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MEMBRANE-STABILIZING EFFECT OF VITAMIN E: EFFECT OF α-TOCOPHEROL AND ITS MODEL COMPOUNDS ON FLUIDITY OF LECITHIN LIPOSOMES*

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Summary: The effects of vitamin E (α-tocopherol) and its model compounds on the fluidity of liposomes composed of dipalmitoylphosphatidylcholin (DPPC) and fatty acids were investigated by the measurement of the fluorescent polarization (P) using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a plobe. Although all tocopherols decreased the fluidity of liposomes which was perturbed by the inclusion of an unsaturated fatty acid having more than one double bond, α-tocopherol was more effective than the others. The fluidity in arachidonic acid-containing liposomes was decreased most in the presence of α-tocopherol and was decreased considerably by the inclusion of model compounds having a side chain at least one isoprene unit or a long straight chain instead of isoprenoid side chain. However the chromanol with methyl group instead of the above side chain, and phytol, having no chromanol moiety, had no effect. These results show that a structural requirement for a membrane stabilization is to be either the chromanol moiety with methyl groups born on its aromatic ring or a side chain of appropriate length; an isoprenoid side chain of full length or one containing 4'a- and 8'a-methyl groups is not necessarily needed.

Vitamin E, especially α-tocopherol, has generally been considered to act as a biological antioxidant in the lipid core of biomembranes (1). This biological property may result from the redox system of α-tocopherol. In contrast, previous experimental data show that certain properties of permiability and fluidity of membranes are related to the presence of α-tocopherol (2). Diplock et al. proposed that these properties may arise from a structural relationship between the isoprenoid side chain of α-tocopherol and the polyunsaturated fatty

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acid residues of the membrane phospholipids (3). Although the physicochemical interaction of of 4'a- and 8'a-methyl groups on the isoprenoid side chain with Z pockets of arachidoyl residues of membrane phospholipids (3) may offer a possible mechanism for vitamin E-controlled molecular mobility in the membranes, no evidence has been reported to show that this interaction exists in biomembranes. Based on this assumption, several attempts to demonstrate this interaction have been carried out by a variety of techniques, such as nuclear magnetic resonance (4), electron magnetic resonance (5), ultraviolet absorption (6), and mono- or bilayer studies (7). Positive proof, nevertheless, has not been obtained. For the elucidation of the ability of vitamin E to stabilize membranes, we investigated the structure-activity relationship of vitamin E on membrane stabilization in liposomes as a simple model of organization of membrane lipids using a fluorescence polarization technique.

MATERIALS AND METHODS

Chemicals. DPPC, DPH and fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were used without further purification. dl-α-Tocopherol and phytol were obtained from E. Merck (Darmstadt, BRD) and further purified by silica gel column chromatography using a mixture of n-hexane and ether (10:1) as an eluent. Tocopherols and model compounds, shown in figure 1, were

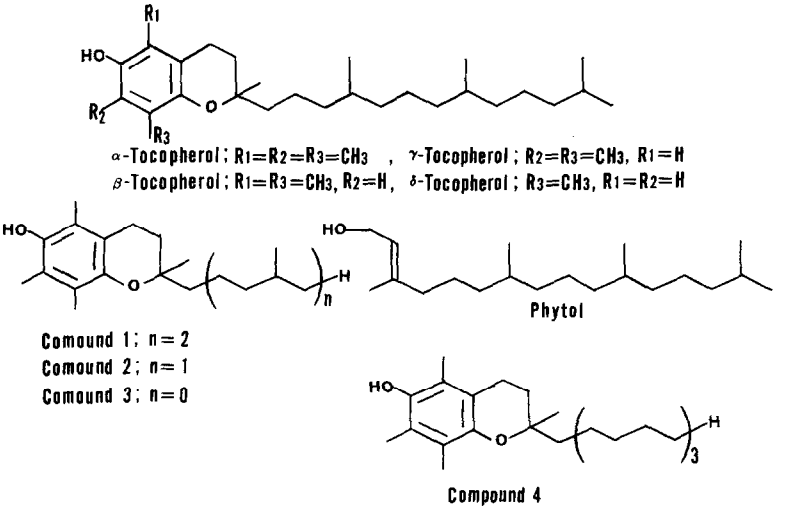

Fig. 1. Structures of tocopherols and the model compounds.

prepared according to previous reports (8). Silica gel C-200 (Wako Chemical Industries, Osaka, Japan) was used for column chromatography. All other chemicals were obtained from well-known laboratory suppliers.

Preparation of liposomes and measurement of fluorescence polarization. Liposomes were prepared according to the method of Batzri et al. (9). In this procedure, DPPC (2.4 mg, 3.3μmol), dicetylphosphate (DCP, 0.18 mg, 0.33 μmol, Sigma Chemical, Co.) and fatty acid (in a given amount) and an additive (if necessary, in a given amount) were dissolved in 1 ml of ethanol. Through a Hamilton syringe, 100 μl of this solution was rapidly injected into 2 ml of phosphate buffered saline (PBS, pH 7.3). The suspension of liposomes was mixed with 2 ml of 4.0×10^{-6} M DPH solution and then incubated at 45°C for 40 min. Fluorescence polarization measurement was carried out using an Elcint microviscosimeter (Elcint Co., Haifa, Israel). The sample compartment was maintained at 45°C. The degree of fluorescence polarization (P) was expressed as $(I_V-I_H)/(I_V+I_H)$, where I_V and I_H are the intensities of vertically and horizontally polarized emissions with excitation of vertically polarized light, respectively.

Measurement of the amount of α-tocopherol and its model compounds incorporated into the liposomes. After the α-tocopherol-contained liposomes were incubated in PBS, suspension was concentrated Amicon (Lexington, Mass.) ultrafitration and applied to a column of Sepharose 4 B using PBS as an eluent. Fractions were monitored at 520 nm, and analyzed α-tocopherol and lipid phosphorus (10). The content of α-tocopherol was determined by HPLC equipped with an Inertsil ODS column (Gasukuro Kogyo Co. Tokyo, Japan) using methanol as an eluent. The liposome suspension was directly injected into column and monitored by an electrochemical detector. The model compounds were also analyzed by almost the same procedure.

RESULTS AND DISCUSSION

Fluorescence polarization (P) is expected to give details about the dynamic nature of membrane lipids (11). An increase in P value is related to a reduction in the fluidity of a lipid core in liposomes. Although it is difficult at present to verify whether there is no direct interaction between the probe for the measurement of P value and the additive molecules in the liposomes, based on the previous reports in which α-tocopherol restricts the movement of the probe in the membrane due to intensified molecular rigidity around the region where the probe molecules are located, and decreases in the lateral diffusion coefficient for the probe in the liposomes (12, 13), it seems likely, in fact, that there is no direct interaction of the probe with the additives in membrane. The relationships of the P value with fatty acid concentrations in liposomes composed of DPPC and one of the fatty acids, which include stearic, oleic, linoleic, linolenic, and arachidonic acids, are shown in Figure 2. With an increase in

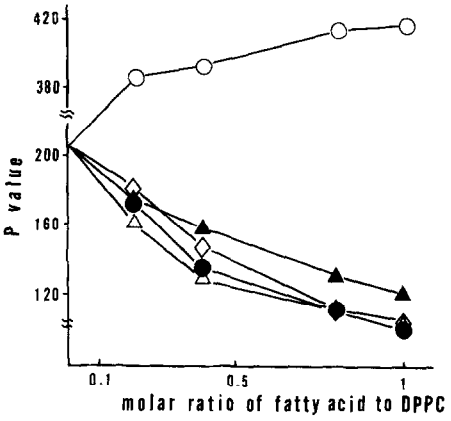

Fig. 2. Effect of fatty acids on the P value in DPPC liposomes. Stearic (18:0, ○), oleic (18:1, △), linoleic (18:2, ◇), linolenic (18:3, ▲) or arachidonic (20:4, ●) acids was incorporated into DPPC liposomes.

concentration of fatty acids, P values decreased except in the case of stearic acid (18:0). This phenomenon can be explained by the fact that when a saturated fatty acid is incorporated into a DPPC bilayer, the ordering of the lipids becomes more compact than in the DPPC liposomes alone, and, hence, the fluidity of the liposomes is decreased; a double bond in a fatty acid molecule creates a "space" in the lipid core of liposomes due to the disordering of micelle by the inclusion of an unsaturated fatty acid, thus increasing fluidity. However, the decrease in the P values of liposomes caused by the addition of unsaturated fatty acids was not significantly affected by the number of double bonds under the experimental conditions used.

The difference in the potency of tocopherols (α-, β-, γ- and δ-tocopherol) on fluorescence polarization of liposomes containing one of the fatty acids is shown in figure 3. The addition of tocopherols to liposomes composed of a mixture of DPPC and fatty acid (molar ratio, 1:1) increased P values with the increase in the number of double bonds in the fatty acid. The largest increases in the P values of linolenic (18:3) and arachidonic (20:4) acid-containing liposomes were caused by α-tocopherol. The other tocopherols had less effective in reducing fluidity of liposomes. Considering that the antioxidant activity of α-

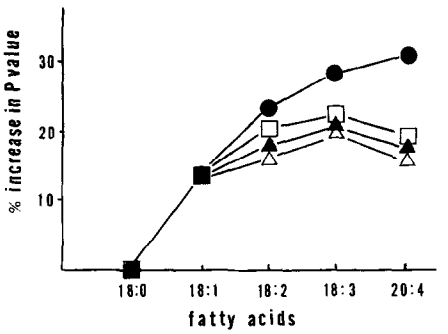

Fig. 3. Increase effect of tocopherols on the P value in DPPC liposomes which was perturbed by each fatty acid. Into DPPC liposomes containing stearic, oleic, linoleic, linolenic, or arachidonic acid was incorporated 8 x 10^{-3} μM α- (●), β- (□), γ- (▲), or δ- (△)tocopherol. A 1 :1 : 0.3 molar ratio of DPPC, each fatty acid, and tocopherol was used.

tocopherol is much higher than that of the other tocopherols (14), it is of great interest that α-tocopherol also apparently dominates in the membrane stabilizing effect of vitamin E. These results show that in liposomes, either the chromanol moiety or the methyl groups attached to the aromatic moiety of α-tocopherol are necessary for optimal packing in the lipid core of liposomes.

Since the presence of methyl groups at the 4'a and 8'a positions of the isoprenoid side chain is essential to the hypothesis of Diplock et al. (3), we investigated the effect of varying isoprenoid side chain of α-tocopherol on the fluorescence polarization of liposomes. As can be seen in figure 4, P values increased and the packing of the micelle became more ordered with an increase in the number of isoprene units. On the other hand, neither compound 3 which has no isoprenoid side chain, nor phytol, a model of the isoprenoid side chain, had any effect on the p value of liposomes. Compound 3 apparently is not retained in liposomes due to the lack of an isoprenoid side chain. This is also supported by the fact that compound 3 incorporated in DMPC (dimyristoylphosphatidylcholine) liposomes can inhibit peroxidation of the other liposomes containing an unsaturated lipid (15). Furthermore, the effect of model compounds having a straight side chain instead of an isoprenoid side chain (compound 4) was almost the same as the

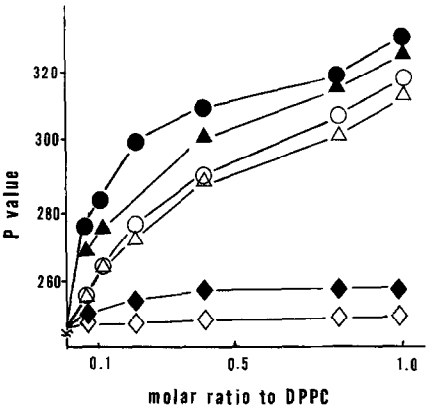

Fig. 4. Effect of α-tocopherol and its model compounds on fluidity of arachidonic acid-containing liposomes. The effect is expressed as p value. An additive was incorporated into liposomes containing arachidonic acid in an amount equimolar to DPPC. As the additives, α-tocopherol (●), compound 1 (▲), 2 (△), 3 (◇) and 4 (○), and phytol (◆) were used.

tocopherols. This result suggests that 4'a- and 8'a-methyl groups of the isoprenoid side chain are not necessarily needed for the interaction of α-tocopherol with unsaturated lipids in liposomes. In all experiments, the retention of the additives incorporated into the liposomes was assured after the experiments by HPLC as shown in figure 5, although compound 3 was not detected.

On the basis of these results, we conclude that α-tocopherol has

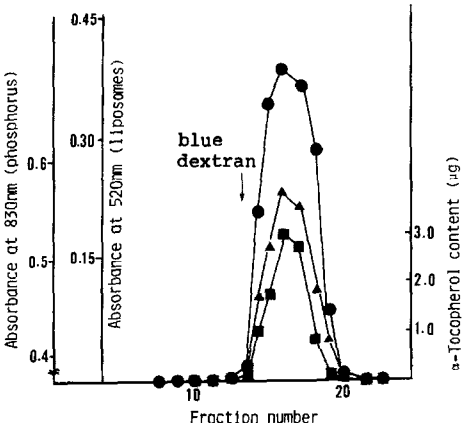

Fig. 5. Gel filtration of the used liposomes on Sepharose 4B. Liposomes (●), lipids phosphorus(▲), and α-tocopherol (■) were measured as described in Materials and Methods.

a strong affinity for unsaturated fatty acids in liposomes that is in good agreement with the affinity observed in homogeneous solution (6). For optimal interaction, the presence of the chromanol moiety with methyl groups born on the aromatic ring moiety, is essential as a "space filler" in liposomes which was perturbed by an unsaturated fatty acid, similarly to the effect of cholesterol in liposomes (16), and the presence of at least one isoprene unit is important for the retention of α-tocopherol molecule in liposomes as a "anchor". These findings are incompatible with the hypothesis of Diplock et al. on the membrane-stabilizing effect of vitamin E (3).

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