CHAPTER III

EXPERIMENTAL

Instruments

1. Ultraviolet - Visible Spectrophotometer. (Milton - Roy Spectronic 3000 Array).

2. Spectrofluorometer. (Jasco FP - 777) connected with Temperature controller (Eyela cool ACE CA 1100).

Materials and Methods

1. Phospholipids

Dilauroylphosphatidylcholine (DLPC , C 12:0), dimyristoylphosphatidylcholine (DMPC , C 14:0) , and distearoylphosphatidylcholine (DSPC , C 18:0) were purchased from Sigma Chemical Co., St. Louis. , dipalmitoylphosphatidyl - choline (DPPC , C 16:0) was from Nippon Oil & Fat Co., Ltd., Tokyo. Thin - layer chromatography of all lipid types on silica gel in a chloroform : methanol : water (65:25:4, by vol) with iodine vapor as a detecting agent shown a single spot, thus they were used without further purification.

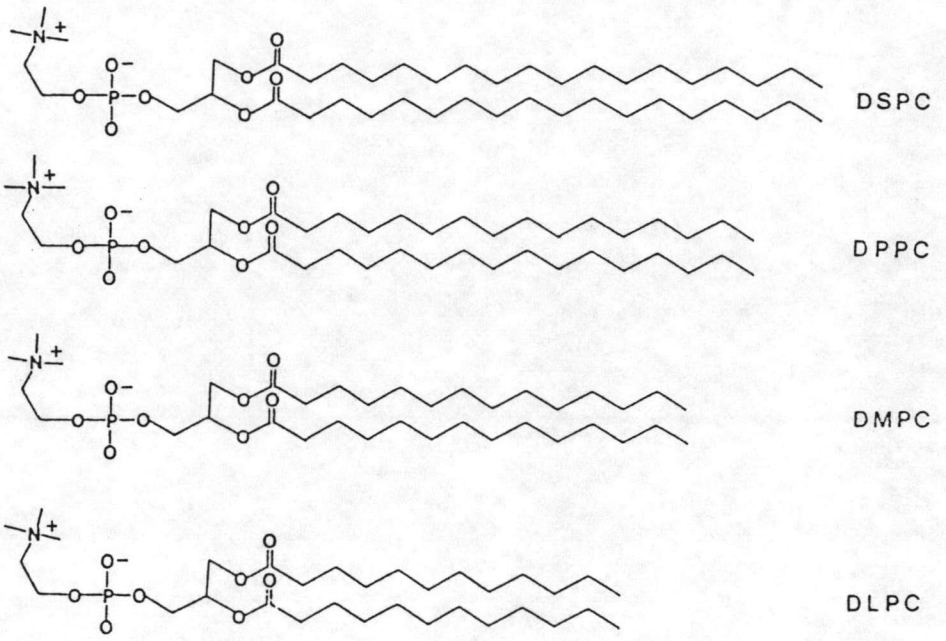


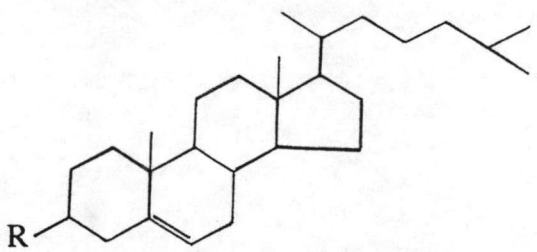
Figure 2. Schematic representation of phospholipids used in this study, illustrating their relative lengths and position in the bilayer, (relative depth of molecules is from Schreier-Mucillo et al., (1976).

2. Cholesterol and hydroxycholesterol derivatives

Cholesterol was obtained from Koso Chemical Co., Ltd., Tokyo., and purified by recrystallization from hot methanol for several times before used to be sure that impurities are no longer present.

All of the cholesterol derivatives used in the experiment were the gift from Dr. Usa Glagasigij (Department of Pharmaceutical Chemistry,Pharmaceutical Sciences,Chulalongkorn University). They were synthesized by the method of Patel et al., (1985) and Glagasigij et al., (1988b).

Cholesterol derivatives having side chain of 1- hydroxy-propyl, 1-hydroxy-ethyl, and 1- hydroxy-3,6 - dioxa-octyl at the 3-position of the cholestene nucleus were named chol I, chol 0, and chol II, respectively.



Where R =

HO	Cholesterol
HO~O	Cholesterol derivative 0
HO~~O	Cholesterol derivative I
HO~O~O~O	Cholesterol derivative II

Figure 3. Chemical structures of cholesterol and its derivatives (chol 0 - chol II) .

3. Charged amphiphile

Dicetylphosphate (DCP) and stearylamine (SL) were used as negatively and positively charged amphiphile respectively, and purchased from Sigma Chemical Co., St. Louis.

4. Bromcresol purple (BCP)

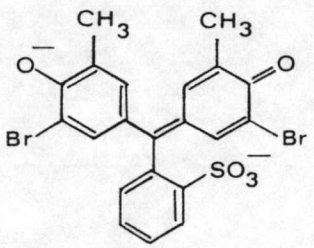


Figure 4. Chemical structure of divalent anion of BCP

Bromcresol purple (BCP , 5, 5′- dibromo-o-cresolsulfonphthalein) was obtained from E. Merck., Germany. 20 mM stock solution of BCP was prepared in water with minimum volume of ethanol to increase solubility and stored at 4 °C , protected from light . BCP solution was freshly prepared from the stock solution of dye to a final concentration of 2 mM in distilled water.

5. 1,6-Diphenyl-1,3,5-hexatriene (DPH)

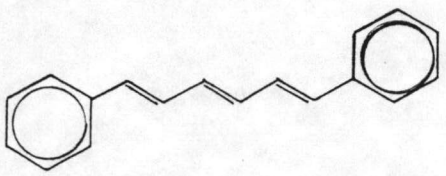


Figure 5. Chemical structure of 1,6-diphenyl-1,3,5-hexatriene.

1,6-Diphenyl-1,3,5-hexatriene , a fluorescence probe for monitoring movement of acyl chain of phospholipid in hydrocarbon region , was purchased from Sigma Chemical Co., St. Louis. Stock solution of DPH was prepared by dissolving in tetrahydrofuran to a final concentration of 2 mM.

6. Tris buffer : 20 mM Tris - HCl , 150 mM NaCl , pH 8.4

Dissolve 2.423 g of Tris - HCl (Tris - (hydroxymethyl)-aminomethane) and 8.775 g of NaCl in 1000 ml. distilled water and adjust to pH 8.4

7. Other reagents

Other reagents used in the experiments were of reagent grade or better.

8. Preparation of large unilamellar liposomes

Large unilamellar liposomes were prepared by method of Glagasigij et al (1988). 20 μmol thin dried lipid film was prepared from phosphatidylcholine or its mixture with other components . Then , mixture of 3 ml. isopropylether (washed three times with water just prior to use in order to eliminate any peroxides) and 1.8 ml. of chloroform was added , followed by 1 ml. of Tris buffer and the mixture was vigorously vortexed and followed by sonication in a bath - type sonicator. This procedure was repeated until good emulsion was obtained. The organic solvent was evaporated off under slightly reduced pressure until a clear suspension was obtained. Traces of organic solvent was eliminated by continuing evaporation under highly reduced pressure for another 1 hr. All of the experiments were performed at temperature above the transition temperature of each phospholipid e.g. , 0 °C for DLPC , 23 °C for DMPC , 41 °C for DPPC , and 58 °C for DSPC. The phospholipid content of each preparation was determined by method of Barlett, [New., R.R.C., 1989].

9. Absorption spectra of bromcresol purple

Absorption spectra were recorded with a Milton - Roy Spectronic 3000 Array. Usual spectra were taken with a 10 mm. path - length cuvette containing 2 ml. of solution at 25 °C.

Dye-binding assay was carried out by addition of 2 mM. of BCP solution to various liposomal preparations to obtained a final concentration of 40 μM dye in Tris buffer. Afterthat incubation was carried out at 25 °C for 90 mins. Absorption spectra were recorded between 480 and 680 nm. The net resulted spectra for BCP binding were acquired from subtraction the BCP - liposome spectrum with the BCP - aqueous spectrum and the liposome spectrum. All of the absorption spectra shown were the representative spectra of at least five determinations. Peak area was achieved by cutting and weighing technique.

10. Fluorescence spectroscopy

An aliquot of 3 μl. of a 2 mM. DPH solution was added to 3 ml. liposomal suspension , followed by Tris buffer for obtaining a final phospholipid concentration of 0.5 μmol / ml. In order to achieve an essentially steady level of fluorescence intensity , DPH - labeled liposomal suspension was incubated for 1 hr. above the transition temperature of lipid used. Polarization value was measured with excitation and emission wavelength at 360 and at 430 nm. , respectively. For all other membrane preparations , the magnitude of the light - scattering depolarization was insignificantly small. Fluorescence polarization and anisotropy were calculated as previously described in Chapter II . Each point of these values in figure involving fluorescence polarization or anisotropy represents the mean of three samples ± S.D.