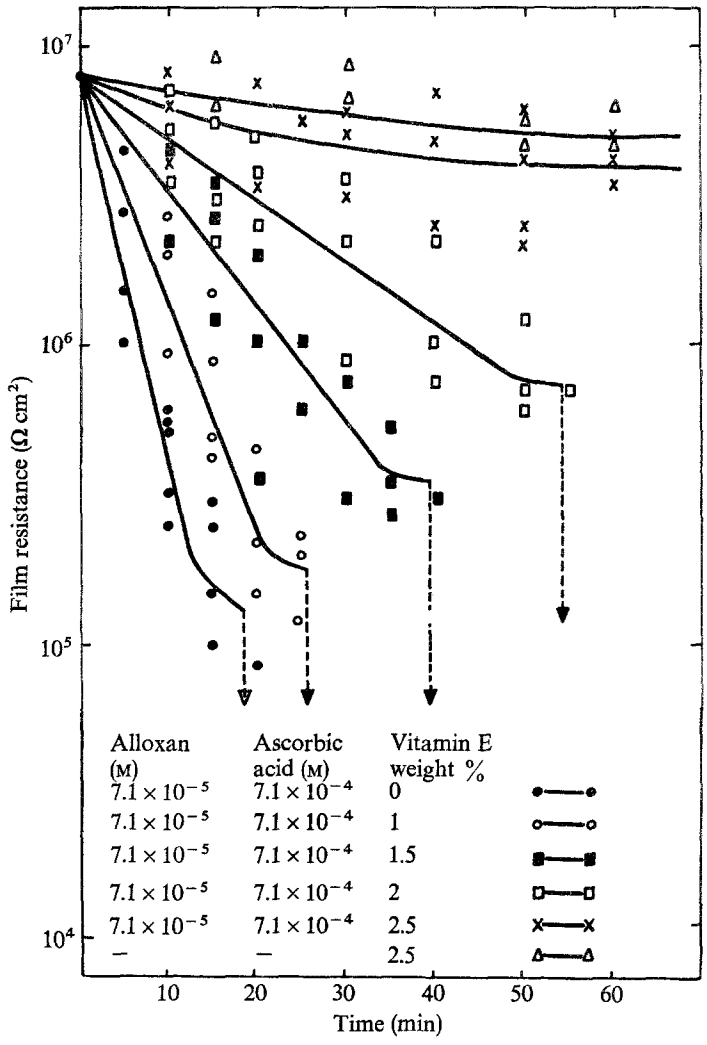

Fig. 3. The effect of 7.1×10^{-5} M alloxan and 7.1×10^{-4} M ascorbic acid on the d-c resistance and stability of lipid bilayers formed from solutions containing egg-yolk lecithin and various weight percentages of vitamin E. The membranes were generated in 0.1 M NaCl and the reagents were added to one chamber of the bilayer cell. Temperature was 24 to 26° C. The weight percentages of vitamin E were calculated on the amount of lecithin. The dotted line indicates the point of film rupture

DL-α-tocopherol. A gradual increase in the amount of α-tocopherol in the film-forming solution had the same effect as decreasing the concentrations of alloxan and ascorbic acid in the aqueous phase (Fig. 2). Films prepared from solutions containing vitamin E were less permeable and more stable in the presence of hydrogen peroxide than films formed from solutions containing

phospholipid alone. Thus, DL-α-tocopherol inhibited the action of alloxan-ascorbic acid on bilayer resistance and bilayer stability just as α-tocopherol inhibited dialuric acid-induced hemolysis [3, 4, 11, 18].

Studies with different concentrations of alloxan-ascorbic acid (Fig. 2) and studies incorporating vitamin E in films (Fig. 3) both showed that bilayer permeability varied directly with the effective hydrogen peroxide concentration. These studies also showed that bilayer stability or film duration varied inversely with the effective hydrogen peroxide concentration. However, the actual d-c film resistance was not a critical factor in the rupture of a film. Under some conditions films ruptured when the d-c resistance decreased to $10^5\ \Omega\text{cm}^2$ while under other conditions films ruptured when the d-c resistance decreased to only $10^6\ \Omega\ \text{cm}^2$. These observations suggested that an initial reaction between lecithin and hydrogen peroxide altered bilayer permeability and that a subsequent reaction altered bilayer stability. Studies with lecithin monolayers supported this hypothesis.

The data summarized in Fig. 4 show surface area as a function of time for lecithin monolayers spread on aqueous subphases containing 0.02 M NaCl or 0.02 M NaCl and various concentrations of alloxan and ascorbic acid. When the films were formed on a subphase containing 7.1×10^{-6} M alloxan and 7.1×10^{-5} M ascorbic acid their surface area increased from about 7 cm^2 to about 13 cm^2 within 80 min. Then the surface area decreased and a value of about 10 cm^2 was reached at 120 min. The rate of film expansion varied directly with the concentration of alloxan and ascorbic acid in the subphase. Maximum film expansion was achieved with the highest concentrations of alloxan and ascorbic acid. On all three subphases used, the surface area measured after 990 min was the same and appreciably lower than the initial suface area of the lecithin films. When lecithin monolayers were spread on 0.02 M NaCl alone, surface area increased slowly and to a limited extent. Similar results were obtained for lecithin monolayers spread on either 0.02 M NaCl and 7.1×10^{-4} M alloxan alone or 0.02 M NaCl and 7.1×10^{-3} M ascorbic acid alone (results not shown). These findings of film expansion-contraction with the oxidation of phospholipid monolayers concur with the results of earlier studies on the oxidation of fatty acid monolayers [1, 12].

Monolayer and bilayer data with lecithin and hydrogen peroxide may be explained by the consecutive reactions which are probably involved in peroxidation [2, 14]. Peroxidation in unsaturated lipids can be represented by the following reaction scheme:

$$R + H_2O_2 \rightarrow R_0 \rightarrow R_c.$$

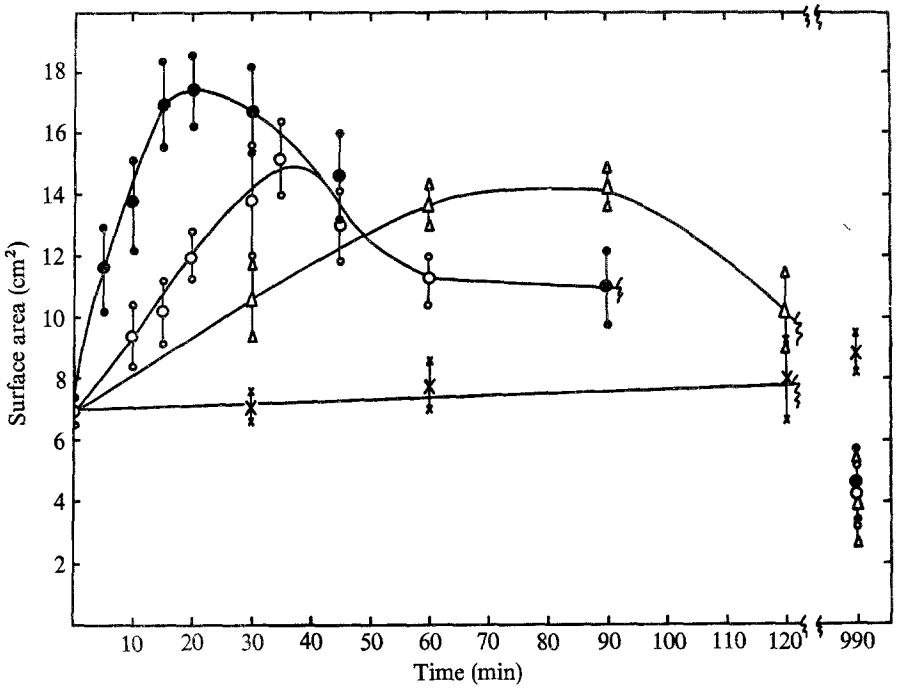

Fig. 4. Surface area as a function of time for lecithin monolayers spread on subphases containing 0.02 M NaCl alone (×——×); 7.1×10^{-6} M alloxan and 7.1×10^{-5} M ascorbic acid (▵——▵); 7.1×10^{-5} M alloxan and 7.1×10^{-4} M ascorbic acid (○——○); 7.1×10^{-4} M alloxan and 7.1×10^{-3} ascorbic acid (•——•). Temperature was 24 to 26° C. Surface areas were measured after a stated time interval (*see* Materials and Methods). Data represent mean ± SD

In the first step the unsaturated aliphatic side-chains, R, are converted into several lipid peroxides, R_0. We assume that the expansion of the monolayer and the increased permeability of the bilayer in the presence of hydrogen peroxide depends directly on the amounts of R_0 formed. In the second step lipid peroxides decompose yielding a mixture of soluble and insoluble cleavage products, R_c. We assume that the partial solubilization of R_c contracts the monolayer. We further suggest that lipid bilayers become unstable when critical amounts of R_c are formed. If peroxidation with H_2O_2 is a consecutive reaction with a transient intermediate [8], a decrease in H_2O_2 concentration will decrease the rate at which the peroxide intermediates, R_0, are formed. Critical amounts of R_c will then be formed at a later time and the film will have a longer duration. Furthermore, less R_0 will be present when critical amounts of R_c are formed and as a consequence the film will be ruptured at a less permeable stage.

Changes in both the permeability and stability of artificial lipid membranes suggest that these effects should be investigated in natural membranes. It is apparent that lipid peroxidation alone can alter the permeability of a membrane.

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