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Deuterium nuclear magnetic resonance studies on the interaction of glycophorin with 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine

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Membrane dynamics of dimyristoylphosphatidylcholine (DMPC) lipid bilayer which contains glycophorin with an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC), was investigated by ^{2}H-NMR technique. For this purpose, both DMPC and DDPC were deuterated at the position of the 8th carbon atom of their acyl chains. Comparing, with DMPC bilayers, DDPC bilayers showed larger deuterium quadrupole splitting ($\Delta\nu_Q$) by approx. 2 kHz. This was explicable in terms of the stabilization of the membrane due to the formation of a strong hydrogen bonding in bilayers. Addition of glycophorin to the DMPC or DDPC single bilayers caused an increase in the $\Delta\nu_Q$ value. The $\Delta\nu_Q$ value of DMPC/DDPC mixed lipid bilayer was smaller than that of each single lipid bilayer. DDPC in the DDPC/DMPC mixed bilayer was not phase-separated but homogeneously distributed. In glycophorin-reconstituted DMPC-d_4/DDPC mixed bilayers, the $\Delta\nu_Q$ of DMPC-d_4 was almost identical to that of the simple DMPC-d_4 bilayer. On the other hand, the $\Delta\nu_Q$ of DDPC-d_4 in the DMPC/DDPC-d_4 mixed bilayer increased significantly upon the reconstitution of glycophorin. Judging from these data, we concluded that, in the DDPC/DMPC mixed bilayer which contains glycophorin, DMPC simply behaves as the matrix lipid, while DDPC surrounds glycophorin and certainly plays a role of the boundary lipid.

Introduction

Boundary lipid is defined as a motionally restricted lipid which is next to a membrane protein with a specific interaction and is distinguished from other bulk matrix lipids. The lipid-protein interaction has been actively investigated in various membranes since Jost et al. [1] have first suggested the existence of the boundary lipids around membranous cytochrome oxidase by ESR technique. In mammalian cell membranes, sphingomyelin has been speculated to be a candidate of the boundary lipids, and a possibility of intra- and/or intermolecular hydrogen bonding formation in sphingomyelin bilayers proposed by Schmidt et al. [2]. It has also been reported that most of membrane-binding enzymes show no activity where no boundary lipids exist [3]. The boundary lipid is considered to be greatly related to the function of biological membranes, and therefore, the investigation on lipid–protein interaction is very important in order to understand the roles of both lipids and proteins in membranes.

Glycophorin is a major and an intrinsic glycoprotein in human erythrocyte membranes. This protein (M_r, 31 000), consists of 131 amino acids [4] and oligosaccharides [5,6]. The hydrophobic domain from Ile-73 to Ile-95 is composed of 23 non-polar amino acids with an α-helical structure and buried in a lipid bilayer [7,8]. The interaction between glycophorin and phospholipids has been investigated by various techniques, such as

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPC-d_4, di([8,8-$^2H_2$]myristoyl)phosphatidylcholine; DDPC, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine; DDPC-d_4, 1,2-([8,8-$^2H_2$]myristoylamido)-1,2-deoxyphosphatidylcholine; DSC, differential scanning calorimetry; ESR, electron spin resonance spectroscopy; NMR, nuclear magnetic resonance spectroscopy; FTIR, Fourier-transform infrared spectroscopy; $\Delta\nu_Q$, quadrupole splitting; T_c, gel-liquid crystalline phase transition temperature; and S_{CD}, segmental order parameter of C-D bond.

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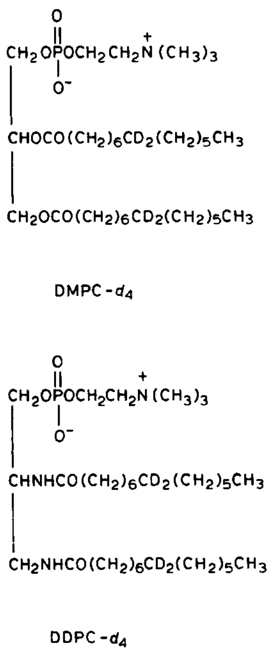


Plate 1. Structures of DMPC-d_4 and DDPC-d_4.

DSC [9], Raman spectroscopy [10], ESR [11], ^{1}H-NMR [12], ^{13}C-NMR [11,13], ^{31}P-NMR [14,15], FTIR [16] and so forth. Through these investigations, approximately 30–40 lipid molecules were proposed to behave as the boundary lipids of glycophorin. Most of lipids considered in these previous studies were, however, phosphatidylcholines bearing various acyl chains.

Very recently, we have prepared a novel artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) to make liposomes more stable and cell recognizable by reconstituting glycophorin into egg PC liposomes [17]. As expected, DDPC was better to keep glycophorin in egg PC liposomal membranes compared with a naturally occurring sphingomyelin [17,18]. It has been confirmed directly by FTIR investigation, in addition, that this better reconstitution efficiency of glycophorin into egg PC liposomes is due to the hydrogen bonding provided by DDPC in lipid bilayers and consequent membrane stabilization [19]. Because, DDPC is bearing an amide group in each of both acyl chains instead of the carboxylic ester group of ordinary dimyristoylphosphatidylcholine (DMPC). The DSC investigation for DDPC indicated the two endothermic peaks. The sharper one observed at 18.0 °C was assigned to the gel-liquid crystalline phase transition and another broad peak at 23.0 °C was ascribed to the release of hydrogen bonding between DDPC molecules [20].

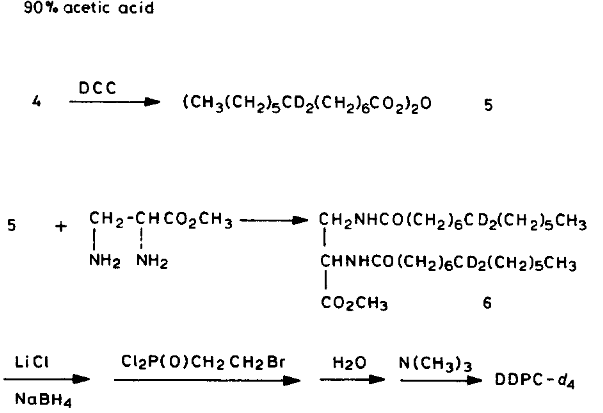
In this work, we carried out ^{2}H-NMR measurements in order to understand microscopic membrane dynamics of the DMPC/DDPC mixed bilayer in the presence or absence of glycophorin. For this purpose, first of all, we synthesized ^{2}H-labelled DMPC (DMPC-d_4) and DDPC (DDPC-d_4). Each acyl chain of both lipids was deuterated at the 8th position. Physical properties of the deuterio-phospholipids are exactly the same as those of the parent non-deuterated phospholipids and do not perturb the parent lipid membranes at all. This means that we do not perturb the parent lipid membranes at all. This means that we do not need to worry about the steric hindrance and changes in the micropolarity, fluidity, and the phase transition of membranes. Such a membrane perturbation is sometimes caused by the probe employed in ESR [21], Raman spectroscopy, or FTIR investigation [10].

Materials and Methods

Materials. Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma Chemical Co. and used without further purification. 1,2-Dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) was synthesized as described elsewhere [20]. The 8th carbon of each acyl residue of DMPC was deuterated according to the processes given in Scheme I.

Ethyl 8-ketomyristate was synthesized as described by Hubbell and McConnell [22]. In order to prepare [8,8-^{2}H$_2$]myristic acid (**4**) from ethyl 8-ketomyristate, we have somewhat modified the synthetic procedure of Heinz et al. [23].

Ethyl 8-hydroxy[8-^{2}H]myristate (1). Ethyl 8-ketomyristate (45.0 g, 0.166 mol) was dissolved in 400 ml of



Scheme I. Processes for preparing DDPC-d_4.

absolute ethanol. To the solution cooled on an ice bath was gradually added 5.80 g (0.139 mol) of $NaBD_4$ (Aldrich). After stirring for 1 h at room temperature, the reaction mixture was acidified to pH 3 with acetic acid, and ethanol was removed under reduced pressure. Diethyl ether (500 ml) and water (250 ml) were added to the residue and the ether phase was separated. The extract was washed twice with 200 ml of water and dried over anhydrous Na_2SO_4. The ester **1** was obtained as a colorless liquid by distillation at 150 C°/0.15 mmHg (yield 35.0 g, 77%) and the purity was inspected on TLC. IR (neat); 3400 (hydroxy) and 1740 cm^{-1} (carbonyl). ^{1}H-NMR; 1.70 ppm (hydroxy).

Ethyl 8-(p-toluenesulfonyl)[8-^{2}H]myristate (2). The ester **1** (33.0 g, 0.120 mol) was dissolved in dry pyridine (35 ml) and the resulting solution was kept at 6°C on an ice bath. *p*-Toluenesulfonyl chloride (26.0 g, 0.136 mol) was gradually added to the solution. After stirring at 10–15°C for 12 h, 327 ml of cold 9% aqueous hydrochloric acid was added. The tosylate was extracted with 300 ml of ether and the extract was washed with 100 ml of water three times. Then the organic phases was dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. The residue was applied to a silica gel column chromatography (Wako Pure Chemical, $\phi 2.5 \times 40$ cm) and eluted with benzene/diethyl ether (7:3, v/v). All the fractions were monitored by TLC (TLC Spot Film, Tokyo Kasei; developed by benzene/diethyl ether (7:3, v/v) and detected by iodine vapor) and the fractions which showed single spot at $R_f = 0.50$ were collected. The solvent was removed in vacuo to give a colorless liquid, **2**, (yield 45.0 g, 87%). IR (neat); 1730 (carbonyl), 1360, and 1170 cm^{-1} (sulfonyl). ^{1}H-NMR; 7.45 and 7.23 ppm (benzene ring).

[1,1,8,8-$^2H_4$]Myristyl alcohol (3). $LiAlD_4$ (Merck, 12.0 g, 0.285 mol) was suspended in dry diethyl ether (200 ml). Then, 500 ml of dry diethyl ether containing **2** (42.0 g, 0.098 mol) was added dropwise under stirring, and the resulting mixture was reacted under reflux for 4 h. After cooling on an ice bath, 280 ml of ether and 25 ml of water were carefully added. After removal of insoluble substances by filtration, the ether phase was separated, washed four times with 200 ml of water, and dried over anhydrous $MgSO_4$. After evaporation of ether, the solid materials obtained were dissolved in a small amount of benzene. Only the fractions containing a component of $R_f = 0.38$ (on TLC with benzene/diethyl ether (7:3, v/v) were collected and concentrated under reduced pressure to give a colorless liquid, 3: yield, 10.0 g (47%). Purity was inspected by IR and ^{1}H-NMR. IR (neat); 3300 cm^{-1} (hydroxy). ^{1}H-NMR; 1.44 ppm (hydroxy).

[8,8-$^2H_2$]Myristic acid (4). Alcohol **3** (10.0 g, 0.046 mol) was dissolved in 500 ml of acetic acid and, then, 180 ml of 90% aqueous acetic acid containing 23.0 g (0.23 mol) of CrO_3 was added. After stirring at room temperature for 18 h, the reaction mixture was poured onto 2 l of water. Precipitates produced were filtered and washed with water. Crude crystalline mass was suspended in 300 ml of water containing 60 g of NaOH and cooled on an ice bath. The produced precipitates were then filtered and washed with water. The residues were resuspended in 500 ml of water, acidified to pH 1 with 0.5 M HCl, and extracted twice with 300 ml of diethyl ether. Diethyl ether extracts were washed three times with 300 ml of water and dried over anhydrous Na_2SO_4. The ether solution was evaporated in vacuo to give solid materials. The crude compound was recrystallized from methanol; yield, 3.18 g (30%) and m.p. 54–55°C. IR (KBr); 1700 cm^{-1} (carbonyl). ^{1}H-NMR; 2.28 ppm (α-methylene of carboxylic acid).

For the synthesis of di([8,8-$^2H_2$]myristoyl)phosphatidylcholine (DMPC-d_4), egg yolk phosphatidylcholine was first deacylated according to the method of Brockerhoff and Yurkowski [24]. The obtained glycerophosphocholine was reacylated by deuterated fatty acid anhydride, **5**, as described by Robles and Van den Berg [25]. 1,2-Di([8,8-$^2H_2$]myristoylamido)-1,2-deoxyphosphatidylcholine (DDPC-d_4) was synthesized with **5** by the same method as that adopted for the synthesis of DDPC [20].

Glycophorin was isolated from human erythrocytes and purified as described in literature [26].

Preparation of samples for ^{2}H-NMR. (A) DMPC-d_4 and DDPC-d_4 bilayers. To the pure deuterated lipids placed in a NMR sample tube (ϕ6 mm) was added the same weight of deuterium depleted water (Aldrich). The tube was flame-sealed and gently centrifuged back and forth several times above the phase transition temperature of the lipid [27]. Thereafter, the sample tube was allowed to stand for 24 h on a water bath at 4°C.

(B) Reconstitution of glycophorin. A given amount of a mixture of lipid and glycophorin (100:1, by mol) were totally dissolved in chloroform/methanol/water (65:25:4, v/v) in a NMR sample tube. The solvent was removed using a stream of gaseous nitrogen and the mixture was allowed to stand overnight in vacuum for completely removing the solvent. Deuterium depleted water (equal amount to the total weight of the lipid-glycophorin mixture) was added and the sample tube was flame-sealed. The sample was then thoroughly suspended by gentle centrifugation and kept at 40°C for 24 h.

(C) DMPC/DDPC mixed bilayers. A mixture of DMPC-d_4 (80 mol%) and DDPC (20 mol%) or that of DMPC (80 mol%) and DDPC-d_4 (20 mol%) was dissolved in chloroform in a sample tube. The solvent was removed off under stream of nitrogen gas and the tube was kept in vacuo overnight to dryness. The same amount of deuterium depleted water to the total amount of lipids was added into the sample tube and the tube

was flame-sealed. The mixed sample was swelled under gentle centrifugation above the phase transition temperature and kept at 37 ° C for 24 h.

(D) Glycophorin-reconstituted DMPC / DDPC bilayers. 1 mol equivalent of glycophorin was mixed with 100 mol equivalents of DMPC-d_4 (80 mol%)/DDPC (20 mol%) or DMPC (80 mol%)/DDPC-d_4 (20 mol%) mixture dissolved in chloroform/methanol (13 : 5, v/v) in a NMR sample tube. After treated by exactly the same manner as that adopted in the method B, the sample mixture was kept at 37 ° C for 5 days to attain the thermodynamically equilibriated sample.

Deuterium nuclear magnetic resonance spectroscopy. ^{2}H-NMR measurements were performed by two different methods. (1) For the simple DMPC-d_4 or DDPC-d_4 membrane system, ^{2}H-NMR was determined on a Nicolet NIC-360 FT NMR spectrometer operating at 55 MHz (corresponding to a magnetic field strength of 8.5 T). Free induction decays (FIDs) were accumulated after 90 pulse duration of 32 μs. Deuterium quadrupole splitting spectra were obtained by Fourier transformation of the FIDs. (2) For the DMPC-d_4/DDPC or DMPC/DDPC-d_4 mixed system, deuterium Fourier-transform NMR spectra were obtained on a Jeol JNM GX-400 FT NMR spectrometer operating at 61.3 MHz (corresponding to a magnetic field strength of 9.4 T). Deuterium quadrupole echo technique was used for data acquisition. The duration of two 90 pulses was 4 μs and the interval between the two 90 pulses was 25 μs. No significant difference was observed in the quadrupole splitting values between the two techniques for the same sample determined on two different instruments.

Samples were incubated at a given temperature for at least 2 h and then inserted into a 10 mm NMR tube. The sample was kept at the same temperature in the NMR sample for another 15 min before measurement. No decomposed materials were detected at all on TLC even after the measurement.

Results and Discussion

Judging from the chemical structure of phospholipid molecule, we considered the terminal end of acyl chains is too flexible to monitor the difference in molecular motion of lipid molecules between the two systems with or without a membrane protein [28]. Similarly, it is also predictable that the position close to the hydrophilic head group of lipid is too insensitive to monitor the change in membrane dynamics upon the addition of a membrane protein [28]. In order to investigate the membrane dynamics and the lipid-protein interaction in bilayers, therefore, we decided to deuterate the 8th carbon atom of both *sn*-1 and *sn*-2 chains of parent DMPC and DDPC. ^{2}H-NMR gives two symmetric signals for lipid bilayers (Fig. 1). The deuterium quadrupole splitting, $\Delta\nu_Q$, is given by the following relationship:

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD} \qquad (1)$$

where (e^2qQ/h) is the static deuterium quadrupole splitting constant and S_{CD} is the order parameter of the carbon-deuterium bond. For a long alkyl chain, e^2qQ/h is approximately 170 kHz [29]. The order parameter, S_{CD}, is thus calculated from $\Delta\nu_Q$. On the other hand, S_{CD} is a function of the angle θ, between the direction of the static magnetic field and the carbon-deuterium bond:

$$S_{CD} = (1/2)\langle 3\cos^2\theta - 1\rangle \qquad (2)$$

where $\langle\rangle$ denotes the time average. Typical examples of the spectra are given in Fig. 1.

DDPC in water shows two endothermic peaks in DSC [20]. The sharper peak at 18.0 ° C was considered to be the gel-liquid crystalline phase transition, while the broad peak observed at 23.0 ° C was considered to relate with the hydrogen bonding provided by the amide bond of DDPC. Since the phase transition temperature, T_c, of DMPC is 23.9 ° C in water [30], there is relatively a large difference in T_c between DMPC and DDPC. The T_c of the deuterated lipid is not different from that of non-deuterated one. Therefore, we are able to discuss the quadrupole splitting of the lipid by using the reduced temperature, $\Theta(=(T-T_c)/T_c)$, where T is the experimental temperature (K) and T_c is the gel-liquid crystalline phase transition temperature (K) [31]. If the quadrupole splittings are plotted as a function of the reduced temperature, therefore, we do not need to con-

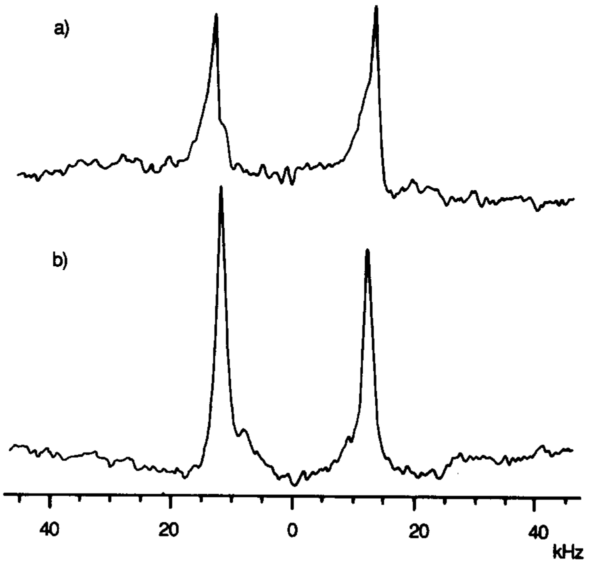


Fig. 1. Deuterium quadrupole splitting spectra of DDPC-d_4 (a) and DMPC-d_4 (b) bilayers at 36.0 ° C: (a) DDPC-d_4 (28 mg/28 mg of ^{1}H-H$_2$O); (b) DMPC-d_4 (35 mg/35 mg of ^{1}H-H$_2$O).

sider the difference in T_c. The correlation between $\Delta\nu_Q$ (or S_{CD}) and the reduced temperature will give information about the net interaction between lipid molecules in bilayers. Fig. 2 shows the quadrupole splittings ($\Delta\nu_Q$) and the order parameters (S_{CD}) of sample DMPC and DDPC bilayers as a function of Θ. It was rather difficult to obtain a clear ^{2}H-NMR spectrum below T_c. At 0.04 of the reduced temperature, the $\Delta\nu_Q$ of DDPC-d_4 bilayer was larger by approx. 2 kHz than that of DMPC-d_4 bilayer. The $\Delta\nu_Q$ of DMPC-d_4 and DDPC-d_4 bilayers becomes closer each other with a decrease in the reduced temperature. Regular lipids bearing carboxylic ester groups, in general, show almost the same $\Delta\nu_Q$ values in the ^{2}H-NMR even if their acyl chains and T_c values are different [31]. Observed data indicate that there certainly exists an additional interaction besides the hydrophobic interaction of hydrocarbon chains and the electrostatic interaction of choline moieties between DDPC molecules in bilayers. The most plausible interaction is the hydrogen bonding brought about by the amide group [19]. The amide group of DDPC can give intermolecular or intramolecular hydrogen bonding with or without water molecules. In any event, the present result indicates that the hydrogen-belt formed by hydrogen bonding makes lipid bilayers more ordered.

The existence of approx. 30–40 lipid molecules which are motionally restricted around a glycophorin molecule has been proposed from the results of ^{1}H-NMR [12], ^{13}C-NMR [11], and ^{31}P-NMR [15] measurements. When the molar ratio of lipid to glycophorin is fixed to 100 : 1, therefore, approx. 30–40 lipid molecules will locate around the protein and excess 60–70 lipid molecules behave as bulk lipids. On the other hand, the outer diameter of the α-helical segment of glycophorin in the hydrophobic domain of lipid bilayer is approx. 10–15 Å [32]. This means that the number of lipid molecules which can directly interact with glycophorin is probably less than 20. It is obvious, therefore, that the glycophorin does not affect only the lipids locating very next to the protein, but also further lipid molecules. In a single lipid bilayer membrane, exchange between lipids bound to the protein and bulk lipids certainly occurs even though the exchange rate is relatively slow [15]. Therefore, the observed quadrupole splitting is an average of those of boundary and bulk lipids. Thus, the higher the content of DDPC as the boundary lipid is, the more difficult the precise observation of the lipid–protein interaction becomes. In order to correctly observe the direct lipid–protein interaction and to obtain a change in the quadrupole splitting as large as possible, in this work, the molar ratio of DMPC to DDPC in the DMPC/DDPC mixed bilayers was fixed at 8):20.

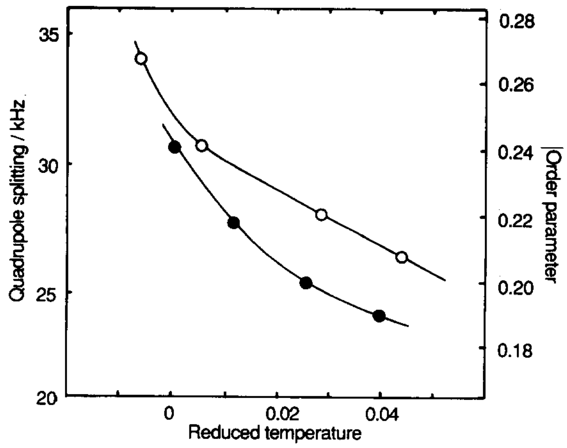


Fig. 2. The quadrupole splittings and the order parameters of deuterated DMPC and DDPC bilayers as a functions of the reduced temperature; ●, DMPC-d_4 and ○, DDPC-d_4.

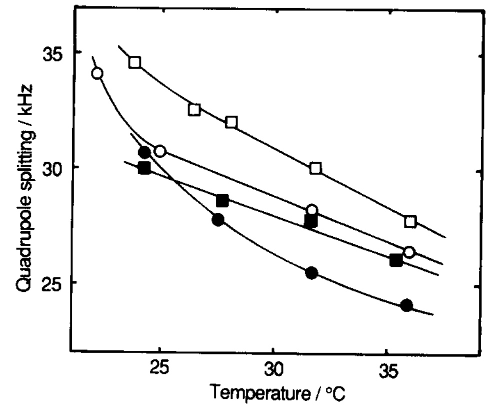


Fig. 3. Effect of reconstitution of glycophorin to DMPC-d_4 and DDPC-d_4 bilayers. The quadrupole splittings and the order parameters as a function of temperature; ●, DMPC-d_4; ○, DDPC-d_4; ■, DMPC-d_4/glycophorin (100 : 1, mol/mol); and □, DDPC-d_4/glycophorin (100 : 1, mol/mol).

Fig. 3 shows the effect of glycophorin on the quadrupole splittings and the order parameters of DMPC-d_4 and DDPC-d_4 bilayers. Above 25.0 ° C, the addition of glycophorin to DMPC-d_4 bilayers caused an increase in the $\Delta\nu_Q$. Below 25.0 ° C, the addition of protein made the $\Delta\nu_Q$ value of DMPC-d_4 bilayers smaller. This is related with the fact that the phase transition temperature of DMPC decreased or is abolished upon the addition of glycophorin [10]. On the other hand, the addition of glycophorin to DDPC-d_4 bilayers increases the $\Delta\nu_Q$ by approx. 2 kHz over the whole temperature region examined. Namely, Fig. 3 reveals that the addition of glycophorin above T_c makes DMPC and DDPC bilayers more ordered.

The acyl chains of membraneous lipids are taking *trans* and *gauche* conformations above T_c. If the bulk matrix lipids are not affected by the addition of the protein, the lipid closest to the protein (namely, the boundary lipid) should show smaller tumbling of the hydrocarbon chain. Consequently, this lipid will take more *trans* conformations than other bulk lipids, leading to an increase in the averaged quadrupole splitting of the lipid bilayers. The ^{2}H-NMR spectra and derived $|S_{CD}(i)|$ values can also be interpreted using a relatively simple statistical method [28,33] to provide an estimate of the average or effective length of the acyl chains, $\langle L \rangle$, projected along the bilayer normal, to a first approximation. The observed $|S_{CD}(i)|$ profiles can be used to estimate the mean chain length $\langle L \rangle$ relative to the all-*trans* reference state:

$$\langle L \rangle = l\left[\left(\frac{n-m+1}{2}\right) - \sum_{i=m}^{n-1} S_{CD}(i) - 3S_{CD}(n)\right] \tag{3}$$

where n is the number of carbon atoms in the fatty acyl chain.

Contributions to the effective chain length from segments m through n are included, so that $\langle L \rangle$ is taken as extending from $(m-1)$th carbon atom to the terminal nth carbon atom. Based on geometrical considerations, the value of the segmental order parameter $S_{CD}(i)$ for the ith carbon atom is presumed to be negative [34]. The projected length of an individual chain segment in the all-*trans* reference state is given by $l = 1.25$ Å [33]. Clearly from Eqns. 1 and 3 and experimental results of Fig. 3, in any event, it is evident that if the $S_{CD}(i = 8)$ of the 8th carbon atom increases upon the addition of glycophorin the effective length of the lipid adjacent to glycophorin in the lipid bilayer.

In order to look at the order of only one of two different lipids, one of the two lipids mixed was deuterated and its $\Delta\nu_Q$ was determined. Fig. 4 shows the respective comparison of the quadrupole splittings and the order parameters of simple DMPC and DDPC bilayers and DMPC/DDPC mixed bilayers. For both DMPC-d_4/DDPC and DMPC/DDPC-d_4 bilayers, their $\Delta\nu_Q$ values decreased over the range of temperatures studied compared with simple DMPC-d_4 or DDPC-d_4 bilayers. In the mixed lipid bilayers, the gel-liquid crystalline phase transition did not exist in this temperature region, since the T_c of DMPC/DDPC (80 : 20, by mol) mixed system was about 22° C [20]. The fact that the phase separation does not take place in the DDPC-DMPC mixed membrane has been also revealed in DSC studies on the same system [20]. These results suggest to us that DDPC homogeneously disperses in DMPC bilayers, and this homogenous dispersion leads to a decrease in the orderness of the hydrophobic domain of the membrane. Because, if we assume a possibility of the phase separation of DDPC from the matrix DMPC in the mixed bilayers, the $\Delta\nu_Q$ values of DMPC-d_4/DDPC and DMPC/DDPC-d_4 bilayers should give almost the same values as those observed in the respective single-lipid bilayers. These present results are completely coincident with those obtained from DSC and fluorescence depolarization measurements [20].

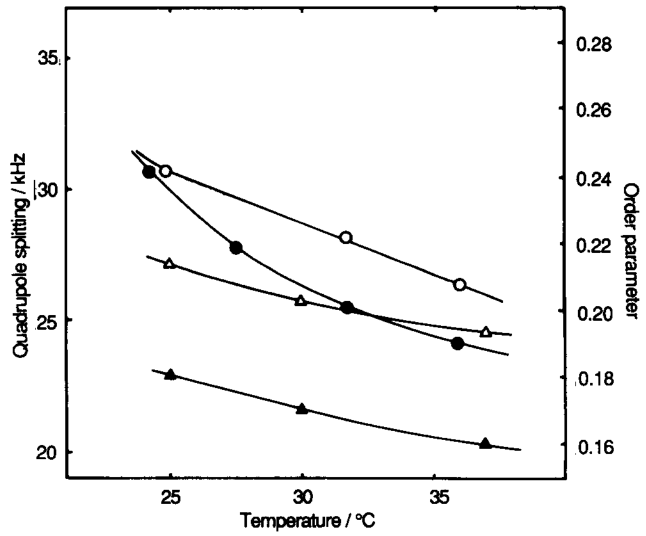


Fig. 4. The quadrupole splittings and the order parameters of single lipid bilayers and DMPC-DDPC mixed bilayers as a function of temperature; ●, DMPC-d_4; ○, DDPC-d_4; ▲, DMPC-d_4/DDPC (80 : 20, mol/mol); and △, DMPC/DDPC-d_4 (80 : 20, mol/mol).

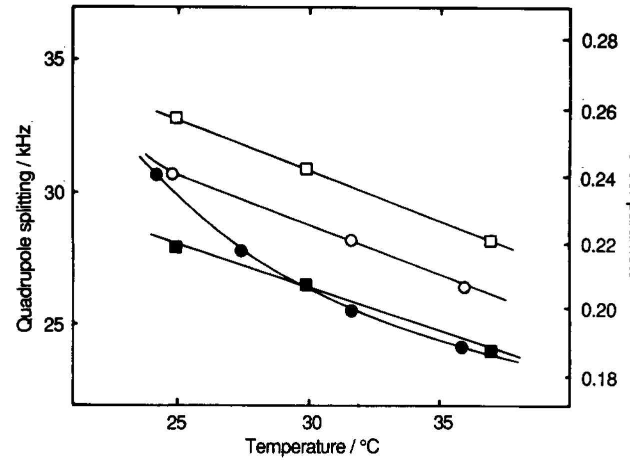


Fig. 5. The quadrupole splittings and the order parameters of pure lipids and DMPC/DDPC/glycophorin as a function of temperature; ●, DMPC-d_4; DDPC-d_4; ■, DMPC-d_4/DDPC/glycophorin (80 : 20 : 1, mol/mol); and □, DMPC/DDPC-d_4/glycophorin (80 : 20 : 1, mol/mol).

Here is another interesting finding that the difference in the $\Delta\nu_Q$ between simple DMPC-d_4 system and DMPC-d_4 (80 mol%)/DDPC (20 mol%) system is larger than that between simple DDPC-d_4 system and DDPC-d_4 (20 mol%)/DMPC (80 mol%) system (Fig. 4). This means that, when we look at the mobility of only one of the two lipids in the mixed lipid system (not the total and averaged mobility), the mobility of DMPC is larger than that of DDPC even if the amount of DMPC is always 4-times excess to that of DDPC and both lipids are homogeneously mixed and distributed in the bilayers.

Fig. 5 shows the quadrupole splittings of glycophorin reconstituted membranes; the DMPC/DDPC mixed bilayer and the simple DMPC or DDPC bilayer. In the DMPC/DDPC-d_4/glycophorin system, the Δn_Q value of DDPC-d_4 at 37°C was larger compared with the simple DDPC-d_4 bilayer system. On the other hand, the $\Delta\nu_Q$ of DMPC-d_4 in DMPC-d_4/DDPC/glycophorin system showed almost the same value as that in the simple DMPC-d_4 bilayer system at 37°C. If the two lipids distribute homogeneously in the DMPC/DDPC mixed lipid system even in the presence of glycophorin, both the two lipids should interact equally with glycophorin. Namely, considering the results in Fig. 3, it is supposed that $\Delta\nu_Q$ values of both lipids will increase only 2 kHz by reconstitution of glycophorin. It seems, therefore, reasonable to consider that, in the DMPC/DDPC mixed bilayer with glycophorin, DDPC predominantly associates with glycophorin and DMPC plays a role as the bulk matrix lipid. Regarding the averaged fluidity of membrane, p value measured by fluorescence depolarization technique also supports this hypothesis [20]. Through the present ^{2}H-NMR experiments, it was revealed that DDPC bearing amide bonds certainly behaves as the boundary lipid for a membrane protein in the DMPC/DDPC mixed lipid bilayer. This is a key to clarify the reason why glycophorin can be effectively reconstituted upon the addition of DDPC in phospholipid bilayers [17,18,20].

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