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THE EFFECTS OF FREE FATTY ACIDS AND TOCOPHEROL ON MEMBRANE ORDER AND ANTIOXIDANT ACTIVITY

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THE EFFECTS OF FREE FATTY ACIDS AND α-TOCOPHEROL ON MEMBRANE ORDER AND ANTIOXIDANT ACTIVITY

A Thesis

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ABSTRACT

Alpha-tocopherol is a lipid soluble Vitamin E species. Vitamin E protects membranes from oxidative damage that can be the result of lipid peroxidation, which is a free radical process that oxidizes membranes and eventually leads to cell destruction. The present paper studies the lipid peroxidation mechanism and its inhibition by Vitamin E.

One of the most common phospholipid components of biological membranes is phosphatidylcholine (PC). PC is an amphipathic molecule which forms a bilayer sheet in preference to micellar structures. When these two-dimensional lipid bilayers fold back on themselves, they form a hollow sphere enclosing an aqueous cavity called a liposome that can be viewed as a simple model for a cell membrane. A method for the purification of soy PC from lecithin tablets via flash column chromatography is utilized to produce soy PC liposomes by extrusion.

In this paper, α-tocopherol is incorporated into soy-PC liposomes, and its physicochemical effects on the liposomes are studied. Using stable free radicals called spin labels and electron spin resonance (ESR) spectroscopy, an order parameter of the membrane is measured. The order parameter measurements demonstrate that incorporation of α-TOH modifies acyl chain ordering and motion within the membrane. This paper measures the effect of α-tocopherol and of free fatty acids on membrane order and relates these to the antioxidant activity of Vitamin E.

LIST OF GRAPHS AND FIGURES

Introduction

I. Vitamin E and Its Effects on the Mechanism of Lipid Peroxidation

Free radicals are species which possess an odd number of electrons and tend to react in a way that will pair their unpaired electrons. They can oxidize membrane lipids to form lipid peroxides. This process is known as lipid peroxidation and eventually leads to cell destruction. Lipid peroxidation has been implicated in the pathogenesis of many disease states, including cancer, atherosclerosis, tissue damage caused by trauma resulting from toxins, myocardial infarction, drugs, surgery, traumatic head injury, damage resulting from air pollution, or the natural aging process (Haliwell *et al.*, 1992). It involves the destruction of polyunsaturated lipids by the following chain mechanism:

Initiation	$RH + \ \bullet OH \text{ -----> } R\bullet + H_2O$
Propagation	$R\bullet + O_2 \text{ -----> } ROO\bullet$
	$ROO\bullet + RH \text{ -----> } ROOH + R\bullet$
Termination	$R\bullet + R\bullet \text{ -----> } RR$
	$R\bullet + ROO\bullet \text{ -----> } ROOR$
	$ROO\bullet + ROO\bullet \text{ -----> } ROOR + O_2$

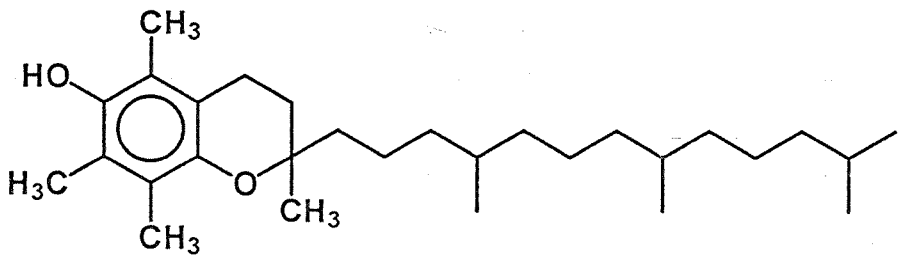
Lipid peroxidation can be initiated by the attack of a hydroxyl radical, as shown above, in a reaction with a high energy of activation. The most reactive and harmful radical in nature is the hydroxyl radical. Transition

metals such as copper (I), cobalt (II), or nickel (II) may react with hydrogen peroxide to yield the hydroxyl radical according to the following formula:

$$M^{(n+1)} + H_2O_2 \text{ ------> } M^{(n+1)} + \ \bullet OH + OH^-$$

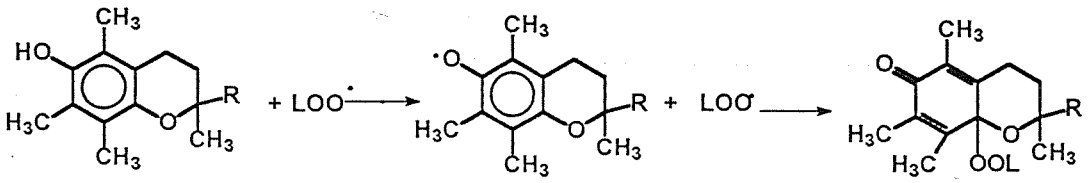
In the first of two chain propagating steps, the radical produced in the initiation step reacts with oxygen to form peroxyl radicals. In the second propagating step, these oxygen containing radicals can abstract a hydrogen from another molecule to form a hydroperoxide and another radical. These peroxyl radicals keep reacting in this cyclical fashion to self-generate. It is during this step that cell damage occurs. The termination step occurs infrequently, but often enough to use up one or both of the reactive intermediates. Membrane lipids that have been peroxidized assume an abnormal configuration to signal for their repair. This abnormal configuration, however, alters the membrane to a degree that causes some cell constituents to leak out of the cell, thus, lysing the cell (Haliwell, 1992).

Vitamin E is the collective name for a group of closely related lipid-soluble species called tocopherols. Tocopherols contain a substituted aromatic ring and a long hydrocarbon side chain:



Vitamin E is one of the most effective peroxyl radical-trapping antioxidants found in the lipid fractions of living organisms (Burton *et al.*, 1983). It is known to protect the lipid bilayer of biological membranes from autoxidation, a process in which organic compounds react in a radical chain mechanism with atmospheric oxygen.

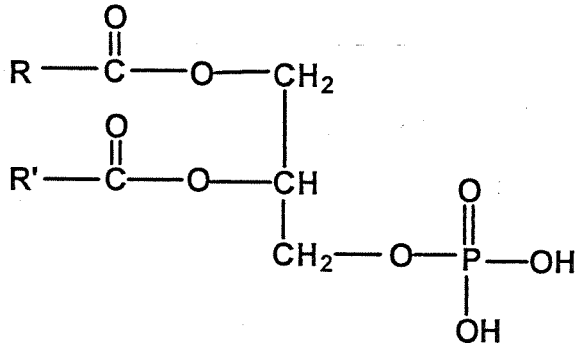
Effects of antioxidants such as α-tocopherol, butylated hydroxytoluene (BHT), and other radical scavengers on lipid peroxide formation have been demonstrated and interest in their use as preventive and curative agents has increased (Chan *et al.*, 1982; Shklar *et al.*, 1982; Perchellet *et al.*, 1985; Saunders *et al.*, 1987). Alpha-tocopherol plays a key role in interrupting the free radical chain mechanism of lipid peroxidation, and thus, protecting unsaturated lipids from further oxidative damage (Burton & Ingold, 1986). α-tocopherol reacts with the peroxyl radicals produced in lipid peroxidation to form more stable tocopheroxyl radicals, thus preventing the destructive chain mechanism to progress further. This reaction is preferred because the tocopheroxyl radical is only mildly reactive and leads to non-radical products, as shown below:



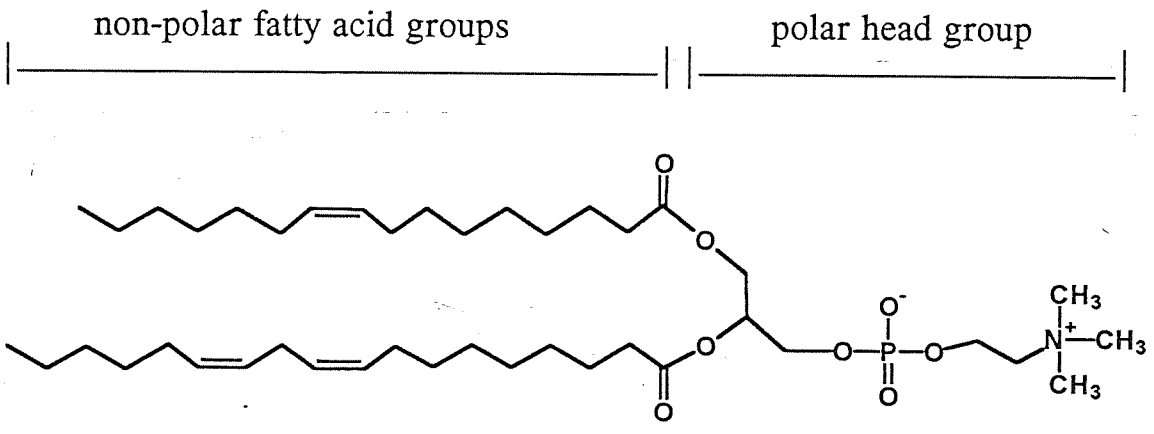
alpha-tocopherol

II. Phospholipid Membranes and Vitamin E

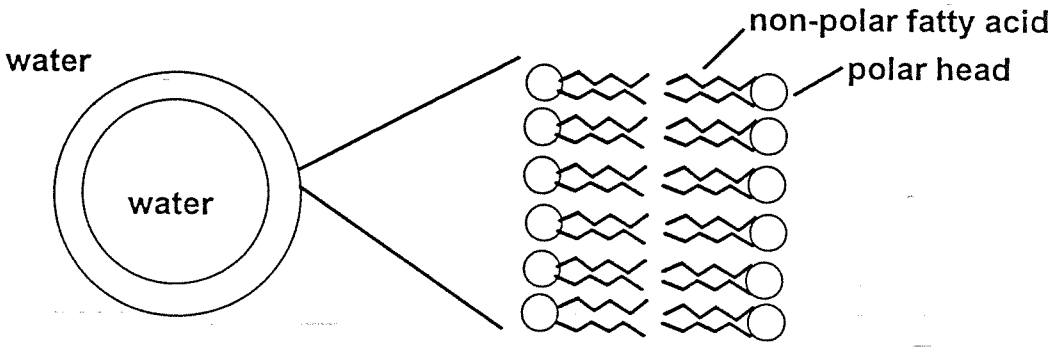
Phosphatidylcholine (PC) belongs to a large class of lipids called phospholipids. Most of them are structurally derived from a diacylglyceryl phosphate (Solomons, 1069):



In the case of PC, the phosphate group is bound through another ester linkage to ethanolamine. The nitrogen of ethanolamine is bound to three methyl groups, giving the nitrogen a positive charge:



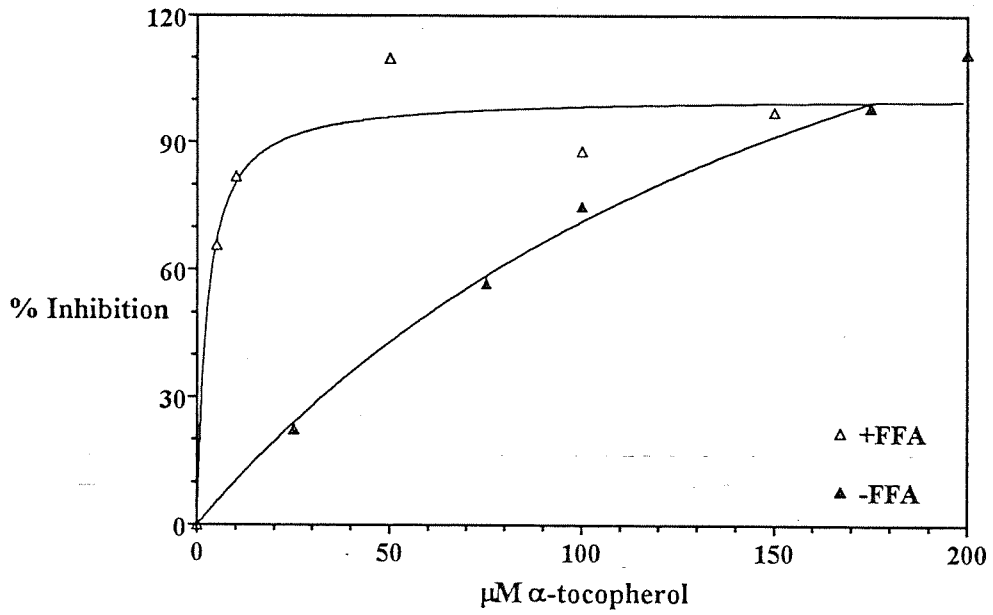
Phosphatides have both a polar head group region, and a nonpolar fatty acid portion. The choline is the "head group", and the long chain hydrocarbon is the "tail". At physiological pH, the PC molecule is a zwitterion, or a molecule with both a positive and a negative charge. The nitrogen bears the positive charge and the phosphate group bears the negative charge. These charges confer surface properties that are characteristic to cell membranes. This amphipathic characteristic allows phosphatides to form bilayers in aqueous media, with the hydrophillic part facing the outside and the hydrophobic part facing away from the water:



When these two-dimensional lipid bilayers fold back on themselves, they form a hollow sphere enclosing an aqueous cavity called a liposome that can be viewed as a simple model for a cell membrane. PC is the most common phospholipid component of biological membranes, and thus, has become an interesting subject for study. In lipid peroxidation, this

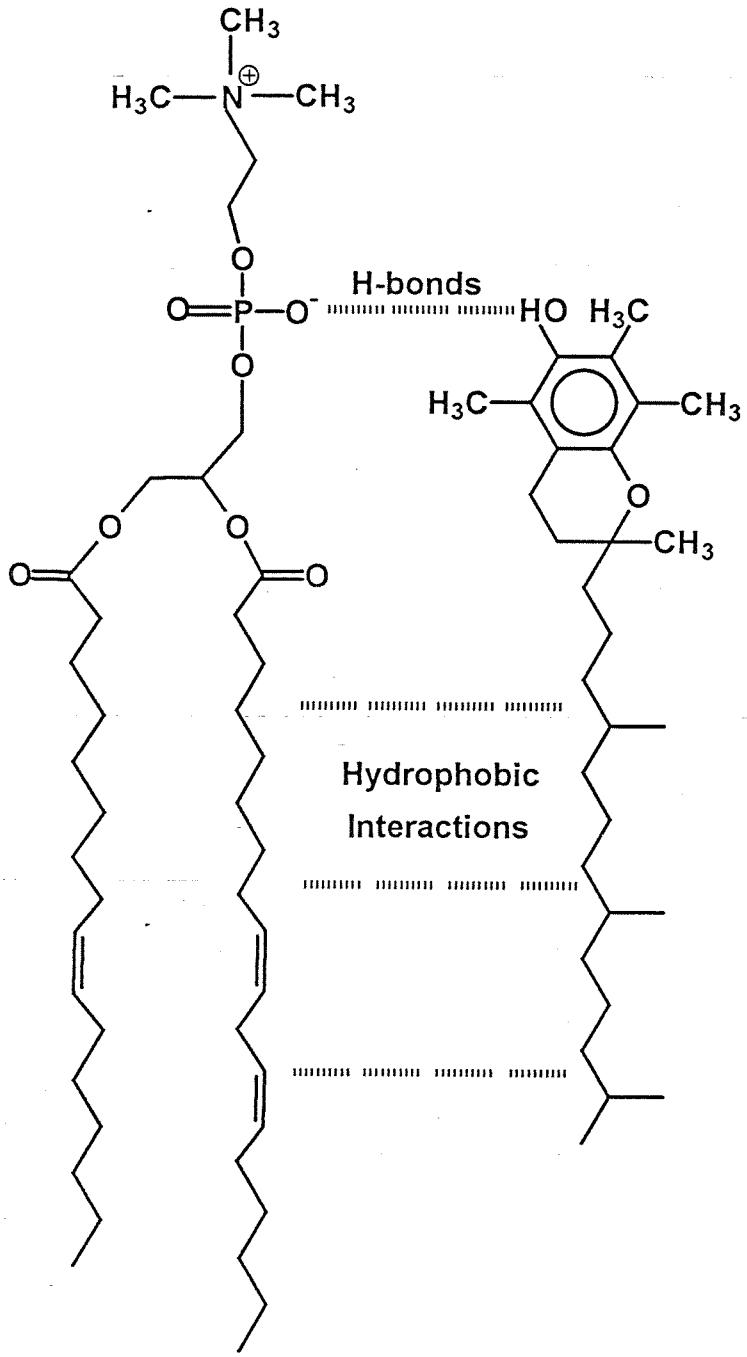
phosphatidylcholine bilayer is damaged, thereby releasing cellular components and toxic products from the affected cell.

Oxidative damage has been shown to release unesterified fatty acids from phospholipids *in vivo* (Lunt *et al.*, 1968). As tissues become damaged, they are oxidized and fatty acids such as arachidonic acid or oleic acid are released. These free fatty acids (FFA) react with the lipid membrane in a way that is believed to increase the antioxidant efficiency of α-tocopherol (Dugas, T.):



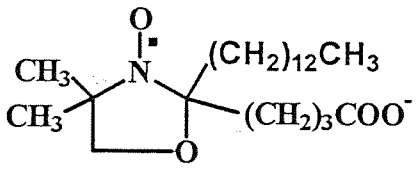
The systems included the initiator ABAP, soy PC, and α-TOH. The graph shows that oxidating PC liposomes containing the FFA oleic acid undergo a higher inhibition percentage than those without FFA. It can be said that without FFA, α-TOH is too unreactive to be an effective inhibitor, and that FFA make α-TOH a more effective inhibitor.

Studies have shown that α-tocopherol modifies the properties of phospholipid model membranes in several ways. The α-tocopherol molecule loses its motional freedom when it becomes strongly bound to the lipid bilayers, both by hydrophobic and hydrogen-bonding type interactions, which results in stabilization of the membrane, as shown below:



This model for inclusion of α-tocopherol in the lipid bilayer suggests a mechanism to explain its antioxidant properties (Srivastava *et al.*, 1983). The rigidity and decreased permeability given to the membrane when α-tocopherol is added would limit the movement of molecules within the bilayer, thus, confining the radical to the membrane. This entrapment of the radical would prevent it from reacting any further in the chain mechanism of lipid peroxidation. Incorporation of α-tocopherol influences chain order and membrane phase behavior in multilamellar dispersions of phospholipids (Wassall *et al.*, 1986). Permeability and fluidity of membranes is also affected by the addition of α-tocopherol (Urano *et al.*, 1988).

When α-tocopherol is added to liposomes, its physicochemical effects on the liposomes can be studied. By examining the motion of stable free radicals called spin labels incorporated into model membranes, an order parameter can be measured using electron spin resonance spectroscopy. 5-Doxyl stearic acid is used as the spin label and also as the FFA in these studies and has the following structure:



This molecule, inserted into the bilayer, is able to monitor the region near the polar interface of the membrane. This work measures the effect of Vitamin E and of free fatty acids on membrane order and relates these to the antioxidant activity of Vitamin E.

Experimental

Materials

Lecithin tablets (Spring Valley brand), used as a crude source of L-α - phosphatidylcholine (PC), were purchased from a local pharmacy and are labeled to contain 19 grains (1.2 g) lecithin from soybeans; PC was purified from these tablets as described below. 5-Doxyl stearic acid, (±)-α - tocopherol, and butylated hydroxytoluene were purchased from Sigma Chemical Company, U.S.A. These and any other incidental chemicals and solvents were of high grade and were used as received.

Methods

I. TBA Assay: Measurement of oxidation of soy PC/TOH models.

Oxidation of membrane models is measured via the thiobarbituric (TBA) assay. This method tests for the production of one of the side products of the oxidation process, malonaldehyde. Malonaldehyde appears to be produced in relatively constant proportion to lipid peroxidation (Greenwald, p. 204). It is therefore a good indicator of the rate of lipid peroxidation. Measurements are made of the absorbance at 532 nm upon reaction of a lipid sample with TBA. This forms a malonaldehyde-TBA

adduct which gives a red color. This color can be correlated with the degree of oxidation. For comparison, one set of samples contained soy PC and Vit E and a second set contained soy PC only. Three milliliters of liposomes were extruded and stored in phosphate buffer (pH 7.4). Glycine was added to the buffer in a final 10 mM concentration in order to complex with the malonaldehyde and give it more weight, therefore making it less volatile and making the measurements more accurate. To initiate oxidation, 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) was added in a 20 mM final concentration. Samples were heated in a 37° water bath with a magnetic stirrer to keep the liposomes suspended in the solution. Three 50 μL portions were taken every ten minutes over a continuous three hour period. 2% of the antioxidant butylated hydroxytoluene (BHT) was added to each portion immediately after being taken out of the water bath. This stops any further oxidation from taking place. One ml of the TBA reagent was added to each sample and heat was added to enhance the appearance of the red color. Each sample is centrifuged at 14,000 rpm for five minutes and the supernatant's absorbance is read at a 532 nm wavelength on a Beckman Model 24 Spectrometer. The TBA reagent contains 0.375% TBA, 15% TCA (trichloroacetic acid) and 0.25 M HCl.

II. Purification of soy phosphatidylcholine from lecithin tablets.

Flash column chromatography is used to extract soy phosphatidylcholine (PC) from lecithin tablets. The column used is 46 cm long and 2.0 cm in diameter. Silica gel, the stationary phase, is poured into the column such that it fills the column 41 to 42 cm. The mobile phase is a solution containing the insides of two lecithin tablets dissolved in 30 ml of chloroform. Methanol and ethyl acetate are used as solvents in a 2:1 ratio. The lecithin tablet/chloroform solution is added to the silica gel column preparation. The first solvent serves to elute less polar compounds, and consists of an 800 ml solution containing 50% ethyl acetate-50% methanol. Nitrogen gas is used to add pressure to the column and make the solvent go through at a faster pace. It is important not to let the silica gel go dry during this process. When the ethyl acetate/methanol solution has almost completely eluted, 350 ml of methanol is added to elute the soy PC. This fraction is collected and evaporated in a Rotor-Vap to a thin film on the flask. The material is dissolved in chloroform to obtain a 20 mg/ml concentration of soy PC. NMR analysis of the substance is performed to evaluate its purity.

III. Extrusion of soy PC liposomes and ESR analysis.

Two sets of thirteen 0.5 ml portions of the purified 20 mg/ml soy PC are taken into separate test tubes. Varying concentrations (ranging from 30 μM to 2000 μM) of 5-doxyl stearic acid are added to each set of solutions. The vitamin E source, a 100 μM concentration of α-tocopherol dissolved in chloroform, is added to one set of samples. The second set is a control and only contains 20 mg/ml PC and 5-doxyl without tocopherol. The solutions in the chloroform solvent are evaporated to a dry film using a Rotor-Vap. Residual solvent is removed by pumping under a vacuum for 2 hours. Appropriate amounts of 50 mM phosphate buffer, pH 7.4, are added to each lipid film.

Each sample is passed through a polycarbonate filter of pore diameter 100 nm using an Avestin liposome extrusion device. This is done approximately fifteen times or until the solution is clear. This process forms the PC liposomes as unilamellar vesicles. Each sample is analyzed by electron spin resonance (ESR) spectroscopy. An ESR from Micro-Now Instrument Company (Spectrometer 8300A Mainframe) is employed. Spectral parameters are set as follows: modulation amplitude 2 G, field center 3410 G, field width 100 G, scan time 1000 s, and time constant 3 ms. Once a spectrum is obtained (see Figure 1), it is analyzed by relating the

order parameter of the membrane to the 5-doxyl concentrations with 100 μM tocopherol and with no tocopherol. The order parameter (S) is calculated with the formula:

$$S = 0.565 \quad \frac{T_{||} - T_{\perp}}{1/3\,(2T_{\perp} + T_{||})}$$

The order parameter (S) is a measure of the amplitude of acyl chain segmental motion about the normal to the membrane surface and falls in the range $0 \leq S \leq 1$ (Wassall et al., 1991). S measures the fluidity of a system. At high S, order in the membrane increases, making the membrane more rigid, or stiff, limiting any movements within the membrane. At low S, order in the membrane decreases, making the membrane more fluid, or increasing the motional freedom of any membrane components.

Figure 1.

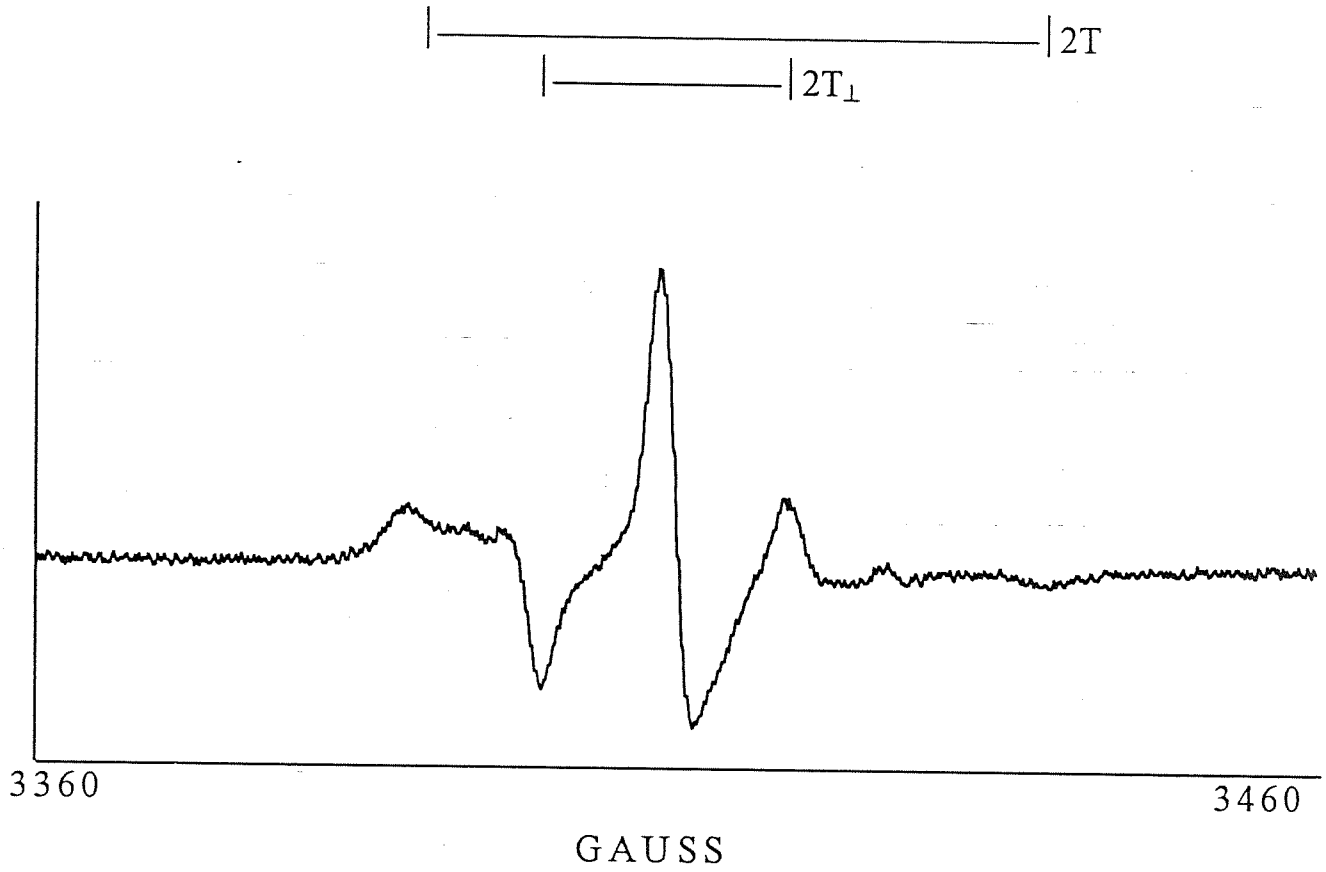


Figure 1. ESR spectrum containing 20 mg/ml soy PC liposomes, 100 μM α-TOH, and 200 μM 5-doxyl stearic acid. $2T_{||}$ and $2T_{\perp}$ are the outer and inner hyperfine splitting (Hubbell and McConnell, 1974), and are used in calculating the order parameter.

Results

I. Purification of soy PC from lecithin tablets.

Soy PC was obtained from lecithin tablets. To examine the purity of the extracted soy PC, an NMR spectrum of the purified substance is compared to a spectrum of soy PC obtained from Sigma Chemical Company. Figure 2 shows that with the exception of a few impurities (marked with an X), both spectra match well. The tall peak in (b) at 2.1 ppm is probably due to acetone present in the glassware used. The characteristic acetone peak is at 2.07 ppm (Silverstein, p. 237). The broad peak in (a) at 3.5 ppm is probably water. The peaks at 7.3 ppm on both (a) and (b) are most likely the residual solvent benzene left on the glassware used. Benzene has a characteristic peak at 7.2 ppm (Silverstein, p. 237). On (a) the peak at approximately 7.5 is probably chloroform. The characteristic peak of deuterated chloroform is 7.25 ppm (Silverstein, p. 237). It should also be taken into consideration that the two phospholipid sources contained different fatty acyl chains, and therefore, slight differences in their spectra should be observed, especially in their relative peak intensities. Overall, the two spectra match well, indicating that the purified soy PC is close in purity and composition to the soy PC obtained from Sigma.

Figure 2. (a) Purified soy PC in $CDCl_3$ and
(b) Soy PC in $CDCl_3$ from Sigma Chemical Company

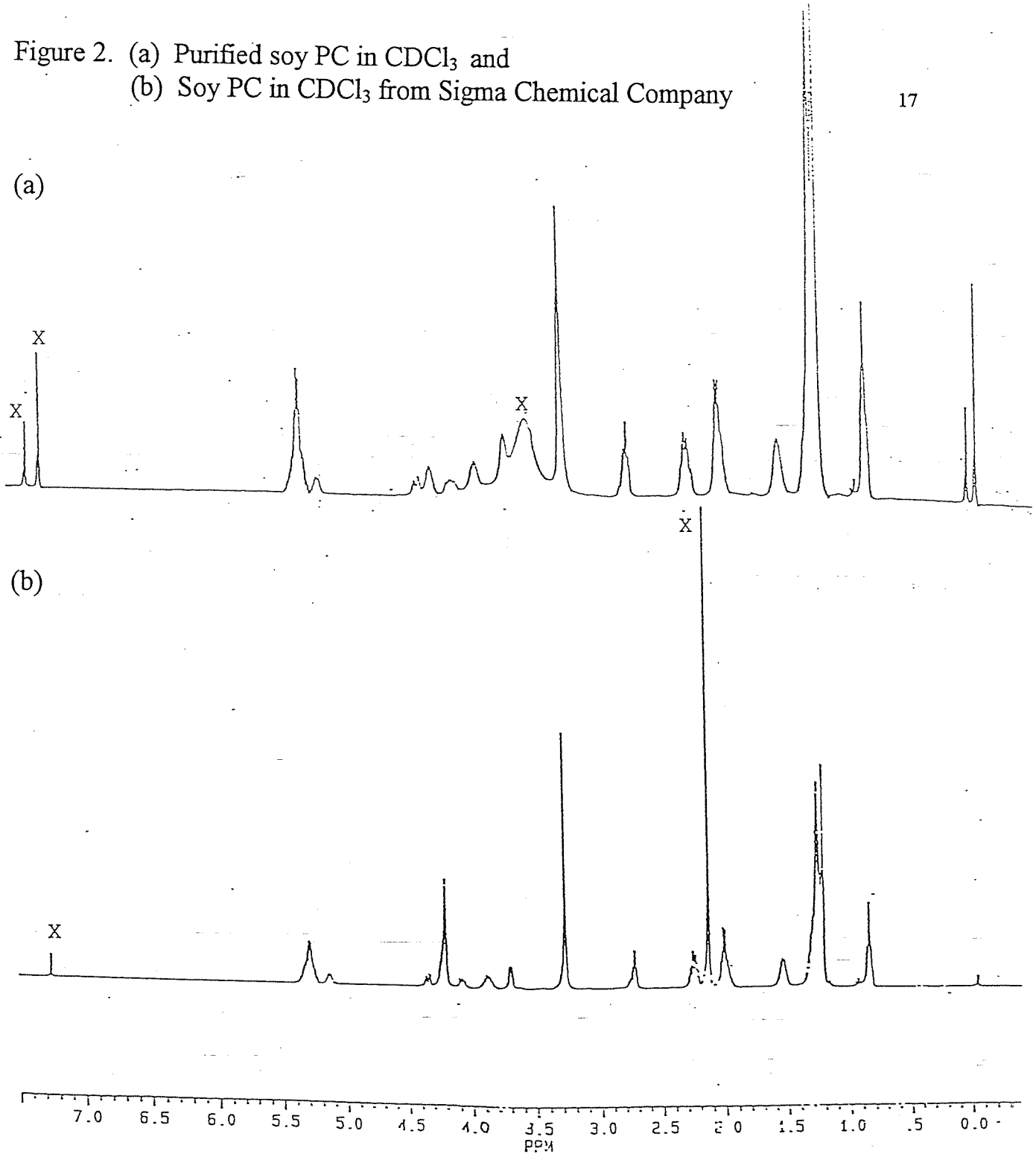


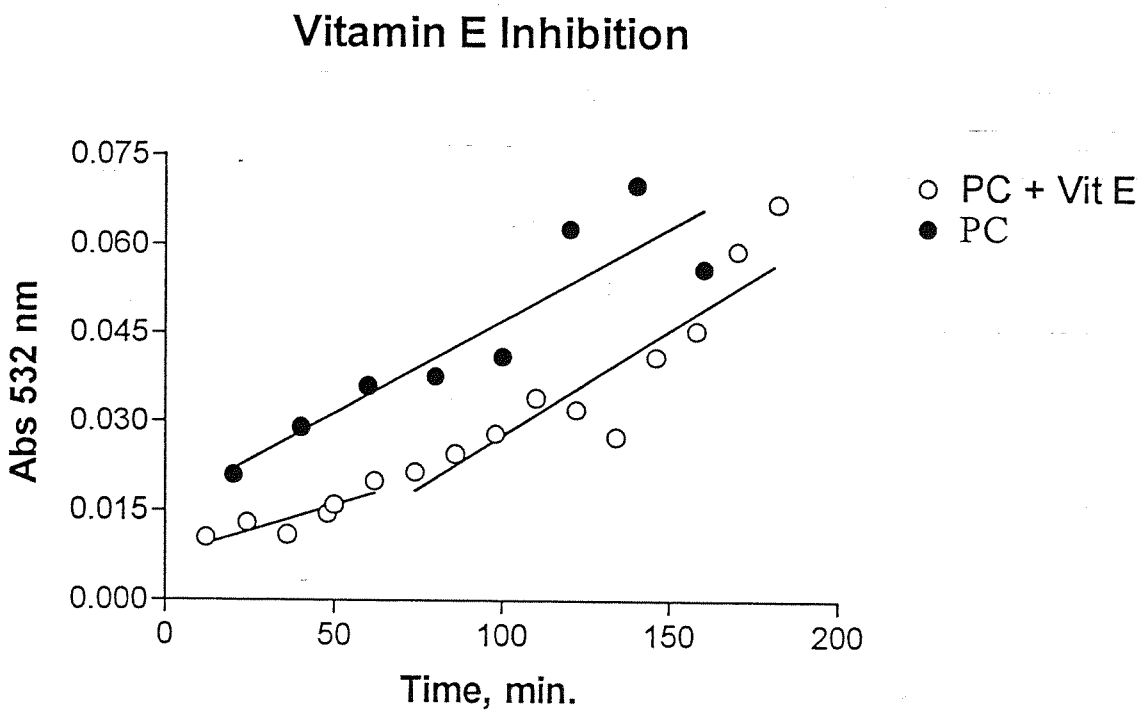
Figure 2. NMR comparison of purified and Sigma soy PC. Impurities such as water, acetone, benzene, and deuterated chloroform occur at 3.5 ppm on (a), 2.1 ppm on (b), 7.3 ppm on both (a) and (b), and 7.5 ppm on (a) respectively. Impurities are marked with an "X".

II. TBA Assay:

Two assays were performed, one with soy PC liposomes and one with soy PC liposomes containing Vit E. This assay measures the production of one of the side products of oxidation, malonaldehyde. Upon reaction of malonaldehyde with TBA, a pink color can be observed. As more oxidation takes place, more malonaldehyde is produced, and substances turn a more intense pink color, therefore having a higher absorbance value. A higher absorbance is significant of more lipid peroxidation taking place. The use of a magnetic stirrer and the glycine buffer allowed for more accurate measurements by keeping the liposomes suspended and decreasing the volatility of malonaldehyde, respectively. Graph 1 shows that the absorbance of the samples containing Vit E increases initially at a faster rate than those containing soy PC only. This shows that, in effect, Vit E inhibits the oxidation process initiated by ABAP in soy PC liposomes. However, after an induction period of approximately 70 minutes, during which Vit E is consumed, the rate of oxidation is the same within experimental error as the system without Vit E. The fact that Vit E is only partially as an effective inhibitor initially shows that there must be other factors contributing to the effectiveness of Vit E in inhibiting lipid peroxidation. Presumably, one of these contributing factors is the concentration of FFA, as shown by the graph

in the introduction which shows that Vit E/PC liposome systems containing FFA have a higher oxidation inhibition rate than systems without FFA.

Graph 1.



Graph 1. TBA assay: inhibition of oxidation by Vit E. • contains only 20 mg/ml soy PC liposomes being oxidized by 20 mM ABAP. ° contains 15 μM Vit E and 20 mg/ml soy PC liposomes being oxidized by ABAP, initially, at a lower rate.

Figure 3. TBA Assay: data analysis

	PC + Vit E		PC only
	0-74 min.	75-200 min.	0-200 min.
slope	0.00017 ±0.00005	0.00036 ±0.00007	0.00032 ±0.00004

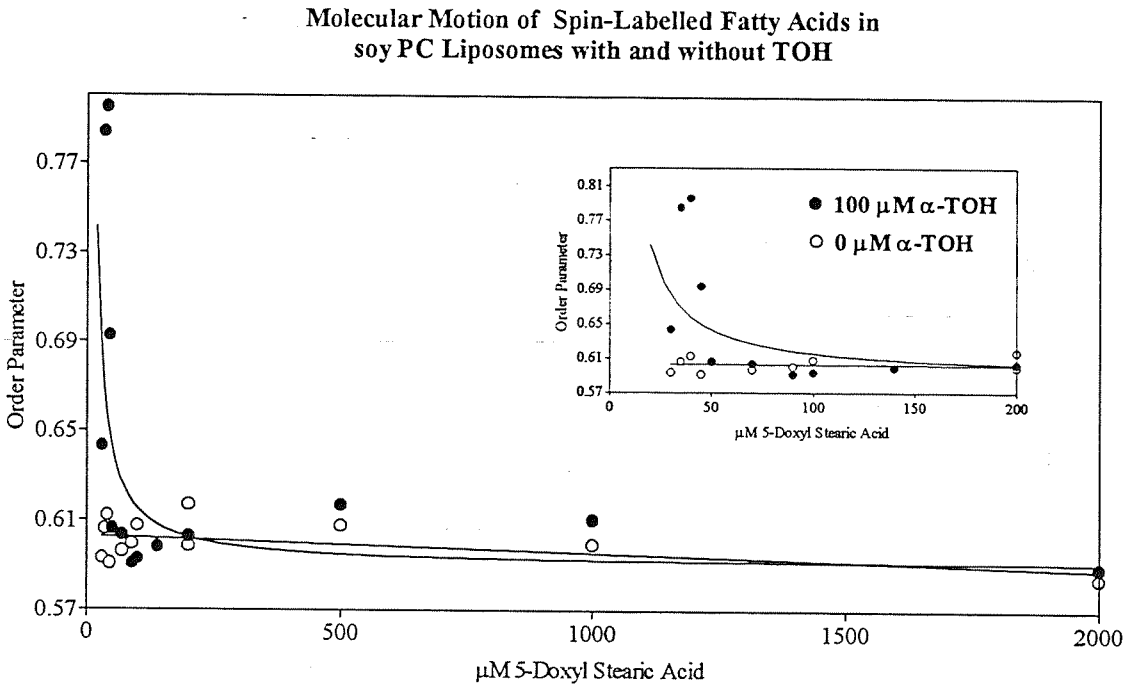
III. ESR order parameter measurements.

The molecular motion of 5-doxyl stearic acid (SA) is measured by ESR. The concentration of 5-doxyl SA is varied from 30 to 2000 μM to see if FFA have any effects on the order of PC liposomes with and without α-TOH. Graph 2 shows that order (S) is increased dramatically in the soy PC liposomes with α-TOH at low FFA concentrations compared to the system without α-TOH. At low FFA concentrations, the order of the membrane is increased in the system containing α-TOH, giving it more rigidity. As the concentration of the FFA increases, order decreases, indicating that the fluidity of the system is increased. When there is no α-TOH present, the order basically stays low at all FFA concentrations tested.

Figure 4. Order Parameters in Systems With and Without α-TOH-- Data Analysis

5-Doxyl Conc. (μM)	ORDER PARAMETER (S)					
	0 α-TOH			100 μM α-TOH		
	Average	Std. Dev.	No. Trials	Average	Std. Dev.	No. Trials
30	0.604	±0.049	3	0.643	±0.059	3
35	0.606	--	1	0.784	--	1
40	0.612	--	1	0.795	--	1
45	0.591	--	1	0.693	--	1
50	0.589	±0.037	4	0.606	±0.027	3
70	0.596	±0.012	3	0.635	±0.055	3
90	0.600	±0.012	3	0.604	±0.034	3
100	0.607	±0.011	3	0.596	±0.004	2
140	0.598	±0.004	3	0.599	±0.001	2
200	0.603	±0.010	4	0.603	±0.003	2
500	0.608	±0.006	3	0.618	±0.011	2
1000	0.599	±0.004	3	0.611	±0.006	2
2000	0.585	±0.012	3	0.590	±0.014	2

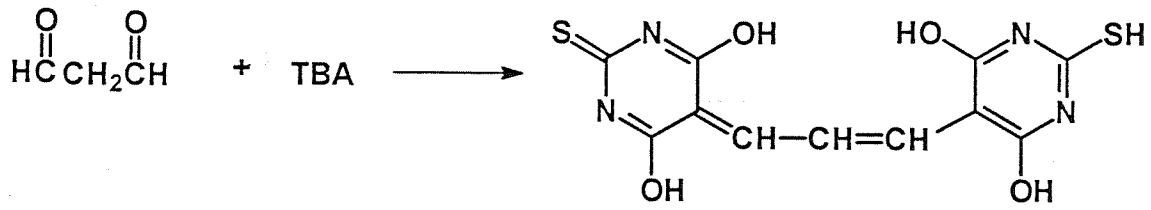
Graph 2.



Graph 2. ESR measurement of the molecular motion of 5-doxyl SA in systems containing 20 mg/ml soy PC, varying concentrations of the SA, with and without α-TOH. Inset shows a magnification of 0-200 µM 5-doxyl area of the graph. In this section, the order increases dramatically in the system containing 100 µM added α-TOH.

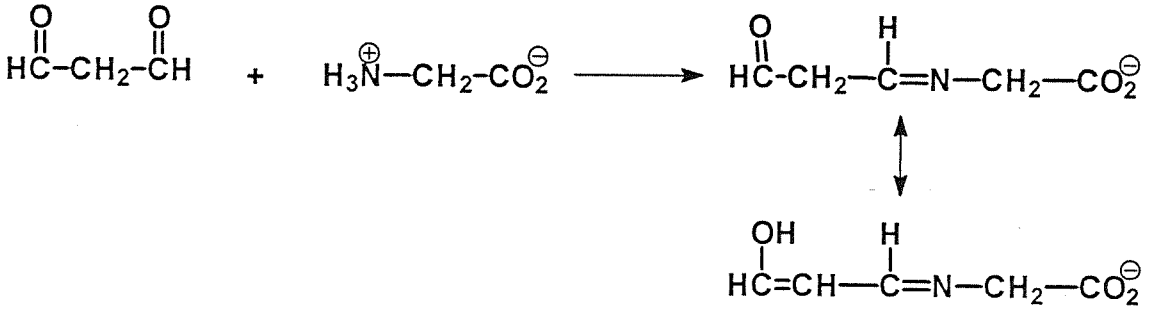
Discussion

From the data gathered, this study has shown several concepts. First, inhibition by Vitamin E is illustrated in unilamellar liposome models oxidized by the initiator ABAP. This inhibition is evident by the TBA assay. Reaction of TBA with the side product of oxidation, malonaldehyde, results in an adduct formation with the following structure (Bhatnagar, p.17):



malonaldehyde-TBA adduct

The absorbance of this adduct can be read at 532 nm. As more lipid peroxidation takes place, more of the side product malonaldehyde is produced to form more adduct. More adduct, or higher absorbance, is indicative of more oxidation taking place. A new modification of this assay is introduced in this experiment, in which glycine is added in order to decrease the volatility of malonaldehyde by forming an imine bond in the following way:



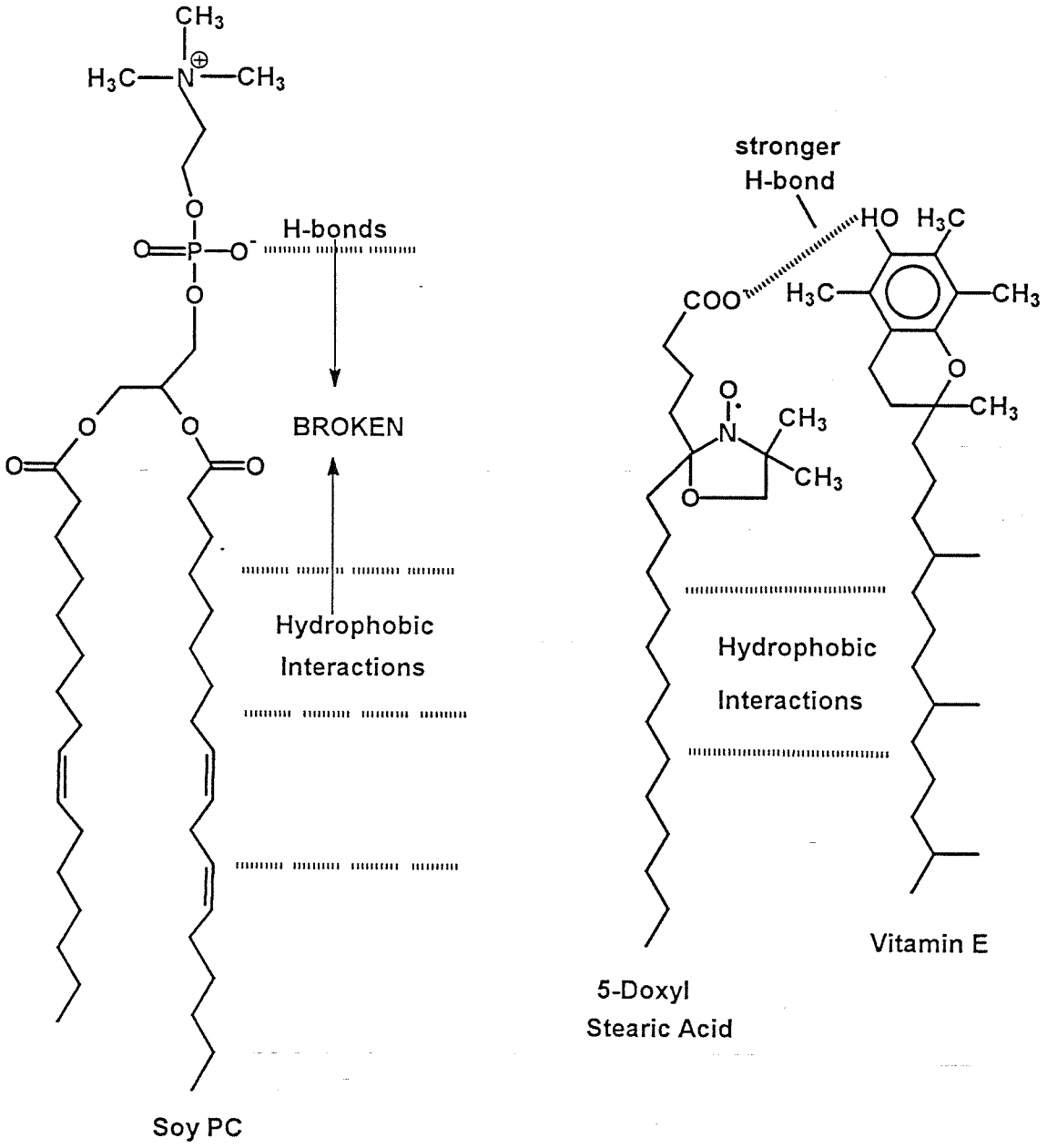
This larger structure confers upon malonaldehyde more weight, making it less susceptible to vaporization. Because more of the total resulting molecule is present in the solution, readings can be made more accurately. Results of the TBA assay demonstrate that absorbance increases initially at a faster rate in soy PC liposomes containing α-TOH than those without α-TOH. This lower absorbance rate is indicative of approximately 50% of the maximum rate of lipid peroxidation taking place in the systems with Vit E. Therefore, Vit E is shown to be only 50% effective in inhibiting oxidation. The lipid to Vit E ratio in this experiment of approximately 1000:1 is similar to the cells in nature. The lipid to Vit E ratio in the heart, liver, and brain and spinal cord is 750:1, 2000:1, and 700:1, respectively (Mitchell *et al.*, 1990). Having a lipid to Vit E ratio close to that in nature shows that this experiment demonstrates Vit E to be an effective inhibitor of lipid peroxidation in nature. However, Vit E is shown to have a higher inhibition rate only initially (0 to approximately 70 minutes). After an induction period in which the limited resource of Vit E is consumed, the rate of oxidation is the same within experimental error to the system containing no Vit E (see Figure 3 for slope comparisons). The effectiveness of Vit E, then, must be linked to other factors affecting the membranes. To try to explain Vitamin E's efficiency in inhibiting lipid peroxidation, the composition and reactions of the PC liposome models with Vitamin E and free fatty acids must be examined.

FFA are known to be released when oxidative damage takes place (Lunt *et al.*, 1968). Results of the present experiment show that without α-TOH, FFA have no effect on soy PC liposomes (see Graph 2). Figure 4 shows that in systems containing no α-TOH and varied FFA, the order of the membrane remains the same within experimental error. Oxidative damage continues to take place uninhibited in such systems. The graph presented in the introduction (Dugas, T.) implies that FFA can play a role in improving the activity of Vit E. Therefore, systems containing Vit E and varied FFA must be considered.

In systems containing Vit E and low fatty acid concentrations, the order of the membrane is shown to increase. By extrapolation of the line (Graph 2), we can conclude that without FFA, Vit E containing membranes are very rigid and highly ordered. When α-TOH is incorporated into the membrane, it is strongly bound to the PC and thus, loses its motional freedom. This means that the overall order in the membrane is increased, or it becomes more rigid. This rigidity comes from both hydrophobic and hydrophillic interaction between the Vit E and PC molecules, and is present in undamaged membranes. The strong interactions between Vit E and PC result in limiting the movement and lowering the activity of Vit E.

In damaged membranes, FFA are released. The present results using 5-doxyl stearic acid as a model for FFA show that when Vit E is present in

membranes, there is an overall decrease in order in the system containing α-TOH, or the membrane becomes more fluid. A possible explanation for Vitamin E's increased activity in the presence of free fatty acids is the creation of a less ordered phase, or a more fluid-like model of the membrane when α-TOH is present in model membranes containing FFA. 5-Doxyl SA bound to Vit E gives the Vit E more freedom to move around the membrane, and thus, enhancing the activity of Vit E. Lower order in the presence of FFA implies that FFA reduce the strong hydrophobic and hydrophillic interactions of Vit E and PC and thus, decrease the rigidity caused by Vit E. FFA disrupting the Vit E-PC bonds could happen in such a way that the FFA interact with the Vit E itself:



These findings supplement those of Severcan and Cannistraro (1990), which indicate a decrease in fluidity in the liquid crystalline phase and an increase in fluidity in the gel phase, when α-TOH is added to dipalmitoyl-L-α-PC.

Oxidative stress has been implicated in the pathogenesis of various diseases, and therefore, there has been an increasing interest in the development of antioxidants. The development of effective antioxidants relies on studies of membrane reorientational dynamics, such as the present study.

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