Interfacial oxidation of α-tocopherol and the surface properties of its oxidation products

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Abstract DL-α-Tocopherol spread on an acidic subphase as a gaseous monolayer was oxidized slowly to a derivative that was identified by thin-layer chromatography as α-tocopherylquinone. The derivative generated the same Π-A isotherm as α-tocopherylquinone. When the subphase contained gold chloride, α-tocopherol was oxidized rapidly and quantitatively to α-tocopherylquinone. DL-α-Tocopherol spread on a basic subphase as a gaseous monolayer was oxidized slowly to a mixture that contained α-tocopherol, a quinone, and a nonpolar derivative. The mixture generated a Π-A isotherm with an inflection point below the equilibrium spreading pressure of either α-tocopherol or α-tocopherylquinone. When potassium ferricyanide was added to the alkaline subphase, α-tocopherol was oxidized rapidly to a mixture that contained both the nonpolar derivative (major product) and the quinone (minor product). The nonpolar derivative was isolated by thin-layer chromatography and identified as the spirodienone ether by ultraviolet, infrared, and chemical ionization mass spectra. The spirodienone ether had a low equilibrium spreading pressure that explained the inflection point in the Π-A isotherm generated by α-tocopherol on an alkaline subphase. Surface area data showed that DL-α-tocopherol formed immiscible films with stearyl alcohol and miscible films with oleyl alcohol. Π-A isotherms showed that α-tocopherol in both immiscible and miscible mixtures was oxidized rapidly on an alkaline potassium ferricyanide subphase to the spirodienone ether. Collapse pressure data showed that the spirodienone ether formed an immiscible film with stearyl alcohol and a miscible film with oleyl alcohol. Interfacial oxidation experiments showed that α-tocopherol is oxidized either to tocopherylquinone (acidic subphase) or to the spirodienone ether (alkaline subphase). The natural occurrence of both tocopherylquinone and the spirodienone ether suggests that several types of oxidant stress are found in biological systems. One type of oxidant stress may involve the peroxy radical generating tocopherylquinone; a second type may involve hydroxyl radical–hydroxide ion generating the spirodienone ether.

Supplementary key words phenoxonium ion · α-tocopherylquinone · spirodienone ether · surface area · equilibrium spreading pressure · gold chloride · acid and alkaline subphases · potassium ferricyanide · stearyl and oleyl alcohol · miscible and immiscible films · peroxy and hydroxyl radicals

Two oxidation products of α-tocopherol are found in liver, tocopherylquinone and a dimer that is identical with the compound obtained by the oxidation of α-tocopherol with alkaline potassium ferricyanide (1, 2). This dimer has been identified as the spirodienone ether (3, 4). These compounds are shown in **Fig. 1**. Both α-tocopherylquinone and the spirodienone ether are formed from the phenoxonium ion, a two-electron oxidation product of α-tocopherol (5). In the presence of water, nucleophilic addition to the phenoxonium ion yields a dienone which rearranges to tocopherylquinone (5). Alternatively, deprotonation of the phenoxonium ion yields the quinone methide which dimerizes to the spirodienone ether (5).

The surface properties of tocopherols and tocopherylquinone in pure and mixed monolayers have been described by several investigators (6–10). Both α-tocopherol and tocopherylquinone are amphipathic molecules containing a hydroxyl group (Fig. 1) and these molecules form stable monolayers, even at relatively high surface pressures. Thus α-tocopherol and tocopherylquinone would be expected to concentrate in biological membranes at the oil–water interface. The spirodienone ether lacks a hydroxyl group and is relatively nonpolar compared to α-tocopherol and tocopherylquinone (Fig. 1); it would be expected to have very different surface and membrane properties. The interfacial oxidation of α-tocopherol to tocopherylquinone and the spirodienone ether, and the surface properties of these oxidation products are examined in the present study.

MATERIALS AND METHODS

DL-α-Tocopherol (ICN Pharmaceuticals, Cleveland, OH), D-α-tocopherylquinone (Eastman Organic Chemicals, Rochester, NY), stearyl (18:0) and oleyl (18:1) alcohols (Applied Science Laboratories, State College, PA) were spread on aqueous subphases as

Abbreviation: TLC, thin-layer chromatography.

n-hexane solutions. *n*-Hexane was purified as previously described (11). Potassium ferricyanide (Baker Analyzed Reagent) was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Specific aqueous subphases are described in Results and Discussion.

Thin-layer chromatography (TLC) was used to establish the initial purity of α-tocopherol and α-tocopherylquinone and to identify these compounds in lipid film. Samples were applied to a TLC plate coated with silica gel G and the plate was developed with benzene–ethanol 99:1 (v/v) (12). R_f values in this system are 0.65 for α-tocopherol and 0.30 for α-tocopherylquinone. Lipid films and lipid droplets were collected from the surface of the Langmuir trough by sweeping with a moving barrier and aspiration. They were then extracted with dichloromethane (Spectroquality, Matheson, Coleman and Bell, Norwood, OH), concentrated in a Büchi Rotavapor, and identified by TLC.

Π was measured by the Wilhelmy plate technique utilizing a Cahn R. G. recording balance as previously described (11). Π-A isotherms were generated on a Teflon Langmuir trough (50 × 10 × 1 cm) utilizing a movable Teflon barrier driven by a high-torque variable speed motor. Constant pressure–variable area measurements were obtained with a floating barrier and piston oil as previously described (13). Castor oil, 17 ± 0.7 dynes/cm, or tri-*m*-tolyl phosphate, 9.5 dynes/cm, was used as the piston oil. Measurements were obtained at the ambient temperature, 22–24°C.

Ultraviolet spectra were obtained with a Beckman Acta II spectrophotometer. Infrared spectra were obtained with a Perkin-Elmer 421 spectrophotometer. Chemical ionization mass spectra were obtained with an A.E.I. MS-9 high resolution mass spectrometer equipped with a chemical ionization ion source. Isobutane was used as the reagent gas.

RESULTS AND DISCUSSION

Π-A isotherms of DL-α-tocopherol on acidic and alkaline subphases

The Π-A isotherm of DL-α-tocopherol spread on an acidic subphase (pH 1.2) is shown in **Fig. 2**. This Π-A isotherm is very similar to that described by Weitzel, Fretzdorff and Heller (7) for the same conditions.

An expanded Π-A isotherm was obtained when DL-α-tocopherol was first spread as a gaseous film (Π approaches 0) and then compressed after 1 hr. The expanded Π-A isotherm is very similar to that

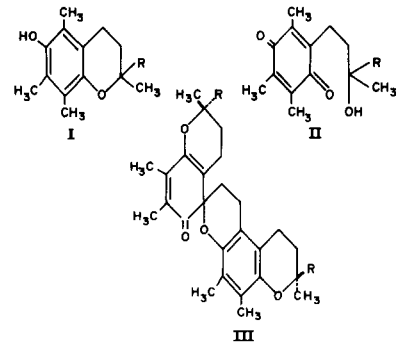

Fig. 1. Structural formulas of α-tocopherol and its metabolic oxidation products in liver: I, α-tocopherol; II, α-tocopherylquinone; III, spirodienone ether.

of authentic D-α-tocopherylquinone (Fig. 2). TLC analysis of film contents at 15 min, 30 min, and 1 hr showed the gradual disappearance of α-tocopherol and the gradual appearance of tocopherylquinone during the 1 hr period. (When oxidation was continued for 16 hr, other more polar products which remained near the origin were found on the TLC plate, indicating further degradation of α-tocopherylquinone.) It is apparent from these data that α-tocopherol in a gaseous film spread on an acidic subphase is oxidized to tocopherylquinone. This reaction was first suggested for β-tocopherol by Moss et al. (6) in an early monolayer study on the structure of vitamin E. The experimental data are consistent with the observation that the α-tocopherol oxidation product rearranges to tocopherylquinone in acid media (5).

Gold chloride has been used for the oxidation of α-tocopherol to tocopherylquinone in the potentiometric determination of α-tocopherol (14). We used gold chloride to confirm the rapid interfacial oxidation of α-tocopherol to tocopherylquinone. α-Tocoph-

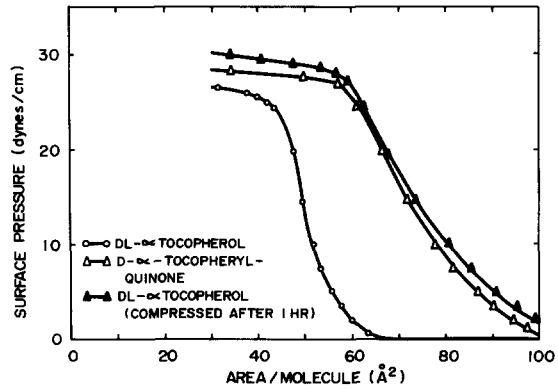

Fig. 2. Π-A isotherms at 22–24°C of DL-α-tocopherol and DL-α-tocopherylquinone spread on a subphase containing 0.1 M sodium chloride and 0.01 N hydrochloric acid. Compounds were spread as gaseous films and compressed immediately or after 1 hr. Films were compressed at 11 Å²/molecule per min (α-tocopherol) and 21 Å²/molecule per min (α-tocopherylquinone).

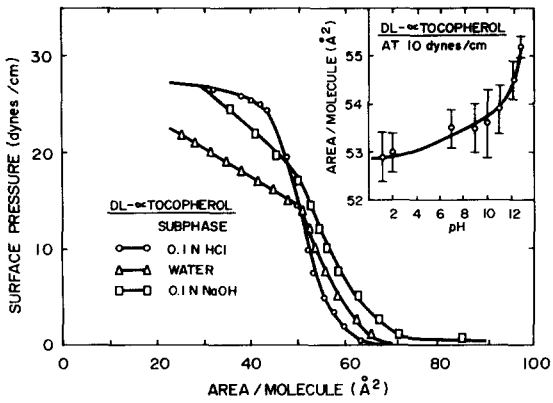

Fig. 3. Π-A isotherms at 22–24°C of DL-α-tocopherol spread on subphases containing 0.1 M sodium chloride alone (pH 7) and 0.1 M sodium chloride with 0.01 N hydrochloric acid (pH 1.2) or 0.1 N sodium hydroxide (pH 12.8). Compounds were spread as gaseous films and compressed immediately at 11 Å²/molecule per min. Insert: pH-A isotherm at 22–24°C (constant pressure-variable area data) for DL-α-tocopherol spread on subphases containing 0.1 M sodium chloride and 1 mM Tris adjusted to the specified pH with concentrated hydrochloric acid or sodium hydroxide. Vertical lines represent the standard deviation for six measurements.

erol was spread as a gaseous monolayer on 0.1 mM gold chloride and compressed immediately at 17 dynes/cm with castor oil. The surface area of this film, 66Å²/molecule, was consistent with the surface area of tocopherylquinone (Fig. 2). Furthermore, film contents were isolated and shown to have the same ultraviolet spectrum and R_f as authentic tocopherylquinone.

Oxidation may explain why several recent investigators (9, 10) have obtained expanded Π-A isotherms for α-tocopherol. For example, Fukuzawa, Hayashi, and Suzuki (10) waited 15 min before generating Π-A isotherms from tocopherol films. We found that "α-tocopherol" expanded over 8 Å²/molecule in this time interval. Studies with expanded films undoubtedly involve a mixture of tocopherol and tocopherylquinone.

When DL-α-tocopherol was spread on subphase at pH 7 and pH 12.8 and the Π-A isotherm was generated, the isotherm was expanded at lower surface pressures (**Fig. 3**). These isotherms may be explained either by oxidation (Fig. 2) or by ionization of the phenolic hydroxyl group. The pK_a of the phenolic hydroxyl group is between 9 and 11 (15). It is difficult to establish a specific oxidation or ionization effect with data from the conventional Π-A isotherm; however, the constant pressure–variable area technique allows the very rapid measurement of A at a specified Π and minimizes the effect of oxidation. Constant pressure–variable area data (see insert in Fig. 3) showed film expansion as a function of pH. These data strongly suggest that the expansion of the Π-A isotherm at lower surface pressures is caused by ionization when the α-tocopherol is spread on the alkaline subphase.

At higher surface pressures, Π-A isotherms of DL-α-tocopherol spread on subphases at pH 7 and pH 12.8 showed an inflection point followed by a decrease in slope (Fig. 3). These unusual characteristics are not explained either by oxidation to the tocopherylquinone or by ionization. Thus tocopherylquinone is expanded and has a higher collapse pressure than α-tocopherol (Fig. 2). Furthermore, the collapse pressure of an ionizable compound generally increases with increasing pH (16).

The inflection point in the Π-A isotherm suggests that a new film component with a lower collapse pressure is formed when α-tocopherol is spread on a neutral or alkaline subphase. The film was collected and analyzed to investigate this possibility. TLC analysis showed that the film actually contained α-tocopherol (R_f 0.65) and two minor components, α-tocopherylquinone (R_f 0.3) and a new component (R_f 0.9). The formation of several oxidation products on an alkaline subphase is consistent with other studies on α-tocopherol oxidation in alkaline media (5). Subsequent experiments showed that the R_f 0.9 component was the spirodienone ether, an oxidation product of α-tocopherol with surface properties that explained the anomalous inflection point.

Interfacial oxidation of α-tocopherol to the spirodienone ether

The surface area of DL-α-tocopherol was 52 Å²/molecule at 17 dynes/cm when a film was spread on a subphase consisting of 0.1 N sodium hydroxide and 0.1 M sodium chloride and was compressed immediately with castor oil. The surface film disappeared and an area of only 5.5 Å²/molecule was generated at 17 dynes/cm when 0.001 M potassium ferricyanide was added to the subphase. Alkaline potassium ferricyanide is an oxidant used to synthesize the spirodienone ether of α-tocopherol (3, 4).

TLC analysis of the surface components after α-tocopherol oxidation with alkaline potassium ferricyanide showed that α-tocopherol had disappeared while a major yellow product, R_f 0.9, and a minor purple product, R_f 0.3, had appeared at the interface. The R_f 0.9 product was eluted with ether, rechromatographed, and characterized.

The ultraviolet spectrum of the R_f 0.9 product in isooctane (maxima at 296 nm and 340 nm) was very similar to that of the spirodienone ether (3, 4). The solvent-free infrared spectrum of the R_f 0.9 product [absence of an OH band and presence of bands characteristic of carbonyl and conjugated double bonds

(5.98μ, 6.05μ, and 6.29μ)] was very similar to that of the spirodienone ether (3, 4). Chemical ionization mass spectra gave an intense peak at *m/e* 857 which was consistent with the protonated molecule ion of the spirodienone ether. Thus ultraviolet, infrared, and mass spectra identified the R_f 0.9 product as the spirodienone ether.

The minor R_f 0.3 product was not isolated in sufficient quantity for purification and characterization. α-Tocopherol is readily oxidized to a number of colored quinones (2), and the purple color and R_f suggested that the minor product was a quinone derivative of α-tocopherol. Surface area data indicated that the minor product accounted for 10% or less of the original α-tocopherol.

Surface properties of the spirodienone ether

Interfacial oxidation experiments (see preceeding section) showed that α-tocopherol, spread on an alkaline potassium ferricyanide subphase, is rapidly converted to the spirodienone ether. The Π-A isotherm of α-tocopherol spread on this subphase will, therefore, reflect the surface properties of the spirodienone ether. The Π-A isotherm (**Fig. 4**) showed that the dimer has a much lower equilibrium spreading pressure (around 7.5 dynes/cm) than α-tocopherol (Fig. 2). The Π-A isotherm was influenced by the compression rate. A new component appeared in the film, generating a significant surface pressure when the compression rate was lowered from 19 Å²/molecule per min to 2.7 Å²/molecule per min (Fig. 4). This new component is most readily explained by decomposition of the dimer to a surface-active component.

The surface properties of the alkaline potassium ferricyanide oxidation product (spirodienone ether) help to explain the anomalous Π-A isotherms of α-tocopherol spread on neutral and alkaline subphases (Fig. 3). TLC showed that these films contained both the spirodienone ether (R_f 0.9) and a tocopheryl-quinone (R_f 0.3). The inflection points in these films may be explained by film loss through dimer formation. The differences in the postinflection slopes of the Π-A curves on neutral and alkaline subphases may be explained by the complex relationship between ionization, dimerization, and dimer decomposition.

Dimerization in mixed lipid films

Monolayers that contain two components may form miscible or immiscible films (17, 18). When both components are good surfactants (high equilibrium spreading pressures), miscibility can be established by the additivity rule for surface area. When the two components have large differences in their equilibrium spreading pressures, miscibility can be established unequivocally by the application of a two-dimensional phase rule. We have used stearyl and oleyl alcohols to investigate the effect of miscibility on surface oxidation and dimer formation.

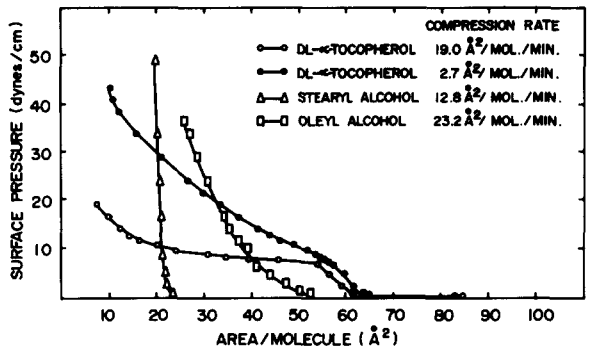

Fig. 4. Π-A isotherms at 22–24°C of DL-α-tocopherol, stearyl alcohol, and oleyl alcohol spread on a subphase containing 0.1 N sodium hydroxide, 0.1 M sodium chloride, and 0.001 M potassium ferricyanide.

Fatty alcohols and α-tocopherol have high equilibrium spreading pressures. When these film components are immiscible, the average surface area of a mixture, $A_{1,2}$, generally obeys the additivity rule (17, 18):

$$A_{1,2} = N_1A_1 + N_2A_2 \qquad \text{Eq. 1}$$

where N_1 and N_2 are the mole fractions of the components and A_1 and A_2 are the surface areas of each component in pure films maintained at the specified Π. When the components are miscible, the average surface area deviates from the surface area calculated by Eq. 1.

Maggio, Diplock, and Lucy (9) found that α-tocopherol formed immiscible films with a disaturated choline phosphoglyceride and miscible films with a diunsaturated choline phosphoglyceride. We found (**Fig. 5**), in agreement with these observations, that α-tocopherol spread on a sodium chloride–sodium hydroxide subphase formed a miscible film with oleyl alcohol (experimental $A_{1,2}$ deviated from calculated $A_{1,2}$) and an apparently immiscible film with stearyl alcohol (experimental $A_{1,2}$ coincided with calculated $A_{1,2}$).

Gaines, Bellamy, and Tweet (19) have shown that additivity is necessary but is not sufficient as the only criterion of immiscibility. A true two-dimensional solution that behaves ideally will also obey the additivity rule. Phase rule predicts (17, 18) that the collapse pressure (inflection point) of a binary mixture will be independent of composition when the film components are immiscible. On the other hand, phase rule predicts (17, 18) that the collapse pressure is

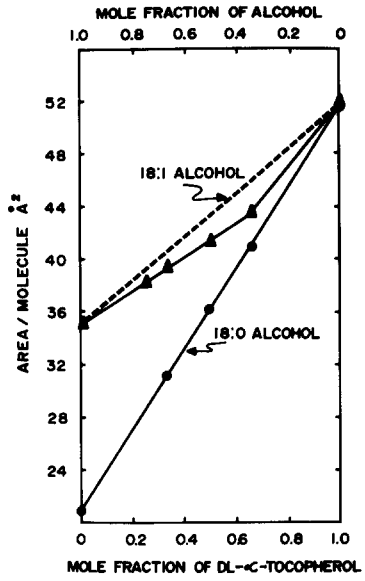

Fig. 5. Surface area (average area/molecule) for DL-α-tocopherol–fatty alcohol mixtures spread on subphases containing 0.1 N sodium hydroxide and 0.1 M sodium chloride. Films were compressed immediately at 17 dynes/cm. Experimental data for the oleyl alcohol mixtures (– ▲ – ▲ –) deviated from calculated values (– – –) obtained by Eq. 1. Experimental data and calculated values coincided for the stearyl alcohol mixtures (– ● – ● –).

dependent on composition, generating an envelope curve when the film components are miscible. We have used these phase rule relationships both to confirm the immiscibility of α-tocopherol–stearyl alcohol mixtures and to study the solubilities of fatty alcohol–spirodienone ether mixtures.

Binary mixtures containing α-tocopherol and stearyl alcohol were spread on an acidic subphase. The Π-A isotherms (**Fig. 6**) had an inflection point near the collapse point of the α-tocopherol monolayer (Fig. 2). The inflection point was independent of film composition (dotted line in Fig. 6), confirming

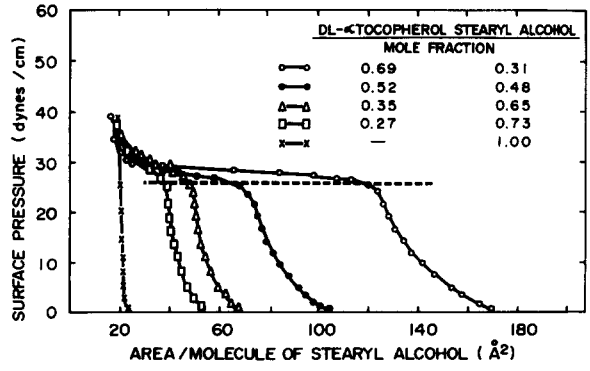

Fig. 6. Π-A isotherms at 22–24°C of DL-α-tocopherol–stearyl alcohol mixtures spread on a subphase containing 0.01 N hydrochloric acid and 0.1 M sodium chloride. Films were compressed at 7 Å²/molecule per min. Collapse pressures for different mixtures are connected by a dotted line.

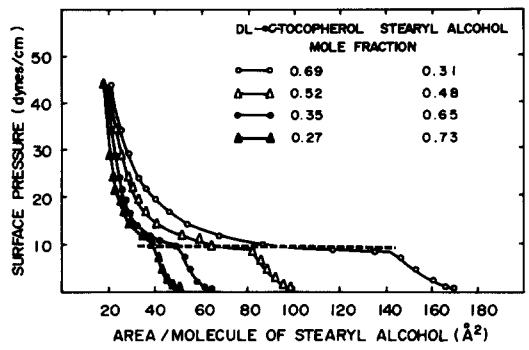

Fig. 7. Π-A isotherms at 22–24°C of DL-α-tocopherol–stearyl alcohol mixtures spread on a subphase containing 0.1 N sodium hydroxide, 0.1 M sodium chloride, and 0.001 M potassium ferricyanide. Films were compressed at 23 Å²/molecule per min. Collapse pressures for the different mixtures are connected by a dotted line.

the additivity data (Fig. 5) which showed that α-tocopherol and stearyl alcohol were immiscible.

The preceding experiments showed that the α-tocopherol–stearyl alcohol mixture served as a model for α-tocopherol in an immiscible film while the α-tocopherol–oleyl alcohol mixture served as a model for α-tocopherol in a miscible film. These mixtures were used in subsequent studies of α-tocopherol dimerization in mixed films at the air–water interface.

Binary mixtures containing α-tocopherol and stearyl alcohol were spread on an alkaline potassium ferricyanide subphase. The Π-A isotherms (**Fig. 7**) had an inflection point near that of the spirodienone ether (Fig. 4). The inflection point was independent of film composition (dotted line in Fig. 7), indicating that the film components were immiscible. It was a function of film composition when binary mixtures containing α-tocopherol and oleyl alcohol were spread on an alkaline potassium ferricyanide subphase (**Fig.**

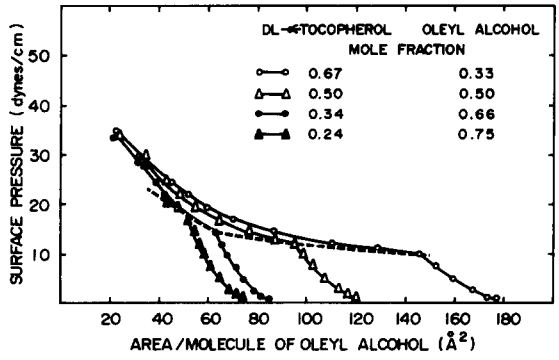

Fig. 8. Π-A isotherms at 22–24°C of DL-α-tocopherol–oleyl alcohol mixtures spread on a subphase containing 0.1 N sodium hydroxide, 0.1 M sodium chloride, and 0.001 M potassium ferricyanide. Films were compressed at 23 Å²/molecule per min. Collapse pressures for the different mixtures are connected by a dotted line.

8). These data showed that the film components were miscible. Thus stearyl alcohol formed immiscible films with both the reactant and the product while oleyl alcohol formed miscible films with both. It should be noted that Π-A isotherms generated on alkaline potassium ferricyanide with mixed lipid films (Figs. 7 and 8) showed larger than expected surface areas (17, 18) at pressures greater than the collapse pressures of the mixtures. Since the alkaline potassium ferricyanide subphase had no effect on the Π-A isotherms of the fatty alcohols (Fig. 4), these increased surface areas are explained by the presence in the film of minor surface-active components formed either initially or through dimer decomposition.

The mixed-lipid film experiments show that the oxidation of α-tocopherol to the spirodienone ether is unaffected by the presence of either miscible or immiscible film components. Furthermore, the spirodienone ether is partially soluble in an alkene environment and small amounts will remain in an unsaturated lipid film (membrane) unless the interfacial tension in the film is very high.

Biological significance of α-tocopherol oxidation at the air–water interface

The film balance experiments described in this study show that both tocopherylquinone and the spirodienone ether may be formed by the oxidation of α-tocopherol at the air–water interface. Tocopherylquinone is formed when oxidation occurs on an acidic subphase and the spirodienone ether is formed when oxidation occurs on an alkaline subphase. These data are consistent with the suggestion (5) that both oxidation products are formed from the phenoxonium ion either through an acid-catalyzed rearrangement to tocopherylquinone or through a base-catalyzed deprotonation to the spirodienone ether.

It is difficult to explain the formation of both tocopherylquinone and the spirodienone ether by the same oxidant stress. We suggest that the natural occurrence of both derivatives (1, 2) indicates that two types of oxidant stress may be found in biological systems. One type of oxidant stress involves a peroxy radical and the formation of tocopherylquinone; a second type involves a hydroxyl radical (·OH) and the formation of the spirodienone ether.

In 1961, Knapp and Tappel (20) showed that tocopherylquinone was formed when α-tocopherol was oxidized in the presence of peroxidizing linoleic acid. Shimasaki and Privett (21) recently isolated tocopherylquinone from erythrocytes incubated with fatty hydroperoxides. The fatty hydroperoxide, a classic example of the peroxy radical (22, 23), evidently oxidizes α-tocopheryl to tocopherylquinone.

Knapp and Tappel (20) noted that a different product was formed when α-tocopherol was irradiated, and Green and McHale (2) later suggested that this product was a spirodienone ether. Hydrogen peroxide and superoxide (O_2^-) are formed during irradiation and they may react to generate both the hydroxyl radical and the hydroxide ion (23, 24):

$$H_2O_2 + O_2^- \rightarrow O_2 + \cdot OH + OH^- \qquad \text{Eq. 2}$$

The hydroxyl radical and hydroxide ion formed in this reaction (Eq. 2) may be involved in the oxidation–deprotonation of α-tocopherol to the spirodienone ether. α-Tocopherol protects the erythrocyte from both oxidative hemolysis and lipid peroxidation (25). Yet these reactions can be separated and there is some evidence that oxidative hemolysis involves ·OH while lipid peroxidation does not (25). These observations suggest that several types of oxidant stress are indeed found in biological systems.

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