Biochimica et Biophysica Acta, 1024 (1990) 209–219
Elsevier

BBAMEM 74827

Synthesis and characterization of 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine as an artificial boundary lipid

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(Received 13 September 1989)
(Revised manuscript received 30 January 1990)

Key words: Boundary lipid, artificial; Lipid–protein interaction; DSC; Fluorescence polarization; Membrane fluidity

The synthesis and characterization of an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC), are described. DDPC has two amide bonds instead of ester bonds of regular lecithins such as 1,2-dimyristoylphosphatidylcholine (DMPC). In differential scanning calorimetry (DSC) measurements, DDPC gave two endothermic peaks: one was at 18.0°C (ΔH = 10.74 kJ · mol^{-1}) and the other at 23.0°C (ΔH = 12.91 kJ · mol^{-1}). The former peak was sharp and considered to be the phase transition of the hydrocarbon region, while the latter was assigned to the melt of the hydrogen-belt formed by the amide groups of DDPC. Addition of DDPC to DMPC made the DMPC membrane less fluid in the region close to the surface, and significantly increased the reconstitution efficiency of glycophorin into the membrane. This effect of DDPC was much larger than that of naturally occurring lipid, sphingomyelin.

Introduction

Liposomes are easily formed by dispersing naturally occurring lipids in an aqueous medium and are considered to be an excellent model of mammalian cells. Hence, liposomes are expected to be an useful materials in biotechnology such as drug delivery in therapies and diagnosis, cell modification in gene technology, or cell culturing. However, liposome has two serious disadvantages to be overcome before they are employed in biotechnology as an artificial cell [1]. Liposome is a noncovalent molecular assembly of naturally occurring lipids and, of course, biocompatible in any sence. Simultaneously, however, these properties confer on the liposome physicochemical (mechanical) and biochemical instabilities against various external stimuli such as changes in pH, ionic strength, and/or osmotic pressure and attacks of proteins in serum and plasma, lipases, and/or lipoxygenases. It is first necessary, therefore, to establish a technique to confer greater stability on the liposome. In this point of view, at first glance, it seems that polymerized vesicles [2,3] might be the best candidate. However, there is still a doubt about the biodegradability and biocompatibility of polymerized vesicles. Another disadvantage of liposomes lies in their lowered functionality. For example, they show less cell specificity and no fusogenicity with intact cells. Hence, secondly, we have to make liposomes more functional. We have recently succeeded in establishing an elegant technique for making liposomes stable [1,4,5]. The basic concept is the formation of liposomes by a noncovalent molecular assembly employing naturally occurring substances. This methodology involves the coating of the outermost surface of liposomes with naturally occurring polysaccharide derivatives [4,5]. The polysaccharide-coating method provided not only stabilization of the liposomes but cell specificity to phagocytes in vitro and targetability to specific tissue in vivo [4–6]. In this paper, we report another methodology for making liposomes both stable and also functional by reconstituting a membrane protein in the liposomal bilayers.

Abbreviations: CF, carboxyfluorescein; DPH, 1,6-diphenyl-1,3,5-hexatriene; DDPC, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, 1,2dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; DSHA, dansylhexadecylamine; egg PC, egg phosphatidylcholine; SM, sphingomyelin.

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Sphingomyelin is speculated to be one of candidates of boundary lipid in cell membranes in nature, because it has an amide bond which can make the hydrogen-bonding network in biomembrane [7]. However, sphingomyelin has a rather complex structure and it is difficult to isolate from animal tissues and purify in a large enough amount. Murakami and his co-workers [8] and Kunitake and his co-workers [9] have independently developed totally synthesized peptide-surfactans and both groups also pointed out the effectiveness of the amide group for making lipid membranes more stable. But, they have not shown any direct evidence of the formation of hydrogen bonding. In this work, we have designed and synthesized an artificial phospholipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC, Fig. 1) and characterized it from the view points of both the hydrogen-belt formation [10] provided by its amide groups and the effective reconstitution of a membrane protein into the DMPC membrane which contains DDPC.

Materials and Methods

Synthesis of 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC). DDPC was synthesized according to the route shown in Fig. 1. A key intermediate, 1,2-dimyristoylamidopropan-3-ol (**4**) was prepared by two different methods.

(I) 2,3-Dibromopropionic acid route. 2,3-Diaminopropionic acid (**1**) was obtained by heating 15 g (0.065 mol) of 2,3-dibromopropionic acid, which was dissolved in 183 g (1.3 mol) of 25% aqueous ammonia, in a glass ampule for 6 h at 100°C; yield, 5.3 g (44.0%). m.p. 242–245°C [11]. Anal.: Calcd. for $C_3H_8N_2O_2 \cdot HBr$: C, 19.47; H, 4.90%. Found: C, 19.50; H, 4.99%. IR (KBr disc); ν_{NH}, 3000 and $\nu_{C=O}$, 1640 cm^{-1}. ^{1}H-NMR (in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the water-soluble standard reference), δ 3.49 (*d*, $J = 6.8$ Hz, 2H, $-N-CH_2-CH-$); 4.03 (*t*, $J = 6.8$ Hz, 1H, $(-CH-CO-)$; and 4.72 ppm (*br*, 4H, $-NH_2$).

Methyl 2,3-diaminopropionate (**2**) was prepared by stirring **1** (1 g, 5.5 mmol) at room temperature for 48 h in 50 ml of absolute methanol saturated with gaseous hydrogen chloride; yield, 0.84 g (61.8%). m.p. 163–164°C. Anal.: Calcd. for $C_4H_{10}N_2O_2 \cdot 2HCl \cdot 5/4H_2O$: C, 23.74; H, 6.60%. Found: C, 23.16; H, 6.29%. IR (KBr); $\nu_{C=O}$, 1725 and ν_{C-O-O}, 1125 cm^{-1}. ^{1}H-NMR (in CD_3OD with TMS), δ 3.60 (*m*, 2H, H_2N-CH_2-CH-), 3.92 (*s*, 3H, $-OCH_3$), and 4.59 ppm (*m*, 1H, $-CH-CO-$).

Myristoyl chloride was synthesized according to the literature [12]. To a suspension of 1.5 g (7.9 mmol) of **2** in 150 ml of dry DMF, 7.9 g (0.078 mol) of dry triethylamine and then 7.8 g (0.031 mol) of freshly prepared myristoyl chloride were added. After stirring the reaction mixture at 65.0°C for 6 h, precipitated ammonium chloride was removed by filtration. The solvent was removed from the filtrate and the crude crystalline mass obtained was recrystallized from methanol to give methyl 2,3-dimyristoylamidopropionate (**3**); yield, 1.22 g (70.0%). m.p. 82–83°C. IR (KBr); ν_{NH}, 3300; $\nu_{C(O)-O-C}$, 1750; and δ_{CO-N}, 1650 cm^{-1}. ^{1}H-NMR (in $CDCl_3$ with TMS), δ 0.83 (*m*, $J = 6.7$ Hz, 6H, $-CH_2-CH_3$), 1.24 (*s*, 44H, $-(CH_2)_{11}-CH_3$), 2.20 (*m*, 4H, $-COCH_2-CH_2-$), 3.52 (*br*, 2H, $-CH_2-NH-$), 3.70 (*s*, 3H, $-O-CH_3$), 4.42 (*br*, 1H, $-CH-NH-$) and 7.32 ppm (*m*, 2H, $-NH-$). TLC (on a Silica gel Spot Film S-194, Tokyo Kasei); developed with methylene dichloride/methanol (4:3, by vol.) and detected with iodine vapor, R_f 0.83 (single spot).

1,2-Dimyristoylamidopropan-3-ol (**4**) was obtained by reducing 1.64 g (3.04 mmol) of **3** with 0.33 g (8.7 mmol) of sodium borohydride at 60°C for 4 h in 20 ml of absolute ethanol in the presence of 0.37 g (8.7 mmol) of lithium chloride; yield 1.0 g (70.0%). m.p. 91–92°C. Anal.: Calcd. for $C_{31}H_{62}N_2O_2$: C, 72.89; H, 12.23; N, 15.48%. Found: C, 73.59; H, 12.36; N, 14.77%. IR (KBr); the absorption at 1725 cm^{-1} ($\nu_{C=O}$) disappeared. ^{1}H-NMR (in $CDCl_3$ with TMS), δ 0.83 (*m*, $J = 6.8$ Hz, 6H, $-CH-CH_3$), 1.22 (*s*, 44H, $-(CH_2)_{11}-$

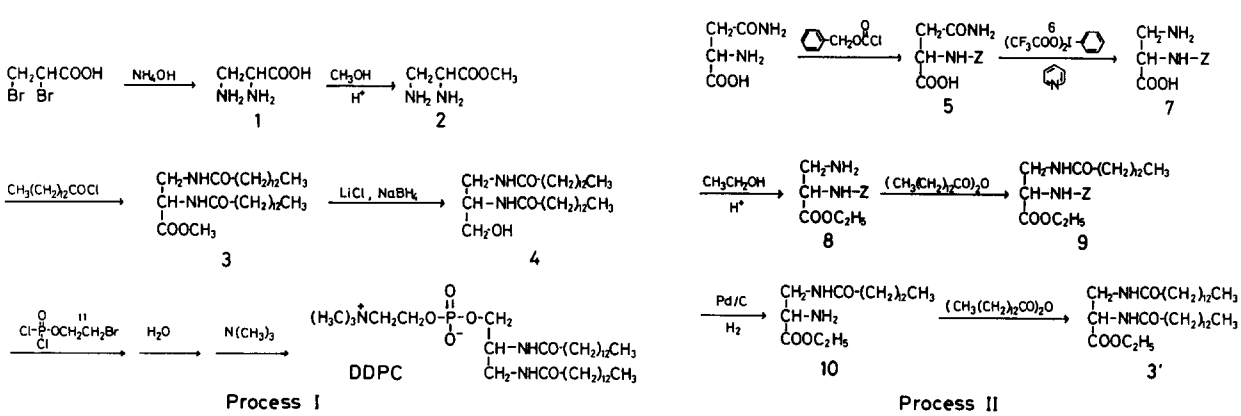

Fig. 1. Processes of DDPC synthesis: I, starting from 1,2-dibromopropionic acid and II, starting from DL-aspargine.

CH_3), (*br*, 4H, $-CO-CH_2-$), 3.60 (*m*, 2H, $-CH_2-NH-$), 3.82 (*m*, $-CH-NH-$), 6.64 (*m*, 1H, $-CH_2-NH-CO-$), and 7.05 ppm (*m*, 1H, $-CH-NH-CO-$). TLC (Silica gel Spot Film S-194, Tokyo Kasei); developed with methylene dichloride/methanol (20 : 1, by vol.) and detected with iodine vapor R_f 0.71 (single spot).

(II) L-Asparagine route. *N*-Benzyloxycarbonyl-L-asparagine (**5**, Z-Asn), bis(trifluoroacetoxy)phenyliodine (**6**) and N^2-Z-diaminopropionic acid (**7**) were prepared according to the method described in the literature [13–15]. After **7** (1.0 g, 4.2 mmol) was reacted with *p*-toluenesulfonic acid monohydrate (1.5 g, 7 mmol) in absolute ethanol (15 ml) for 3 h under reflux, the solvent was removed in vacuo. Recrystallization of the obtained crystalline mass from ethanol/diethyl ether (1 : 25, by vol.) gave the ethyl ester of **8** as *p*-toluenesulfonate; yield 1.6 g (87%), m.p. 126–128°C. To the ethyl ester **8** (500 mg, 1.1 mmol) dissolved in dry chloroform (20 ml) were added triethylamine (0.3 ml, 2.15 mmol) and myristic anhydride (0.5 g, 1.1 mmol). After reacting for 6 h at 70°C under stirring, the reaction mixture in chloroform was washed with 4% aqueous sodium bicarbonate and, then, 10% citric acid solutions, and the chloroform layer was dried over anhydrous sodium sulfate. Recrystallization of obtained crude mass from ethanol gave ethyl N^1-myristoyl-N^2-Z-diaminopropionate (**9**); yield 0.5 g (95%), m.p. 87–89°C. IR (neat); ν_{CH} 2910 and 2840; ν_{CO_2Et}, 1720; $\nu_{C=C}$, 1690; and ν_{CONH}, 1650 cm^{-1}. Anal.: Calcd. for $C_{27}H_{44}N_2O_5$: C, 68.03; H, 9.30; N, 5.88%. Found: C, 68.07; H, 9.23; N, 5.79. ^{1}H-NMR ($CDCl_3$ with TMS), δ 0.95 (*t*, $J = 6.8$ Hz, 6H, CH_3), 1.34 (*s*, 22H, $-(CH_2)_{11}-CH_3$), 2.39 (*t*, $J = 7.5$ Hz, 2H, $-NH-CO-CH_2-$), 4.20–3.75 (*m*, 5H, CO_2CH_2-, $-CH_2-NH-$, and $-CH-NH-$), 5.18 (*s*, 2H, $-CH_2-C_6H_5$), and 7.4 ppm (*s*, 5H, benzene ring protons).

In order to eliminate the 2-protecting group, compound **9** was reduced by H_2 over Pd/C (the activity, 25%) in dry methanol to give ethyl N^2-myristoylamidopropionate. The ethyl ester so obtained was further reacted with myristic anhydride to yield ethyl N^1,N^2-2,3-dimyristoylamidopropionate (**3′**). The compound **4** was also obtained from the ethyl ester precursor **3′** (vide supra) by essentially the same procedure as that adopted to the methyl ester 3.

(III) Final procedure for preparing DDPC. β-Bromoethylphosphoryldichloride (**11**) was obtained as follows. Phosphorousoxychloride (36.4 g, 237 mmol) was dissolved in 30 ml of dry trichloroethylene, to which 7.3 g (137 mmol) of 2-bromoethanol was added dropwise over 30 min and the resulting mixture was reacted at 25.0°C for 12 h under bubbling of dried gaseous nitrogen to remove hydrogen chloride produced; yield, 31.9 g (95.9%). IR (neat); $\nu_{P=O}$, 1300 and ν_{P-O-C}, 1070 cm^{-1}.

A 1.6 g sample (6.5 mmol) of **11** was dissolved in 10 ml of dry chloroform, to which 0.66 g (6.5 mmol) of triethylamine and then 0.8 g (1.8 mmol) of **4** dissolved in 10 ml of chloroform were added and then the reaction was carried out at 4°C for 4 days under stirring. Completion of the reaction was confirmed by disappearance of the spot at R_f 0.80 corresponding to the starting material **4** on TLC developed with chloroform/methanol/water (65 : 25 : 4, by vol.) and detected by iodine vapor. The reaction mixture was washed with 4% aqueous sodium bicarbonate, 10% citric acid, and then water. The organic phase was distilled off and the solid materials remained were dissolved in 20 ml of acetonitrile. To the resulting solution were added 6.6 ml of 0.5 M aqueous sodium acetate solution (to adjust pH to 8.0) and 3 ml of 0.5 M EDTA · 4Na solution. After completion of the hydrolysis of the phosphorylchloride to the phosphate diester at room temperature, the resulting mixture was extracted with chloroform. The chloroform layer was washed with 4% aqueous sodium bicarbonate and then 10% citric acid, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue so obtained was recrystallized from 70 ml of chloroform/acetone (1 : 7, by vol.). The precipitates were collected by filtration and the crystalline mass was dissolved in a 200 ml of a mixture of chloroform/2-propanol/acetonitrile/40% aqueous trimethylamine (3 : 5 : 5 : 7, by vol.). The conversion of the bromide to trimethylammonium was carried out for 72 h at 25°C. After removing the solvent, the resulting mixture was redissolved in 5 ml of chloroform/methanol (5 : 4, by vol.) and submitted to an ion-exchange chromatography for desalination (Amberlite, IRA-45 and IRC-50 mixture, ϕ 2 cm × 15 cm developed by the same solvent). All the solvents were again removed. The material so obtained was redissolved in a small amount of chloroform and reprecipitated with seven-fold volumes of acetone. The precipitates obtained were dissolved in a minimum amount of a mixed solvent (chloroform/methanol/water (65 : 25 : 4, by vol.)) and submitted to a silica gel chromatography column (Wakogel C-200, ϕ 1.0 cm × 30 cm) developing with the same solvent. Fractions containing DDPC were collected and lyophilized. When developed with the same solvent as that used for column chromatography, R_f of DDPC was 0.30 on a TLC (Silica gel Spot Film, S-194, Tokyo Kasei). The obtained crystalline mass was purified by HPLC (Silica gel CPS-HS-221-1, Kusano Scientific Co., with methanol at 1 ml/min). DDPC was eluted out 40 min after application; yield, 160 mg (12.0%). m.p. 165–167°C. Anal.: Calcd. for $C_{36}H_{74}N_3O_6P \cdot 2H_2O$: C, 60.63; H, 11.02; N, 5.89%. Found: C, 60.42; H, 10.84; N, 5.85%. IR (KBr); ν_{CH}, 2960; $\nu_{P=O}$, 1220; and ν_{P-O-C}, 1050 cm^{-1}. ^{1}H-NMR (in $CDCl_3$/DMSO-d_6 (9 : 1) with TMS), δ 0.87 (*t*, $J =$ 7.0 Hz, 6H, $-CH_2-CH_3$), 1.25 (*s*, 40H, $-(CH_2)_{10}-CH_3$), 1.54 (*m*, 4H, $-CO-CH_2-(CH_2)_{10}-$), 2.13 (*m*, 4H,

CH_2–CH_2–CO–), 3.34 (*s*, 9H, –$\overset{+}{N}(CH_3)_3$), 3.68–3.95 (*br*, 1H, –NH–CH_2–CH(N)–; 2H, –CH_2–$\overset{+}{N}(CH_3)_3$; 2H, –CH(N)–CH_2–O–P–), and 4.30 ppm (*br*, 4H, –P–O–CH_2CH_2–NX; 1H, –CH–NH–CO–). ^{13}C-NMR (in $CDCl_3$/DMSO-d_6 (9:1) with TMS, 135 deg DEPT), 14.0 (+, –CH_3), 22.4 (–, CH_2–CH_3), 25.5 and 25.6 (–, –CH_2–CH_2–CH_3), 29.2 (–, –CO–CH_2–CH_2–$(CH_2)_8$-CH_2), 31.6 (–, –CO–CH_2–CH_2–$(CH_2)_8$–), 36.3 and 36.2 (–, –CO–CH_2), 39.9 (–, –CH_2–NH–), 50.3 (+, –CH–NH–), 53.8 (+, –$\overset{+}{N}(CH_3)_3$), 58.9 (–, –CH_2–NX), 63.8 (–, –CH–CH_2–O–P–), and 66.0 ppm (–, –N-CH_2–CH_2–O–P–).

Other lipids. Isolation of sphingomyelin (SM). A lipid mixture was extracted from 300 g of adult pig brains with chloroform/methanol (2:1, by vol.) and treated by the procedure of Folch et al. [16]. TLC was carried out on a silica gel plate developed by chloroform/methanol/water (65:25:4, by vol.) and the product was detected by Dragendorf reagent; a single spot at R_f 0.32 [17]. The obtained SM showed a single phase transition at 32°C [18].

Dimyristoyl-DL-α-phosphatidylcholine (DMPC) was purchased from Sigma Chemical Co., St. Louis, MO and egg phosphatidylcholine (egg PC) was isolated from fresh egg yolk [19] and purified according to the method already established [20].

Isolation of glycophorin from human erythrocytes. Human erythrocyte ghosts were first obtained using essentially the same procedure described in literature [21]. Lyophilization of the protein-containing fractions upon the phosphocellulose chromatography (P-cellulose, Seikagaku Kogyo, ϕ 1.7 cm × 20 cm) gave approximately 100 mg of glycophorin A [22].

Fluorescent probes. N-Dansylhexadecylamine (DSHA) was the same as that used in our previous study [23]. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich, and used without further purification.

Liposomes. DDPC itself forms very stable liposome without coexistence of any other lipids and/or cholesterol. A thin film of DDPC (10 mg) was formed at the bottom of a small round-bottom flask according to the established technique by ourselves [23] and dispersed in 4 ml of an aqueous buffered solution (20 mM Tris-HCl containing 200 mM NaCl, pH 8.6) on a Vortex mixer for 20 min to give multilamellar vesicles (MLVs) of DDPC. By the same procedure, DDPC-egg PC and DDPC-DMPC mixed liposomes were prepared. The formation of vesicles was visualized by electron microscopy using a JEOL JEM-100V electron microscope (vide infra).

Glycophorin or pig mucin (a kind gift from Nippon Koutai Kenkyusho, Takasaki) was reconstituted in a mixed liposome such as DDPC-egg PC, DDPC-DMPC, SM-egg PC, or SM-DMPC liposomes by two different methods.

In method A, which has been developed by MacDonald and MacDonald [24], the membrane glycoprotein was dissolved in a chloroform/methanol/water mixed solvent (120:60:4.5, by vol.) together with the lipids and a thin film was formed at the bottom of a round-bottom flask under reduced pressure using a rotary vacuum evaporator. This method was adopted when glycophorin was reconstituted into small unilamellar vesicles (SUV) and reversed evaporation vesicles (REV) [25].

In method B, glycophorin or pig mucin (5 mg/ml) dissolved in an aqueous buffered solution was added to a liposomal suspension previously prepared. In this procedure, the glycoproteins are reconstituted only in the outermost leaflet of MLV [26]. In all the preparations, the lipid concentration in a liposomal suspension was determined as inorganic phosphate using an Assay Kit Clinical (Phospholipids-Test Wako, Wako Pure Chemical Ind., Ltd., Osaka). The amount of glycophorin reconstituted in liposome was determined from both the total protein content after complete destruction of liposome with deoxycholic acid and the assay of the sialic acid residues of glycophorin on the surface of intact liposome [26].

Electron microscopic observation. To the resulting milky suspension of MLV was added an equal volume of 2% sodium phosphotungstate. The mixture was kept for 1 h at 0°C to ensure the negative staining, the placed onto the carbon-deposited copper sheet-mesh, submitted to an electron microscopic observation on a JEOL JEM-100V or a JEM-100SX.

Fluorescence depolarization measurements. The fluorescence depolarization serves as a measure of fluidity arround the fluorophore in membranes [27]. Fluorescence depolarization spectra were measured by essentially the same procedure as that described before [23]. The fluorescence polarization, p, is calculated by Eqn. 1:

$$p = \frac{I_{VV} - C_f I_{VH}}{I_{VV} + C_f I_{VH}} \quad (1)$$

where I is the fluorescence intensity and subscripts V and H refer to the vertical and horizontal orientations of the excitation (first) and analyzer (second) polarizer, respectively. $C_f (= I_{VH}/I_{HH})$ is the grating correction factor. Depolarization measurements were run on a Union Giken fluorescence polarization spectrometer FS-501 using sharp cut-off filters Y-46 (for DSHA) and L-36 (for DPH) (Hoya Glass Works, Tokyo) and the cell compartment was connected to a Komatsu-Yamato Coolnics Model CTR-120. A Sord Microcomputer M200 Mark II system was adopted to control the measurement conditions and to collect the data.

Differential scanning calorimetry (DSC) measurements. The DSC investigation provides valuable infor-

mation about membrane dynamics and physical properties of membranes such as phase transition, phase separation and membrane fluidity [28]. For DSC measurements, lipids (2.0 mg) were dissolved in 150 μl of chloroform in an aluminum sample pan. The solvent was removed over 4 h under reduced pressure and 50 μl of an aqueous Tris-HCl buffered solution (pH 8.6) was then added. After capsule was sealed, the sample capsuled was kept in the pan at least for 1 h above the phase transition temperature, T_c, of the sample.

Alternatively, 10 mg of DDPC was dissolved in 4 ml of chloroform and a thin lipid film was formed according to the established procedure [26]. The thin film so obtained was dispersed in 2 ml of an aqueous Tris-buffered solution on a Vortex mixer followed by ultrasonication for 40 min at 40°C under nitrogen atmosphere. The resulting liposome suspension was concentrated to 300 μl using an ultra membrane-filter (Amicon, the pore size of 0.01 μm). After determination of the lipid concentration, 100 μl of the sample solution were transferred to a DSC pan.

All the measurements were run on a Daini Seikosha SSC/560U at a scanning rate of 1.0 C°/min. For all the aqueous systems, very stable instrumental base lines were attained and remained constant in both the sample and reference cells over the temperature range between 4°C and 70°C.

Measurements of carboxyfluorescein (CF) release from liposome. The CF-release technique is one of useful and convenient methodologies for invetigating the barrier function of liposomal membranes [29]. Effect of addition of DDPC and/or glycophorin on stabilization of a conventional liposome was examined in the presence and absence of serum or plasma by the CF-release technique previously employed [25].

(1) Serum and plasma preparation. 50 ml of human blood was transferred to a vacuum test tube in the presence (for plasma) or the absence (for serum) of heparin. After incubation for 1 h, the test tube was centrifuged at 150 × *g* at room temperature for 10 min. Plasma or serum was collected in the supernatant.

(2) Preparation of CF-containing liposomes. Liposomes, which are encapsulating CF in the interior water phase, were prepared by the method described elsewhere [25].

(3) Measurements of CF leakage. Measurements were carried out on a Hitachi 650-10S fluorospectrophotometer equipped with a thermo-regulated cuvette cell compartment. The release of CF from liposomes was followed by monitoring an increase in the fluorescence intensity (I_t) at 520 nm by excitation at 470 nm. The total amount of CF (I_B) encapsulated in liposomes was determined by totally destroying the liposomal membranes with either 10% (v/v) aqueous Triton X-100 solution for a simple liposomal suspension or 1.3% deoxycholic acid-phosphate buffered saline for a sample which contains serum or plasma. Leakage of CF (%) was calculated as follows;

$$\% \text{ leakage of CF} = \frac{I_t - I_0}{I_B - I_0} \times 100 \quad (2)$$

CF-containing liposomal suspension ($1.3 \cdot 10^{-4}$ M, 0.8 ml) were mixed with serum, plasma, or a buffer solution (0.2 ml) in a cuvette cell, and spontaneous and plasma- or serum-induced leakages of CF were followed.

Results and Discussion

We first planned the research for understanding the relationship between the structural stability of the lipid bilayer membrane and the chirality of lipid molecule [30,31]. As described under Materials, hence, we have synthesized DDPC by two different processes. In the route I started from diaminopropanol, the racemic form of DDPC is produced, while in the route II started from L-asparagine, only the L-isomer of DDPC is regiospecifically prepared. Nevertheless we carefully studied the difference in their physicochemical properties of liposomal membranes between and L- and DL-isomers by differential scanning calorimetry, electron spin resonance spectroscopy, infrared spectroscopy, and fluorescence polarization, we could not find any significant difference between the two (though data are not shown). In this paper, therefore, we gave only the data obtained by employing the DL-isomer. Arnett and Gold [30] have reported that there is no significant difference in NMR characteristics between D- and L-dipalmitoylphosphatidylcholine membranes. Ghosh and his collaborators [31] also have found that no difference was observed between D- and L-dipalmitoylphosphatidylcholine liposomes even when cholesterol was added to.

DDPC forms a very stable and large multilamellar vesicle (MLV) by itself as shown in Fig. 2. It was, however, relatively difficult to obtain small unilamellar vesicles (SUVs) unless another natural lipid such as DMPC, DDPC, or egg PC was added.

DSC measurements

Under regular experimental conditions for DSC measurements (2.0 mg of DDPC in 50 μl of an aqueous buffered or unbuffered solution is equivalent to 2.44 wt.% of water), DDPC gives two characteristic endothermic peaks: one with $\Delta H = 10.58$ kJ · mol^{-1} is relatively sharp at 18°C, while another peak with $\Delta H =$ 12.73 kJ · mol^{-1} is broad at 23° C (the top curve in Fig. 3). When 10 mol% of DMPC was mixed with DDPC, however, DSC gave only one broad peak (the second curve from the top in Fig. 3). This peak becomes sharper gradually and shifts toward the characteristic phase transition peak of DMPC itself [32] with an increase in the amount of DMPC mixed in. This result

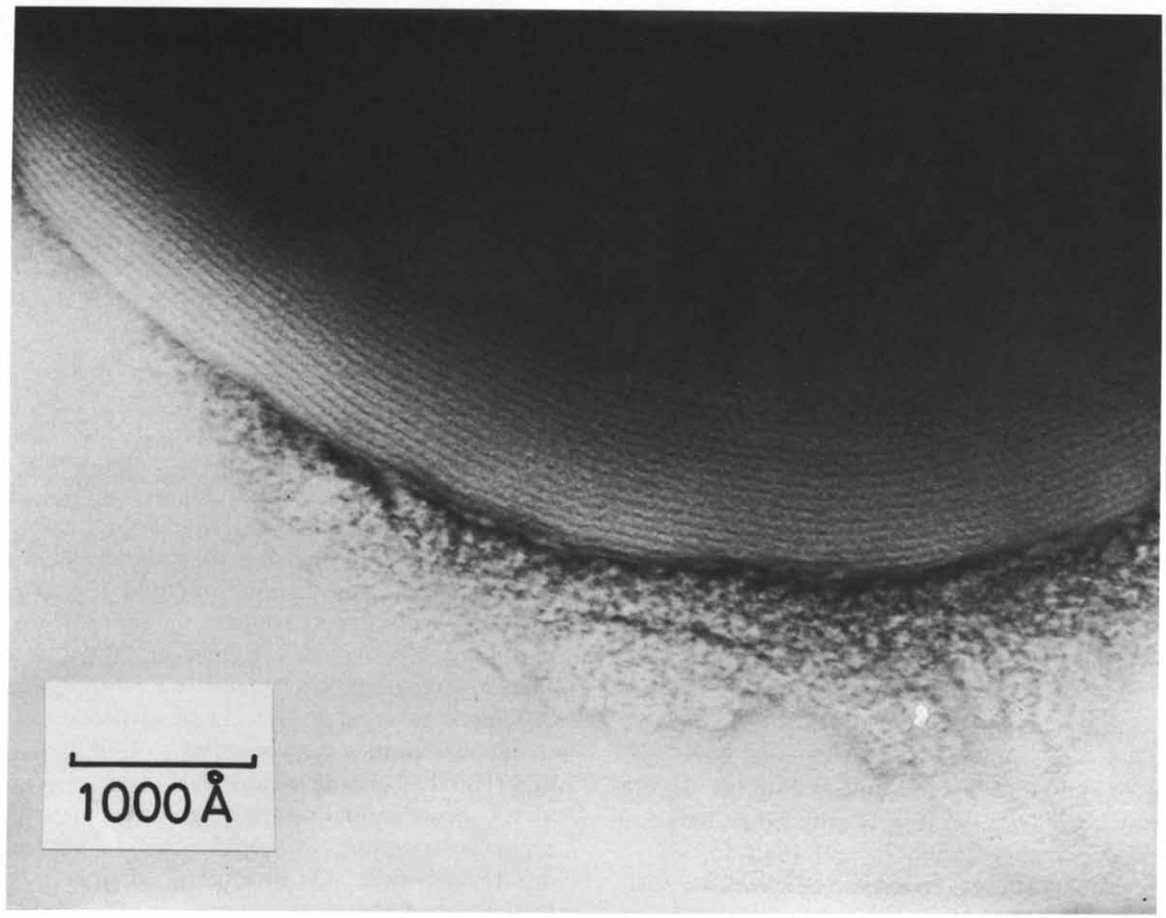

Fig. 2. An electron microscopic picture of multilamellar liposome formed by DDPC after ultrasonication and negatively stained by 2% phosphorous tangstate.

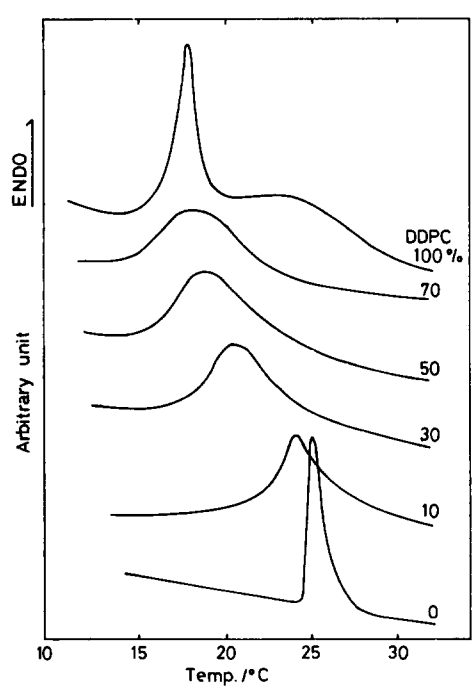

Fig. 3. DSC curves of DDPC-DMPC system.

suggests that the original two peaks observed in the DSC measurement of DDPC are not from the phase separation of two different lipids or any contaminated impurity. It is clear, in addition, that mixing of DDPC with DMPC is able to form a very homogeneous mixed-lipid bilayers without any phase separation.

Fig. 4 shows the effect of calcium ion on the phase transition of DDPC. When calcium ion is added to a mixed lipid system in which a phase separation is taking place [33], one of the two peaks, at least, is certainly affected because of the difference in the binding ability to the calcium ion between the two lipids [34]. However, even after 10 mM calcium chloride was added to a DDPC suspension and incubated for 1 h at 40°C (curve b in Fig. 4), the two peaks of DDPC were still clearly separated and the endothermic temperatures of both peaks also unchanged. Only the peak which appeared at the lower temperature was broadened; namely, the ΔH value increased with a decrease in the membrane fluidity upon the calcium ion binding [35]. When an aqueous 12 mM EDTA solution was further added into the 10 mM calcium ion containing system and incubated for another 30 min at 40°C (the bottom, Fig. 4), the peak at

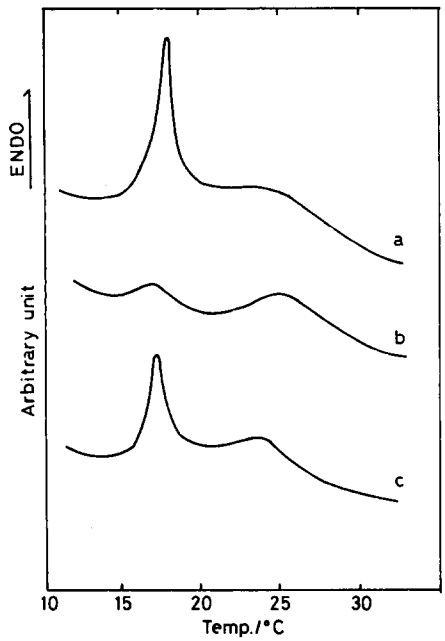

Fig. 4. Effect of calcium ion on DSC of DDPC: (a) without calcium ion; (b) in the presence of 10 mM $CaCl_2$; and (c) in the presence of 10 mM $CaCl_2$ and 12 mM EDTA.

the lower temperature became sharper again [36,37]. This indicates that the calcium ion certainly binds to the phosphate moiety of DDPC and the addition of EDTA makes the calcium ion free from this binding, which leads to recovery in the sharpness of the peak. The less effect of the calcium ion on the peak with higher endothermic temperature indicates that this peak comes from a part which does scarcely concern with the metal ion binding. Because, the binding ability of the amide group to the metal ion is weaker than that of the anionic phosphate moiety.

Addition of 20 mol% of cholesterol to DDPC almost completely abolished both peaks, while the addition of 2 M sodium chloride did not cause any significant change in both peaks (although data are not shown).

Urea is well known to perturb the ice-berg structure of bulk water. The addition of urea brought about broadening of both peaks, especially of the peak at higher endothermic transition. By adding 0.5 M urea, ΔH of the endothermic transition at 18°C decreased from 10.7 to 7 $kJ \cdot mol^{-1}$. Addition of more urea (5.0 M) caused a further decrease in enthalpy of both endothermic peaks, down to 6.6 $kJ \cdot mol^{-1}$ for the peak at lower temperature and to 1.65 $kJ \cdot mol^{-1}$ for that at higher temperature. In cases of natural phosphatidylcholines such as DMPC or DPPC, we did not observe any significant change in their phase transition phenomena by the addition of urea.

Judging from these results, we suggest that the sharper endothermic peak at 18°C is most probably from the gel-liquid crystalline phase transition upon the *trans-gauche* conversion of hydrophobic alkyl chains [38], which is observed normally in natural phosphatidylcholines. On the other hand, the broader peak at 23°C may be relating with intramolecular and/or intermolecular hydrogen-bondings formed by amide bonds in a region close to the surface of lipid bilayer membranes.

Uniracht and Shipley [39] have reported that a mixture of lecithin and bovine sphingomyelin greater than 33 mol% gives two endothermic peaks in its DSC under the conditions containing 42 wt.% of water. They believe that phase separation of sphingomyelin occurs under these conditions [39]. Cuaratolo et al. [40] have recently synthesized 1-palmitoyl-2-tridecanoylcarbamyloxy-*sn*-phosphocholine which bears an urethane bond (–NH–C(O)–O–) in one of the acyl chains, and they have found that this artificial lipid gives two endothermic peaks at 9°C with $\Delta H = 4.1–8.2$ $kJ \cdot mol^{-1}$ and at 38°C with $\Delta H = 53.5$ $kJ \cdot mol^{-1}$. They pointed out that the former peak at lower temperature may reflect the hydrogen-bonding formed by its urethane functionality [40]. Considering that our DDPC has two amide bonds in contrast to the lipid synthesized by them, it seems reasonable that DDPC shows the higher endothermic temperature and the larger enthalpy change because of increased and strengthened hydrogen-bondings.

Another interesting finding in the DSC measurements of DDPC is the solvent isotope effect. When DDPC was dispersed in D_2O and its DSC measurement was carried out just after the preparation of the sample, only the peak which appeared at 23°C in H_2O, shifted to 26°C and became broader; namely, ΔH increased to 31.3 $kJ \cdot mol^{-1}$ in D_2O from 12.8 $kJ \cdot mol^{-1}$ in H_2O. When the sample was kept in D_2O for 3 weeks at 0°C for aging, this peak shifted further up to 31°C ($\Delta H =$ 31.31 $kJ \cdot mol^{-1}$). Further aging of the sample for another three weeks at the same temperature did not show any additional change in both the transition temperature and the enthalpy change. The most important finding is that, for the peak at lower endothermic transition, we did not observe any significant isotope-induced change in both the phase transition temperature and the enthalpy of transition. Since the amide hydrogen (–NH–) of DDPC is certainly exchangeable to –ND–, as ascertained by FTIR investigation [41], the hydrogen-belt formed in the DDPC bilayer membranes may partly involve water molecules (Fig. 5B) as well as the direct interaction between neighboring lipids (Fig. 5A). Considering that the hydrogen-bonding in O–D $\cdots$ O is stronger than that in O–H $\cdots$ O [42], the increase in the enthalpy change and the phase transition temperature in D_2O compared with those in H_2O seem reasonable.

Stabilization of membrane structure with DDPC

(i) Reconstitution of human glycophorin into DDPC-egg PC liposomes. Reconstitution efficiency of a membrane

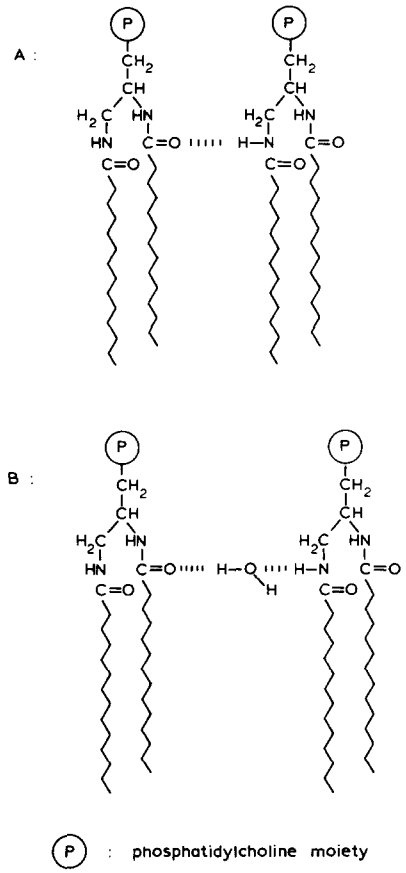

Fig. 5. Schematic representation of hydrogen bonding formed by DDPC; (A) without water molecule and (B) via water molecule.

protein into an artificial lipid membrane or liposome is rather low even if sphingomyelin was employed [26]. Apart from whether a boundary lipid certainly exists or not [43,44] it will be expected that if the physicochemical stability of liposomal membranes is 'increased by employing DDPC, a membrane protein must be effectively reconstituted in DDPC containing liposomes.

First of all, we tried to reconstitute human glycophorin, which was isolated from erythrocytes, into a DDPC-egg PC mixed liposome and the reconstitution efficiency was compared with the case of naturally occurring sphingomyelin which was isolated from pig brain. In the case of simple egg PC liposome, the reconstitution efficiency was only 9% at most. When 20 mol% of sphingomyelin was mixed with egg PC, the efficiency increased up to 40 mol%. However, even if the content of sphingomyelin was increased up to 60% the reconstitution efficiency for the protein decreased because of the membrane instability. In contrast to sphingomyelin, when DDPC was added to egg PC, the reconstitution efficiency of glycophorin was greatly enhanced: up to 80% in the DDPC(20)-egg PC(80) liposome and more than 95% in the DDPC(40)-egg PC(60) liposome [26] (Table I).

TABLE I

Reconstitution efficiency of glycophorin [a] into egg PC liposomes [a]

	Egg PC	Egg PC/SM, SM content (mol%)		Egg PC/DDPC, DDPC content (mol%)		
		10	20	10	20	40
Reconstitution efficiency (%)	9±1	21	40±10	80±5	80±5	95±5

[a] Glycophorin (0.1 mol% to total lipids) was reconstituted only in the outermost leaflet of MLV by Method B (see text).

(ii) Fluorescence polarization investigation. Membrane fluidity is closely related with barrier function and structural stability of the membrane. First, the effect of DDPC or sphingomyelin added to DMPC bilayers on the membrane fluidity was investigated by using fluorescence polarization technique. DPH and DSHA were employed as the probes [23]. The former gives information about the very hydrophobic domain of the bilayer membranes, while the latter reports on the microenvironment around the region close to the surface. Measurements were carried out both below and above the phase transition temperature of the system. Clearly from the relationship between the fluorescence depolarization (p value) and the membrane fluidity around the probe [23], the larger the p value is, less fluid the membrane is. Table II shows that an increase in the molar ratio of DDPC to DMPC causes a decrease in the fluidity at the region close to the surface both below and above the phase transition temperature of DMPC. The formation of a hydrogen-belt upon the more addition of DDPC to DMPC more decreases the fluidity of the membrane

TABLE II

p values of mixtures of DMPC and DDPC or SM at different temperatures [a]

DMPC:DDPC	p value			
	DPH		DSHA	
	15°C	30°C	15°C	30°C
100:0	0.358	0.148	0.201	0.125
90:10	0.356	0.146	0.203	0.128
70:30	0.338	0.128	0.221	0.142
50:50	0.355	0.129	0.242	0.154
30:70	0.359	0.137	0.257	0.183
0:100	0.361	0.184	0.264	0.203
DMPC:SM	DPH		DSHA	
	15°C	35°C	15°C	35°C
70:30	0.318	0.106	0.098	0.068
50:50	0.267	0.110	0.100	0.071
30:70	0.241	0.124	0.101	0.076
0:100	0.234	0.148	0.104	0.090

[a] [Total lipids] = $1.3 \cdot 10^{-4}$ M, [DPH] = $1.3 \cdot 10^{-7}$ M, and [DSHA] = $1.3 \cdot 10^{-7}$ M.

TABLE III

p values of DMPC in the presence of DDPC or SM and glycophorin at different concentration ratios below or above T_c [a]

Composition [b] (μg)							
DMPC	DDPC	SM	glycophorin [c]	DPH		DSHA	
				15°C	30°C	15°C	30°C
264.4	105.6	–	–	0.338	0.128	0.221	0.142
246.4	105.6	–	7.4	0.348	0.130	0.120	0.054
246.4	105.6	–	14.6	0.362	0.135	0.196	0.083
246.4	105.6	–	28.1	0.348	0.141	0.204	0.087
246.4	–	106.8	14.6	0.317	0.106 [d]	0.111	0.064 [d]

[a] [Total lipids] $= 1.3 \cdot 10^{-4}$ M, [DPH] $= 1.3 \cdot 10^{-7}$ M, and [DSHA] $= 1.3 \cdot 10^{-7}$ M.
[b] The molar ratio of DDPC or SM to DMPC was kept constant at 30 mol%.
[c] Glycophorin was reconstituted by the Method A (see text).
[d] At 35°C.

surface. In the very hydrophobic domain of the membrane, however, the fluidity first increases upon the addition of DDPC up to approx. 50 mol%. Further addition of DDPC (more than 50 mol%) decreases the membrane fluidity again. However, in contrast to DDPC, the addition of sphingomyelin to DMPC causes more complex changes. Above the phase transition temperature of DMPC, an increase in the sphingomyelin content caused a decrease in the fluidity of both the membrane surface and the hydrophobic domain. Below the phase transition temperature (at 15.0°C), however, the increase in the sphingomyelin content brought about an increase in the fluidity of the hydrophobic domain and a decrease in that of the membrane surface (Table II). Sphingomyelin also has an amide bond in its acyl residue at the position close to the headgroup and a hydrogen-bonding network must be somewhat formed, leading to a decrease in the membrane fluidity. In any event, the present artificial lipid DDPC is much more able to make the lecithin membrane more rigid and stable than the naturally occurring lipid, sphingomyelin.

Furthermore, in order to understand the higher efficiency of DDPC in the reconstitution of glycophorin, fluorescence depolarization measurements were carried out in several systems which contain glycophorin in DDPC-DMPC or sphingomyelin-DMPC liposomes (Table III). First, when glycophorin was reconstituted into the DMPC liposome, the fluidity at the membrane surface was even if DDPC was present, increased both considerably below and above the phase transition. Second, the fluidity decreased with an increase in the glycophorin content. This trend was the same also in the hydrophobic domain of the membrane. On the other hand, the addition of sphingomyelin showed a signifi-

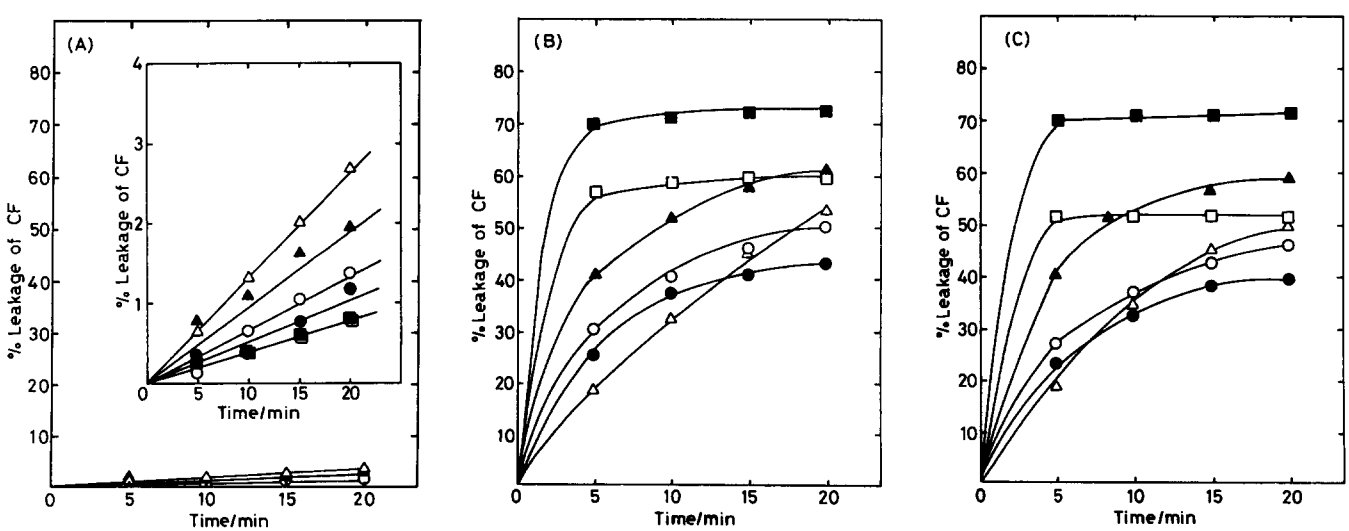

Fig. 6. Percent CF leakage from SUV ([total lipid] $= 1.3 \cdot 10^{-4}$ M) at 37.0°C and pH 7.4 (20 mM Tris-HCl containing 200 mM NaCl): △, egg PC; ▲, egg PC(60)/DDPC(40); ■, egg PC(60)/SM(40); ○, egg PC/glycophorin(0.2); ●, egg PC(60)/DDPC(40)/glycophorin(0.2); and □ egg PC(60)/SM(40)/glycophorin(0.2). (A) Spontaneus release of CF; (B) in the presence of serum (20% by vol.); and (C) in the presence of plasma (20% by vol.).

cant increase in the membrane fluidity. We, of course, understand that rationalization between fluorescence depolarization and microviscosity is somewhat difficult when the fluorescent probe employed behaves anisotropically. However, these results obtained in this work are totally coincident with those observed in the reconstitution efficiency of glycophorin [26] and the barrier function to CF release (vide infra).

(iii) Barrier function of DDPC-egg PC bilayer membranes. Physicochemical stabilities of DDPC-egg PC and sphingomyelin-egg PC bilayer membranes were investigated in the presence or absence of glycophorin using carboxyfluorescein (CF)-release technique [25] (Fig. 6).

In spontaneous release of CF from liposomes, as expected, the glycophorin-reconstituted DDPC-egg PC liposome depressed effectively the CF release under the controlled conditions. Even sphingomyelin-egg PC liposome was better than the simple egg PC liposome. Gupta and his co-workers also reported that a lecithin which has a carbamoyloxy group at the C-2 position instead of ester group is very effective for depressing transfer of lipids from liposome to high density lipoprotein in serum [45,46]. In the presence of human serum or plasma, both sphingomyelin-egg PC and DDPC-egg PC liposomes showed less barrier function than the conventional egg PC liposomes. Though the actual extent of CF-release is certainly increased in the presence of serum or plasma, the trend of depression in the CF-release was kept by the addition of both DDPC and glycophorin to egg PC. The reason why the barrier function of sphingomyelin-egg PC and DDPC-egg PC membranes in the presence of serum or plasma decreased is obscure at present. One possibility may be ascribed to the increased interaction between serum or plasma proteins and these modified liposomes.

As the result, DDPC was found to be effective for making egg PC or DMPC liposomes more physicochemically stable. In addition, a membrane protein is effectively reconstituted into liposomes by employing DDPC.

Acknowledgments

This work was supported by the Ministry of Education (Nos. 62604594, 62116003, and 61550681) and by Special Aid from the Asahi Glass Foundation for Industrial Technology (1987–1988).

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