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A Study of Stabilization Effect of α-Tocopherol Incorporated into Liposomal Phospholipid Membrane

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Abstract□The stabilization effect of α-tocopherol incorporated into liposomal phospholipid membrane was investigated by fluorospectrophotometry and UV-visible spectrophotometry. The release of entraped carboxyfluorescein from liposomes was markedly retarded by the presence of α-tocopherol in the bilayer of liposomal phospholipid membrane relative to cholesterol-containing liposomes and pure phospholipid liposomes. α-tocopherol-containing liposomes prolonged the oxidation of liposomes-embedded heme as an oxygen carrier. The oxidation index of α-tocopherol-containing liposomes was lower than those of cholesterol-containing liposomes and pure phospholipid liposomes. Thus α-tocopherol-containing liposomes may be useful for the carrier systems of nutrients and drugs to phospholipid bilayer and stabilized liposomes.

Keywords□Stabilizing effect, carboxyfluorescein, lipid-bilayer, liposome-embedded heme, oxidation index

Biological membrane organize living matter in the cells, and allow for the controlled transport of solutes. Membranes enclose barriers from the environment and also make possible internal compartmentalization of metabolic activities within cells. They contain many important enzymes and transport systems. Moreover, on the other surface of cell membranes are located many different recognition sites, which bind certain hormones and transmitters such as insulin, dopamine and acetylchloline[1,2]. A large number of solutes to exert their effects. Their modes of action involve passage of biomembranes.

It is the proteins that are responsible for the specific transport properties and many of the enzymatic functions of the membranes[1,2]. In many cases these activities have been found to be sensitive to the physical state of the membrane lipids. The perturbation of the biomembranes by some compounds could reslt in a drastic change in the permeability of membrane to ions and small molecules, and also result in some changes in the properties of enzyme, recognition sites or receptors on the membrane.

Membrane mimetic systems are extensively used in a large variety of fields[3-5]. In reality, no such perfect system has been developed. Meaningful design of suitable systems will have to await the elucidation of factors affecting drug entrapment and retainment in liposomes. The defects of bilayers in liposomes lower the entrapment efficiency of liposomes. It has also been reported that oxidation of phospholipid induces destabilization of its liposomes, resulting in defects of bilayers of liposomal membranes[6].

In this research, the condensing effect and antioxidation effect of α-tocopherol on the bilayers of dimyristoyl phosphatidylcholine (DMPC) liposomes and egg phosphatidylcholine (egg-PC) liposomes-embedded heme were observed by the fluorescence self quenching (FSQ) method and a relative measurement of lipid peroxidation. FSQ method is based on the loss of fluorescence due to probe-probe interaction when florophore molecules are present at high concentration[7,8], which is monitored by the fluorescence of liposomes containing a concentrated solution (100 or 200 mM) of a highly water soluble fluorophore such as 5 (6)-carboxy-fluorescence (CF). So the CF technique, when applied properly, is an extremely sensitive tool to measure leakage of the aqueous contents of liposomes *in vitro* and to determine their stability *in vivo* upon injection into animals.

The nutritional relationship between α-tocopherol and dietary polyunsaturated fatty acid (PUFA) is considerably more complex than is im-

plied by the antioxidant hypothesis. Oxidation of PUFA is accompanied by increased UV absorption. Changes in 230 to 260 nm range are a measure of diene unsaturation and around 270 nm of triene unsaturation. These change can be used as a relative measurement of lipid peroxidation[9, 10] 215 nm as reference wavelength was the lowest wavelengh where the absorption depended linearly on the amount of lipid used peroxidation until $A_{233\ nm}/A_{215\ nm}$ of 0.8[10]. Heme forms its reversible oxygen abduct and carries oxygen under physiological conditions. Oxygen-binding capability of the liposome-embedded heme was comparable to that of hemoglobin in red blood cell[11, 12]. On degradation of the heme-oxygen abduct, it is considered that slight amounts of superoxide and hydrogen peroxide are formed, which weaken the liposomes.

From the release of CF from DMPC liposomes and the oxidation index of Egg-PC liposomes-embedded heme, the comdensing and the antioxidizing effect of liposomes by α-tocopherol were discussed.

MATERIALS AND METHODS

Materials

Dimyristoyl phosphatidylcholine (DMPC), egg phosphatidylcholine (Egg-PC), α-tocopherol, 5 (6)-carboxyflorescence (CF). The remaining chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A).

Preparation of CF-containing liposomes

Either α-tocopherol or cholesterol in chloroform was mixed with chloroformic DMPC and dried under N_2 gas as usual. After adding Tris buffer/0.2 M CF soltion was adjusted to pH 7.4, vortexed for 1 min, 5 times and sonicated at 37°C. Small unilamellar vesicles obtained were filtered through a Sephadex G-50 column (2×20 cm) to remove external dyes. The final concentration of phospholipid was 0.2 mg/ml Tris buffer.

Preparation of hemolysate

Concentrated human hemolysate was prepared by lysis and membrane extraction of washed red cells by the addition of an equal volume of tetrachloroethylene. This extraction was repeated twice for the final dialysis. The hemoglobin concentration was determined according to the standard method[11].

Preparation of egg-PC liposomes-embedded heme

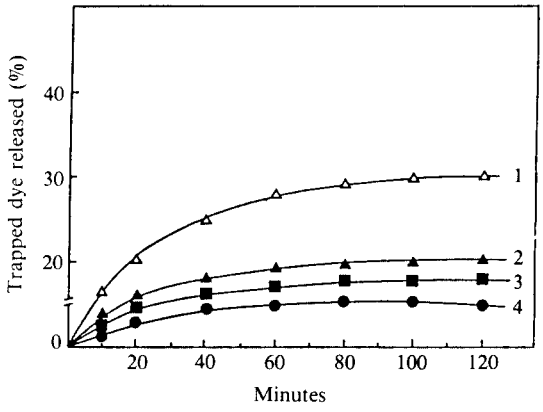

Fig. 1. The release of CF from DMPC liposomes was measured at 37°C.

1. DMPC liposomes, 2. 10 mol% Cholesterol-containing DMPC liposomes, 3. 10 mol% α-Toco pherol-containing DMPC liposomes, 4. 15 mol% α-Tocopherol-containing DMPC liposomes.

Egg-PC liposomes were prepared as described by Gregoriadis[4], and was stored in benzene at −20°C under nitrogen. α-Tocopherol-containing egg-PC liposomes-embedded heme was prepared by the usual method[13]. Heme used in this research is a potent catalyst of lipid peroxidation and induces the oxidation of phospholipid in liposomes.

Fluorospectrophotometry

Fluorescence was measured with a spectrophotometer (Perkin-Elmer, LS-5-Luminescence Spectrometer) at 37°C, 460 nm excitation and 520 nm emission (5 nm slit width). At the end of experiment, 0.1 ml of 20% triton X-100 solution was added to disrupt the liposomes and release the dye remaining within them. The percent of dye released at time t, R (t), was calculated according to the following formular[13]:

$$R(t) = 100\ (F(t)-F_0)/((F_{max}\times 1.1)-F_0)$$

where F_0 is the intensity of fluorescence in 1 ml buffer plus 10 μl dye-containing liposomes at time 0, F (t) is the fluorescent intensity of 1 ml buffer at time t, and F_{max} is the fluorescent intensity of the above mixtures after the addition of 0.1 ml of triton X-100 solution.

Estimation of lipids oxidation in Egg-PC liposomes

The oxidation state of lipids in egg-PC liposomes was estimated by the ratio $A_{233\ nm}/A_{215\ nm}$ which is called "oxidation index"[10] with a Varian DMS-9-Spectrophotometer.

Table I. Remaining efficacy of CF-containing DMPC incubated for 2 hrs at 37°C

Liposome	Composition (mol%)	Fraction (Remains of CF)
DMPC:Cho		0.70
DMPC	5	0.78
	10	0.82
	15	0.84
DMPC: α-Toco	5	0.80
	10	0.88
	15	0.93
	20	0.95

*DMPC, dimyristoylphosphatidylcholine; Cho, cholesterol; -Toco, α-tocopherol.

RESULTS AND DISCUSSION

Efficacy of encapsulation

CF-labelled α-tocopherol-containining DMPC were prepared and compared to CF-containing liposomes composed of either pure DMPC liposomes liposomes composed of either pure DMPC liposomes or 5 to 15 mol% cholesterol-containing liposomes as shown in Fig. 1. All liposomes showed a slow increase in dye-release with time. Pure DMPC liposomes released about 30% of their dye contents within 2 hr. Ten mol% cholesterol-containing liposomes released about 18% of their dye-contents, 10 mol% α-tocopherol-containing liposomes released about 12% of their dye-contents 15 mol% α-tocopherol-containing liposomes released only 7% of their dye-contents after 2 hr.

As R (t) means the percent of CF-dye released at time, the entrapment efficacy such as remaining efficacy of CF of liposomes that has smaller R (t) is lowered.

Remaining efficacy of CF in α-tocopherol-containing liposomes is higher than that in cholesterol-containing liposomes. Twenty mol% of α-tocopherol-containing liposomes have the highest levels of remaining efficacy of CF in liposomes as shown in Table I. The large loss of dye exhibited by the pure DMPC liposomes probably reflects a fast outward diffusion of CF through the phospholipid bilayer. The diffusion of CF is markedly retarded by the presence of α-tocopherol in liposomes. The vesicular size of α-tocopherol-containing liposomes, which result from the condensed bilayer of liposomes by α-tocopherol, might be larger than that of pure DMPC liposomes[7]. The efficacy of metabolic materials, ntrients and drugs in α-tocopherol-containing liposomes could be higher than those in the absence of α-tocopherol. From these results, the appropriate contents of α-tocopherol in liposomes could be assmed to be 15 mol% for encapsulation of metabolic materials, nutrients and drugs. It has been shown[6, 14, 15] that 15 mol% α-tocopherol-containing liposomes increase neuronal life-span and decrease gliosis in an *in vitro*, neuroglial culture system.

Table II. Oxidation index of multilamellar liposomes of Egg-PC incubated for 2 days at 37°C.

Liposome	Absorbance $A_{215\ nm}$	Absorbance $A_{233\ nm}$	Oxidation Index
Egg-PC	0.32	0.19	0.58
Egg-PC + Heme (1 μM)	0.39	0.32	0.81
Egg-PC + α-Toco (10 mol%)	0.38	0.18	0.47
Egg-PC + Heme (1 μM) + α-Toco (10 mol%)	0.36	0.23	0.65

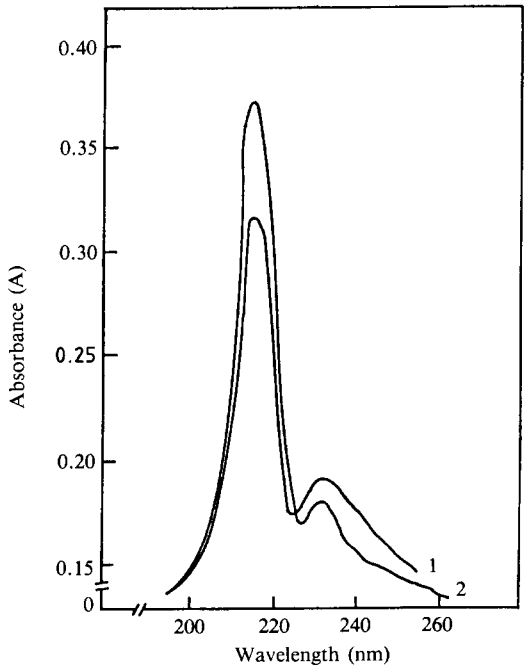

Fig. 2. UV absorption spectrum of Egg-PC liposomes incubated for 2 days at 37°C.
1. Egg-PC liposomes, 2. 10 mol% α-Tocopherol-containing Egg-PC liposomes.

Oxidation of phospholipid liposomes-embedded heme

Table III. Oxidation index of multilamellar liposomes of various concentrations of α-tocopherol in Egg-PC

Composition (mol% of α-Toco)	Oxidation Index
0	0.58
5	0.50
10	0.47
15	0.40

Lipids are known to be easily oxidized by hydrogen peroxide and superoxide. It has also been reported that oxidation of phospholipid induces distribution of the bilayer and that the oxygen abducts of lipids destabilize the strcture of the liposomes[11].

In accordance with the suggestion of Klein[9], increased absorbance at 233 nm is an indication of the appearance of conjugated dienes, hydroperoxides and peroxides. We used 215 nm as a reference wavelength, which was the shortest wavelength that the absorbance depends linearly on the amount of lipid used. The ratio of these absorbance ($A_{233\ nm}$/$A_{215\ nm}$) is defined as the oxidation index, and can be used as a relative measurement of lipid peroxidation. α-Tocopherol lowered the oxidation index of egg-PC liposomes-embedded heme. As shown in Table II, the oxidation index of 10 mol% α-tocopherol-containing Egg-PC liposomes is the lowest and expected to stabilize phospholipid in liposomes as shown in Fig. 2. Twenty α-tocopherol-containing liposomes lowered the oxidation index more than any other concentrations of α-tocopherol in liposomes as shown on Table III.

Thus, α-tocopherol-containing liposomes may be useful for stabilizing phospholipid liposomes from lipid oxidtion.

Much of the biological activity of α-tocopherol is mediated by its function as a lipid antioxidant. α-Tocopherol-containing liposomes may prove useful for the treatment of neurogical symptoms associated with certain disease states in which α-tocopherol depletion is prominent[6, 14, 15]. Further, the interaction between phospholipid bilayers and α-tocopherol might reslt in changes in changes in membrane permeability and stability.

Liposomes which are very similar to biomembrane may be useful as carrier-systems. In order to develope liposomes as carrier systems for metabolic materials nutrients and drugs, the encapsulation efficacy and the stability of phospholipids in liposomes must be considered.

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