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LIPOSOMAL MEMBRANES. XV

IMPORTANCE OF SURFACE STRUCTURE IN LIPOSOMAL MEMBRANES OF GLYCEROGLYCOLIPIDS

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To elucidate the importance of the headgroup structure of glycolipids in the physicochemical properties of liposomal membranes of glycolipids, two diglucosyldialkylglycerols containing an $\alpha(1' \rightarrow 4')$ or a $\beta(1' \rightarrow 4')$ glucoside linkage, 1,2-dihexadecyl-*O*-β-D-maltosyl(1′→3)-*rac*-glycerol (MAL-DG) and 1,2-dihexadecyl-*O*-β-D-cellobiosyl(1′→3)-*rac*-glycerol (CEL-DG) were employed. The fluorescence spectra and steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in the deep hydrophobic domain of these liposomal bilayers and dansylhexadecylamine in the vicinity of glycerol backbone were measured, respectively. Compared with dipalmitoylphosphatidylcholine (DPPC) liposomes, the phase-transition temperatures (T_c) of the present two glycolipid liposomes were about 11–15°C higher and the fluidity at the surface of these glycolipid liposome was considerably lower. This means that the interaction between neighboring diglucoside headgroups may be stronger than that between phosphatidylcholine headgroups. Fluorospectrometric and CPK model studies suggested that the structural difference in anomerization and epimerization of the disaccharide moiety of glyceroglycolipids is an important factor for determining the physicochemical properties of these glycolipid liposomes.

Introduction

Glyceroglycolipids are known to be widely present in plant tissues [1], bacteria [2] and mycoplasma membranes [3]. For example, the chloroplast membranes contain mono- and digalactosyldiacylglycerols as major constituents [1,4,5], while *Acholeplasma laidlawii A* membranes involve mono- and diglucosyldiacylglycerols [3,6] (see Fig. 1). Unlike phospholipids, however, the individual role of these glyceroglycolipids in membranes is not yet fully understood [1,7–10]. This may be the reason for the small amount of information about the precise chemical structure of those lipids and about the phase behavior of pure glyceroglycolipid membranes. The phase behavior of unsaturated mono- and digalactolipids extracted from thylakoid membranes was investigated by X-ray diffraction and freeze-fracture electron microscopy, which showed that in water they form a hexagonal-type and lamellar structure [11,12]. Recently, Williams and his co-workers have demonstrated that mono- and digalactolipids bearing distearoyl moieties also afford an open sheet-like structure of bilayers and multilamellar liposomes like phosphatidylcholines [13]. Similarly,

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Abbreviations: MAL-DG, 1,2-dihexadecyl-*O*-β-D-maltosyl(1′→3)-*rac*-glycerol; CEL-DG, 1,2-dihexadecyl-*O*-β-D-cellobiosyl(1′→3)-*rac*-glycerol; DPPC, dipalmitoyl-DL-α-phosphatidylcholine.

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mono- and diglucosyldiacylglycerols containing an $\alpha(1' \rightarrow 2')$ glucoside linkage (see Fig. 1) were found to form hexagonal and liposomal bilayer structures in an aqueous system [14]. To clarify the correlation between the structure of the saccharide moiety and physicochemical properties of bilayer membranes of glycolipids, three of the present authors (T.E., K.I. and S.N.) have recently synthesized two diglucosyldialkylglycerols containing an $\alpha(1' \rightarrow 4')$ or a $\beta(1' \rightarrow 4')$ glucoside linkage, MAL-DG (1,2-dihexadecyl-*O*-β-D-maltosyl(1′ → 3)-*sn*-glycerol) and CEL-DG (1,2-dihexadecyl-*O*-β-D-cellobiosyl(1′ → 3)-*sn*-glycerol) (Fig. 1) [15]. In this work, the physicochemical properties of liposomal bilayers of these diglucosyldialkylglycerols were investigated by the steady-state fluorescence depolarization and fluorescence spectroscopy. For this purpose, two different kinds of fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene and dansylhexadecylamine, were employed. The former is known to be localized in the very hydrophobic domain of bilayers in biological and artificial membranes [16–18], while the dansyl moiety of the latter probe is close to the surface of membranes; namely, in the vicinity of glycerol backbone of the lipids [19–21].

Recently, a nanosecond time-resolved fluorescence anisotropy method has been developed and it has been pointed out that the conventional and simple interpretation in terms of 'microviscosity' for the fluidity of lipid membranes must be revised, since the steady-state fluorescence anisotropy of the lipid bilayer membranes implies both the dynamic term (viscosity) and the static term (order) of the probe [22–26]. Later, however, it has been proposed that steady-state fluorescence anisotropy measurements of diphenylhexatriene in biological and liposomal membranes can be still utilized to estimate approximately the order parameter (S) [27–29]. On the other hand, fluorescence spectra and steady-state fluorescence anisotropy of dansylhexadecylamine are able to provide information about the microscopic polarity and fluidity around the probe [19,20]. Hence, in this work we adopted these fluorescence spectroscopic techniques to elucidate the importance of the interaction between neighboring disaccharide moieties for glyceroglycolipids to maintain the stable bilayer structure.

Materials and Methods

Materials Detailed procedures for the synthesis and purification of two diglucosyldialkylglycerols, MAL-DG and CEL-DG, are described elsewhere [15]. Dipalmitoyl-DL-α-phosphatidylcholine (DPPC) and sodium dihexadecylphosphate were purchased from Sigma Chemical Co., St Louis, MO. 1,6-Diphenyl-1,3,5-hexatriene was obtained from Aldrich Chemical Co., Milwaukee, WI. Dansylhexadecylamine was the same as that used in a previous work [21].

Preparation of liposomes. Diphenylhexatriene or dansylhexadecylamine encapsulated liposomes were formed and isolated by the same procedures as those described previously [21,30,31]. An appropriate amount of dry chloroform solution containing lipid, dihexadecylphosphate, and fluo-

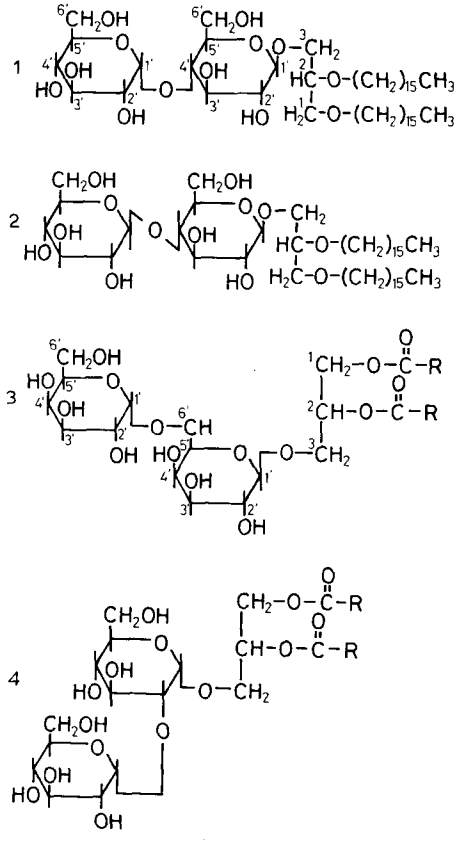

Fig. 1. Structures of various glycolipids. 1, 1,2-dihexadecyl-*O*-β-D-maltosyl(1′→3)-*rac*-glycerol (MAL-DG); 2, 1,2-dihexadecyl-*O*-β-D-cellobiosyl(1′→3)-*rac*-glycerol (CEL-DG); 3, digalactosyldiacylglycerol (from thylakoid membrane); 4, diglucosyldiacylglycerol (from *A. laidlawii*).

rescent probe was placed in a pear-type flask and the solvent was removed under reduced pressure using a vacuum rotary evaporator. Usually, an amount of dihexadecyl phosphate was maintained at 10 mol% to that of lipids. The molar ratios of the fluorescent probes to lipids were maintained at 1 : 200 for diphenylhexatriene and 1 : 100 for dansylhexadecylamine, respectively, through all the runs. Under these conditions, undesirable effects of these fluorescent probes on the fluorescence depolarization measurements were not observed at all, since it is well established that a ratio of lipid to diphenylhexatriene or dansylhexadecylamine as low as 50 has no effect on the fluorescence depolarization results [20,21,31,32]. After evaporating the solvents, the remaining thin film was dispersed in pure water by shaking on a vortex mixer with glass beads. For glyceroglycolipids, addition of any inorganic salts had to be avoided because it gave rise to the precipitation of lipid aggregates. Liposome suspensions were subjected to ultrasonic irradiation under nitrogen atmosphere using a Tomy UR-200P probe-type sonifier at 25 W for 10 min at 1 min intervals at a temperature about 10°C higher than the corresponding phase-transition temperature of the lipids employed. The resulting liposome suspensions were filtrated at 20°C by passing through a Sephadex G-50 column ($\phi 0.8 \times 21$ cm) pre-equilibrated with pure water, by which almost optically clear suspensions of small unilamellar liposomes were obtained. The concentration of liposomes of DPPC was determined as inorganic phosphate according to the procedure of Allen [33].

Fluorescence measurements. For steady-state fluorescence anisotropy measurements, the concentration of the probe entrapped liposome suspensions was kept constant at $1.6 \cdot 10^{-5}$ M on the basis of the probe concentration estimated from the fluorescence intensity, where no interference caused by light scattering on fluorescence anisotropy measurements was observed [34,35]. All the fluorescence measurements were essentially the same methods as those described before [21,31,36,37], and were carried out on a Hitachi 650-10S fluorescence spectrophotometer equipped with a thermoregulated cell compartment connected to a Toyo Thermo Electric TE-104S and on a Union Giken FS-501S fluorescence polarization spectrophotometer combined to a Komatsu-Yamato Coolnics Model CTR-120. A Sord Microcomputer M 200 Mark-II system was interfaced to the instrument to control measurement conditions and collect all the data. Emission at 430 nm from diphenylhexatriene excitation at 360 nm was collected for fluorescence anisotropy measurements using a sharp cut-off filter L-39 (Hoya Glass Works, Tokyo) to eliminate the light of wavelength below 370 nm. Though the emission maximum of dansylhexadecylamine was changed sensitively due to the microenvironment around the probe, for fluorescence anisotropy measurements a Y-46 cut-off filter was employed to cut out the light below 440 nm.

The steady-state fluorescence anisotropy of diphenylhexatriene, r_s, can be obtained from Eqn. 1:

$$r_s = \frac{I_{VV} - C_f I_{VH}}{I_{VV} + 2C_f I_{VH}} \quad (1)$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of the excitation (first) and analyzer (second) polarizers, respectively. C_f ($= I_{HV}/I_{HH}$) is the grating correction factor. r_s can be alternately described by Eqn. 2 [18,19]:

$$r_s = \frac{r_0 - r_\infty}{1 + (\tau/\phi)} + r_\infty \quad (2)$$

where τ and ϕ are the fluorescence lifetime and rotational relaxation time, respectively. r_0 and r_∞ stand for the maximal and limiting fluorescence anisotropies, respectively. Hence, the first term of Eqn. 2 represents a dynamic part (r_f) concerning with a fast decaying or kinetic component and is proportional to the 'microscopic viscosity', while the second term is an infinitely slowly decaying component and proportional to the square of the lipid order parameter (S) as determined by the membrane anisotropy (structural order) [27,28]:

$$r_\infty = r_0 \cdot S^2 \quad (3)$$

For diphenylhexatriene, r_0 has been estimated to be 0.395 by Kawato et al. [22] using nanosecond time-resolved fluorescence technique. In addition, very recently, Van Blitterswijk and his co-workers [29] have proposed that there exists a linear correlation between r_∞ and r_s over a range of $0.13 < r_s <$

0.28 as given by Eqn. 4:

$$r_\infty = \tfrac{4}{3} r_s - 0.10 \quad (4)$$

Namely, they showed a possibility of the empirical resolution of the steady-state fluorescence anisotropy (r_s) of diphenylhexatriene entrapped in liposomes into the kinetic (r_f) and the structural (r_∞) components as shown in Eqn. 2. Under the condition where Eqn. 4 is valid, thus, r_∞ can be estimated from r_s. Hence, in this work, we adopted Eqn. 4 to estimate the order parameter, S, of MAL-DG, CEL-DG, and DPPC liposomal membranes from the steady-state fluorescence anisotropy of diphenylhexatriene embedded in these liposomes.

Results

As in the case of digalactosyldiacylglycerol and $\alpha(1' \rightarrow 2')$-diglucosyldiacylglycerol [13,14], synthesized diglucosyldialkylglycerols MAL-DG and CEL-DG were found to form a closed bilayer structure (liposome) in water and to be able to trap glucose in their interior core [15]. In contrast to liposomes of phosphatidylcholines, however, those of synthesized glycolipids show the tendency to precipitate spontaneously. Thus, we had to introduce 10 mol% of dihexadecyl phosphate to glycolipids in order to cause an electrostatic repulsion between liposomes, by which no aggregation of liposomes themselves was observed. Of course, adding such an amount of dihexadecyl phosphate produced no significant effect on their phase-transition temperature (T_c), the size of liposomes (200–500 Å), and the fluorescence depolarization [15]. In addition, no significant difference was observed in the leakiness and trapped volume among three liposomes of DPPC, MAL-DG, and CEL-DG [15].

The steady-state fluorescence anisotropy (r_s) of diphenylhexatriene embedded in the synthesized glycolipid liposomes was determined as a function of temperature compared with that in DPPC liposomes (Fig. 2). For all the three liposomes, an abrupt change in r_s was observed around the respective phase-transition temperature. Differential scanning calorimetric (DSC) measurements revealed that gel-liquid crystalline phase-transition temperatures of DPPC, MAL-DG, and CEL-DG

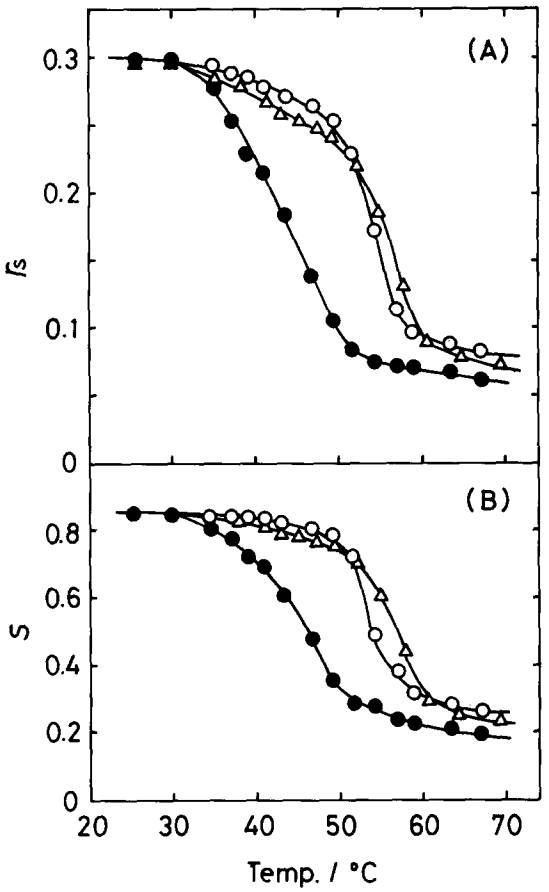

Fig. 2. Steady-state fluorescence anisotropy (r_s) (A), and order parameter (S) (B) of 1,6-diphenyl-1,3,5-hexatriene in three liposomal membranes as a function of incubation temperature. All the liposomes contain 10 mol% of dihexadecyl phosphate to control the surface charge of membranes throughout all the experiments (see text). ○–○, MAL-DG; △–△, CEL-DG; ●–●, DPPC.

liposomal membranes were 41°C (with $\Delta H = 8.65$ kcal · mol^{-1}), 52°C (5.95 kcal · mol^{-1}) and 54°C (5.95 kcal · mol^{-1}), respectively, and thermal phase transitions of the latter two were broader than that of DPPC [15]. Hence, their respective T_c values as estimated from the r_s-temperature profile were in good agreement with those obtained from the DSC measurement. The order parameter S calculated from r_s according to the procedure of Van Blitterswijk also showed an inflection point around the phase transition temperature for all the three liposomes investigated (Fig. 2). In the liquid-crystalline state the order parameter of synthesized diglucosyldialkylglycerol liposomes was larger than

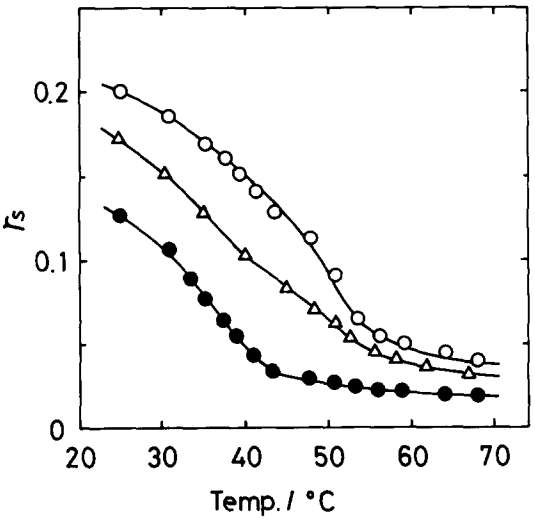

Fig. 3. Steady-state fluorescence anisotropy (r_s) of dansylhexadecylamine in three liposomal membranes as a function of incubation temperature. ○–○, MAL-DG; △–△, CEL-DG; ●–●, DPPC.

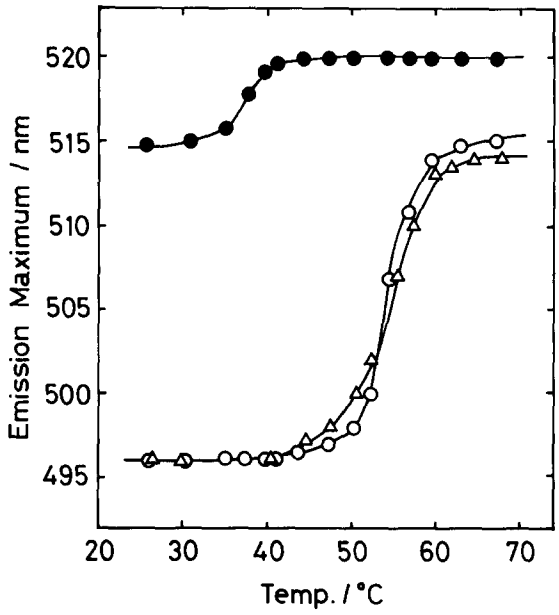

Fig. 4. Emission maximum from dansylhexadecylamine in three liposomal membranes as a function of incubation temperature. ○–○, MAL-DG; △–△, CEL-DG; ●–●, DPPC.

that of DPPC liposomes, while in the gel state below 30°C no significant difference in S values was observed between phosphatidylcholine and glycolipid liposomes.

Fig. 3 shows the steady-state fluorescence anisotropy of dansylhexadecylamine intercalated in three liposomal membranes as a function of incubation temperature. Obvious changes in r_s were again observed around their respective phase-transition temperatures, though they were smaller than those observed for diphenylhexatriene. The r_s values of DPPC liposomes were always smaller compared with those of other two glycolipid liposomes over a whole range of incubation temperatures adopted. Interestingly, unlike in the case of the probe diphenylhexatriene, the r_s values obtained by dansylhexadecylamine revealed a difference in phase behavior between MAL-DG and CEL-DG liposomes. In both the gel and liquid-crystalline states, r_s of the former liposome was larger than that of the latter (Fig. 3).

The emission maximum of dansylhexadecylamine embedded in liposomes was also determined for all the three liposomes as a function of incubation temperature (Fig. 4). The fluorescence characteristics of dansylhexadecylamine are known to be sensitive to the microenvironment around the probe and the dansyl fluorophore is located in the vicinity of glycerol backbone of lipids in liposomal bilayers [19–21]. Therefore, it is expected that the emission maximum of dansylhexadecylamine in liposomes will inform the micropolarity around the surface of liposomal membranes. The emission maxima of dansylhexadecylamine entrapped in two diglucosyldialkylglycerol and DPPC liposomes were 496 and 515 nm, respectively, in their gel state (Fig. 4). This means that the micropolarity around the probe in glycolipid liposomes is comparable to that of ethanol and is more hydrophobic than that in DPPC liposomes [21].

Discussion

As summarized in Table I, for the present diglucosyldialkylglycerol and DPPC liposomes, their phase-transition temperatures estimated by three different ways, namely, from r_s using two different fluorescent probes, from the fluorescence maxima of dansylhexadecylamine, and from DSC measurement [15], are in good agreement irrespective of the method. This means that the fluorescence spectroscopic techniques used in this work are reliable enough for study of the phase behavior of lipo-

TABLE I

PHASE-TRANSITION TEMPERATURES OF THE TWO DIGLYCOSYLDIALKYLGLYCEROL AND DIPALMITOYLPHOSPHATIDYLCHOLINE LIPOSOMES BY DIFFERENT TECHNIQUES

Method	Phase-transition temperature of liposomes (°C)		
	MAL-DG	CEL-DG	DPPC
Emission maxima of dansylhexadecylamine	54	55	39
Fluorescence anisotropy of dansylhexadecylamine	52	53	38
Fluorescence anisotropy and order parameter of diphenylhexatriene	53	55	42
Differential scanning calorimetry	52	54	41

somal membranes. The phase-transition temperature of DPPC liposomes was about 11–15°C lower than those of the two glycolipids, MAL-DG and CEL-DG; nevertheless, all the three lipids bear hydrophobic legs almost comparable in length and size. Recently, Bittman et al. [38,39] have reported that the replacement of the ether bond for the ester bond in DPPC does not cause a significant change in either the permeation property or the phase-transition temperature of the liposomes, where the ether analogue showed only about 1.8°C higher phase transition compared with the ester lipid. Our synthesized glycolipids carry the diether glycerol linkage for the diester glycerol one in DPPC. However, their phase-transition temperatures higher than that of DPPC must be ascribed to the difference in the headgroup structure, not to the fashion of the linkage between the sugar and glycerol moieties. The interaction between neighboring diglucoside headgroups may be stronger than that between the phosphatidylcholine headgroups. It has been already demonstrated that the phase transition of 1,2-distearoyldigalactosyldiacylglycerol, which is prepared by reducing the unsaturated galactolipid isolated from chloroplasts of bean leaves, is observed at about 51°C, close to the phase-transition temperature of distearoylphosphatidylcholine (54.2°C) [13] and the phase behavior of digalactosyldiacylglycerol from *Pelargonium* leaves also resembles that of

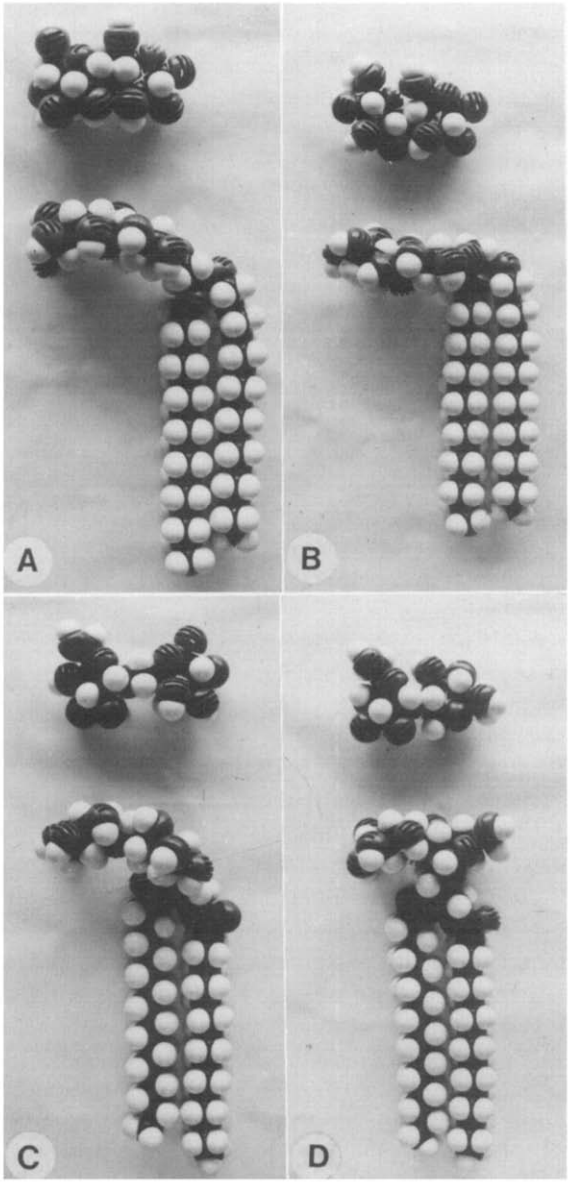

Fig. 5. The CPK molecular model building of various glycolipids: (A) MAL-DG, (B) CEL-DG, (C) digalactosyldiacylglycerol from thylakoid membrane, and (D) diglucosyldiacylglycerol from *A. laidlawii* (see Fig. 1). In models C and D, acyl residues are built as a heptadecanoyl group. The upper picture is a top view of the disaccharide headgroup, while the lower is a side view of the glycolipids, respectively, in each frame.

phosphatidylcholines [12]. On the other hand, the phase-transition temperature of diglucosyldiacylglycerol (extracted from *Acholeplasma laidlawii*) containing an elaidic acid residue in its acyl chain is close to that of dielaidoylphosphatidylethanolamine, but about 30°C higher than that of the phosphatidylcholine bearing an acyl chain of similar length [14]. Considering these previous and present results, if all these lipids carry acyl chains of similar length and size, the diglucolipids such as diglucosyldiacylglycerol isolated from *A. laidlawii* may afford the most rigid surface structure resulting in the highest phase-transition temperature. In addition, it is also known that the hydration capacity of diglucosyldiacylglycerol from *A. laidlawii* (13% w/w) is substantially lower than that of digalactosyldiacylglycerol (22% w/w), but close to that of phosphatidylethanolamine [14]. Taken altogether, it may be considered that the diglucoside part of glycolipids resembles the phosphatidylethanolamine moiety in phase behavior such as phase transition and/or hydration capacity, while the digalactoside structure resembles the phosphatidylcholine residue. Such a difference in phase behavior arising from the headgroup structure very probably comes from the structural dissimilarity in the stereoisomers: namely, anomer and epimer.

The CPK-molecular model helps us to elucidate the relationship between the structural characteristics of the headgroup and phase behavior (Fig. 5). It has been proposed that the diglucoside residue of 1,2-diacyl-[*O*-D-glucopyranosyl-(1′ → 2′)-*O*-α-D-glucopyranosyl(1′ → 3)]-*sn*-glycerol extracted from *A. laidlawii* membranes aligns approximately parallel to the bilayer surface [14,40], similar to the alignment in the phosphatidylcholine headgroups [41]. Certainly, disaccharide headgroups of MAL-DG, CEL-DG, and digalactosyldiacylglycerol all seem to prefer a configuration to align parallel to the bilayer surface (Fig. 5). At the surface of digalactosyldiacylglycerol liposomes compared with the diglucosyldiacylglycerol liposomes, however, more hydroxyl groups can be exposed to the bulk aqueous phase because of an epimerization at the C-4′ position of the glucose unit, which should bring about a difference in the hydration capacity [12,14]. As expected, the micropolarity around the membrane surface estimated from the emission maximum of dansylhexadecylamine indicates less hydration for the two glycolipids, MAL-DG and CEL-DG, in both the gel and liquid-crystalline states compared with that of DPPC (Fig. 4). In addition, the red shift in the emission maximum of dansylhexadecylamine above the phase-transition temperature indicates greater hydration of the membrane surface with the phase transition from the gel to liquid-crystal. These interpretations are strongly supported also by the amount of bound water to various saccharides estimated from ultrasonic velocity [42] and heat capacity measurements [43] in an aqueous solution containing saccharides. Previous results indicate that the sequence of the extent in binding of water to saccharides (glucose < galactose ≤ maltose < cellobiose) is consistent with our present assumptions and results.

We must consider several additional points in the structural characteristics affecting the phase behavior. The molecular model also suggests that the disaccharide headgroup structure, in which two sugar units are linked with a 1′ → 6′ bond, is rather flexible, unlike the structure which has a 1′ → 4′ or a 1′ → 2′ linkage, even though they all have the same linkage between the sugar and glycerol units (a 1′ → 3 linkage). The flexibility in the headgroup structure of digalactosyldiacylglycerol must be closely related to the relatively high hydration capacity and low phase-transition temperature of this lipid [13]. In the present diglucosyldialkylglycerols, MAL-DG and CEL-DG, the sugar units of which are linked by a 1′ → 4′ bond, the total distance between the glycerol backbone and terminal glucose moiety is longer than that in those with a 1′ → 2′ linkage. As a result, the diglucoside headgroup with an α(1′ → 2′) linkage seems to provide more compact and rigid surface structure, resulting in the lower hydration capacity and higher phase-transition temperature. When the difference in the phase behavior is elucidated, the problem of the anomeric form of sugar may be taken into account. DPPC liposomes are certainly more fluid than MAL-DG and CEL-DG. But, CEL-DG showed a slightly higher fluidity than MAL-DG in all the experiments. Cellobiose has a β(1′ → 4′) linkage between glucose units, while maltose has α(1′ → 4′). In Fig. 5 all the molecular models are built considering that hydroxyl groups will possibly face the bulk aqueous phase. Clearly from the model building, the anomeric isomeriza-

tion leads to the difference in the surface alignment between the two lipids (Fig. 5). A slight difference in the phase behavior between MAL-DG and CEL-DG might arise from the anomerization at the sugar-sugar linkage.

When diphenylhexatriene was employed to monitor the phase behavior of liposomal membranes, there existed no significant difference in r_s and S among the three liposomes under consideration, especially below their phase-transition temperatures. On the other hand, when dansylhexadecylamine was used, even below the phase-transition temperature a relatively large difference in the fluidity of liposomal membranes was observed. This means that the headgroup structure of lipids may not greatly affect the packing in the very hydrophobic domain of liposomal bilayers.

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