Thermodynamic Effects of Hopanoids on Synthetic and Bacterial Phospholipid Membranes

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This paper reports on the study of the influence of hopanoids, bacteriophopane-32-ol (Monol) and bacteriophopane-32,33,34,35-tetrol (Tetrol) on the phospholipid membranes formed from dimyristoylphosphatidylcholines (DMPC) or bacterial phospholipids (BPL). Maximum splitting values and rotational correlation times were evaluated from the ESR spectra of 5- and 16-doxylstearic acids incorporated into these membranes. The effects of Monol on the DMPC membranes depended on both the phase transition temperature of DMPC (T_m: 23 °C) and the distance from the membrane surface. These effects of Monol were similar to those of cholesterol (Chol). The effects of Tetrol and Chol on the BPL membranes were similar to those of Monol and Chol on the DMPC membranes. However, the effects of Monol on the BPL membranes were different from those of Tetrol and Chol. It was found that the values of activation energy (E_a) and activation entropy change (ΔS^*) in the Monol–DMPC, Chol–DMPC, Tetrol–BPL and Chol–BPL systems were smaller than those in DMPC or BPL alone. Such characteristic effects of Monol and Tetrol on the liposomal membranes were compared with those of Chol.

Key words hopanoid; cholesterol; membrane fluidity; ESR spectrum; dimyristoylphosphatidylcholine; bacterial phospholipid

Cholesterol (Chol) is a major constituent of the membranes of mammalian cells. It modifies the packing of phospholipids of membranes and has a key role in many biological phenomena.[1] In contrast with eukaryotes, the membranes of prokaryotes contain no Chol. However, the latter contain a variety of hopanoids, and the contents of hopanoids can be as high as 30 mg/g of dry cells under certain strict conditions.[2-4] It is considered that primordial hopanoids, such as bacterihopane-32,33,34,35-tetrol (Tetrol) and its glycosides, act as membrane reinforcers in prokaryotic membranes, as Chol does in the membranes of eukaryotes.[5-9] These hopanoids may regulate the thermodynamic and mechanical properties of the membranes in a manner similar to Chol.[3] However, the details are unknown.

Previously, we reported that bacteriophopane-32-ol (Monol), a semi-artificial hopanoid derived from hopanepolyols, was also able to modulate the fluidity and stability of the membranes composed of dipalmitoylphosphatidylcholines (DPPC) in a peculiar manner.[10a,b] Further, it was observed that Monol was cytotoxic to several mouse leukemia cells,[10c] and that Monol inhibited a phospholipid secretion from the Hep G2 cell line.[10d] On the other hand, Tetrol affected only the fluidity near the polar head groups of the DPPC membranes.[10e] The effects of hopanoids on lipid membranes are dependent on the kinds of fatty acids involved and acyl-chain length. Here, we report the results of an ESR study on the thermodynamic effects of Monol and Tetrol on membranes composed of dimyristoylphosphatidylcholines (DMPC) or bacterialphospholipids (BPL). Then, the effects of hopanoids were compared with those of Chol.

Materials and Methods

Materials Monol and Tetrol were prepared from *Acetobacter aceti* according to the method previously reported.[3b,10a] DMPC was bought from Nippon Oil & Fats Co., Ltd. Stearic spin labels, 5-doxylstearic acid (5-SASL) and 16-doxylstearic acid (16-SASL), were from Aldrich Chemical Co., Ltd. BPL were extracted from freeze-dried cells of bacteria *Acetobacter aceti* I-6 according to the method of Bligh and Dyer.[11] Freeze-dried cells were kindly gifted by Nippon Del Monte Co.

Preparation of Liposomes Liposomes (SUV; small unilamellar vesicle) were prepared by the following method[10b,12]: DMPC or BPL (4 mM), spin probe (0.7 mol% of phospholipid) and various amounts of Monol, Tetrol or Chol were dissolved in methylene chloride–methanol (2 : 1, v/v), and then the solvents were removed under a stream of nitrogen, followed by vacuum pumping overnight to form a thin homogeneous film in a test tube. After 1 h of hydration with 0.2 ml of phosphate buffered saline (pH 7.4) at 35 °C, the mixtures were vortexed for 30 min, followed by the sonication, until they became homogeneous opalescent dispersions.

Spectral Measurements ESR spectra were recorded with a JEOL JES-FE1X spectrometer with a field intensity of 3280 G (X-band, 100 kHz field modulation, 0.63 mT modulation width) equipped with a temperature controller. The microwave was kept at 4 mW, at which no power saturation was observed.

Results

Effects of Monol on DMPC Membranes The mobility of 5- and 16-SASL does not depend on the alkyl-chain length of the host phosphatidylcholine, except for dilauroylphosphatidylcholine.[13a] Thus, in spin-label studies of membranes, SASL spin labels have been widely used as analogues for phospholipids. An ESR spectrum of 5-SASL incorporated into the DMPC membranes is anisotropic, and the fluidity of the membrane can be estimated from the outermost separation between the spectral extrema ($2T_{//}$). The value of $2T_{//}$ reflects the rotational motional freedom of the phospholipids close to the polar head groups in the membrane. This value increased with the decrease in fluidity.[10b,13,14] Figure 1a represents the profiles of the change in $2T_{//}$ values of 5-SASL incorporated into the DMPC, DMPC–Monol or DMPC–Chol membranes as a function of temperature. The $2T_{//}$ values progressively decreased with an increase in temperature in all three cases. A phase transition was observed at about 23 °C for the DMPC membrane. The $2T_{//}$ values were increased by incorporating 33.3 mol% of Monol or Chol at all temperatures examined. Furthermore, the phase transition became vague by the addition

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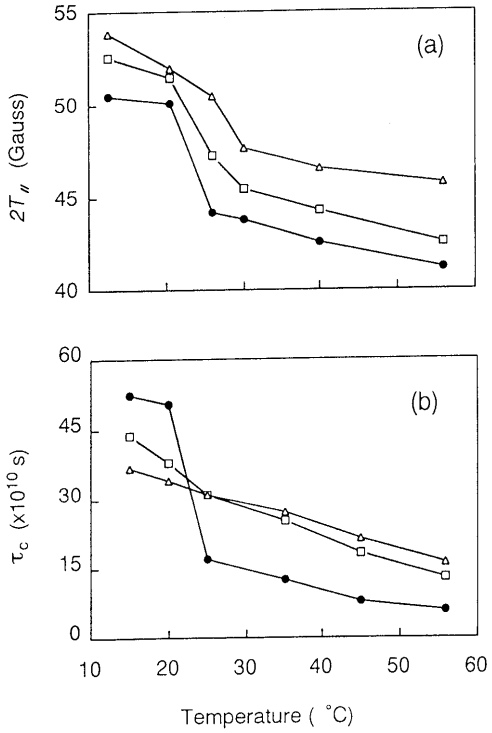

Fig.1. Changes in Maximum Splitting Values ($2T_{//}$) of 5-SASL (a) and the Rotational Correlation Times (τ_c) of 16-SASL (b) in the DMPC Membranes as a Function of Temperature

(●), DMPC alone; (△), DMPC containing 33.3 mol% of Monol; (□), DMPC containing 33.3 mol% of Chol.

of Monol or Chol, corresponding to a reduction in cooperation during the transition. The extent of the packing or condensing effect was markedly enhanced at temperatures higher than the phase transition temperature (T_m). These observations were consistent with the results reported on DMPC–Chol and DPPC–Chol systems.[15-17]

The spectra of 16-SASL incorporated into DMPC membranes reflected an isotropic motion of the acyl-chain of DMPC. In this case, the rotational correlation time, τ_c, of the motion of the phospholipid acyl-chains near the hydrophobic end can be estimated from the linear term of the line width parameter in the ESR spectra as the following Eq. 1[18]:

$$\tau_c = 6.5 \times 10^{-10} \Delta H_0 [(h_0/h_{+1})^{1/2} + (h_0/h_{-1})^{1/2} - 2] \quad (1)$$

where ΔH_0 is the peak-to-peak width of the central line in gauss, and h_{+1}, h_0 and h_{-1} are the heights of the low-, central- and high-field peaks, respectively. Figure 1b shows the changes in τ_c calculated from the ESR spectra of 16-SASL incorporated into the DMPC membranes containing 33.3 mol% of Monol or Chol as a function of temperature. As can be seen from Fig. 1b, the phase transition became vague with the addition of Monol or Chol. It should be noticed that below T_m, the addition of Monol decreased the τ_c values, indicating that Monol relaxes the lipid close-packing or has a fluidizing effect on the acyl-chains near the hydrophobic end of DMPC membranes. On the other hand, above T_m, the addition of Monol increased the τ_c values, indicating that Monol increases the packing of phospholipids or has a condensing effect. Such effect is attributable to the formation of specific Monol–DMPC complexes.

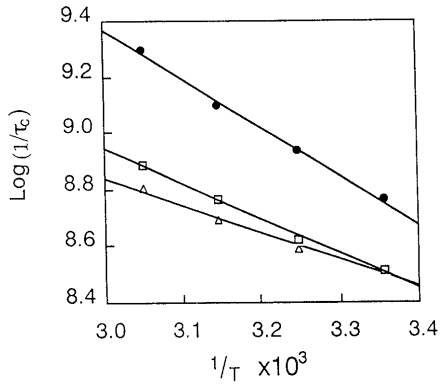

Fig. 2. Arrhenius Plots of $\log 1/\tau_c$ *vs.* $1/T$ for 16-SASL Incorporated into the DMPC Membranes

(●), DMPC alone; (△), DMPC containing 33.3 mol% of Monol; (□), DMPC containing 33.3 mol% of Chol.

Table 1. Thermodynamic Parameters for the Rotational Motion of 16-SASL Embedded in Various DMPC Membranes

Membranes	E_a (kcal/mol)	ΔS^* (cal/mol·degree)	ΔF^* (kcal/mol)
DMPC	7.9	8.0	5.5
DMPC–Monol (4:2)	5.6	−0.8	5.8
DMPC–Chol (4:2)	4.3	−5.0	5.8

Figure 2 represents Arrhenius plots of the data shown in Fig. 1b. From this figure, the activation energy, E_a, of the rotational motion of the nitroxide moiety of 16-SASL in the DMPC membranes containing Monol or Chol was calculated (Table 1). The value of E_a for the DMPC membranes is in fair agreement with the value reported by Subczynski *et al.*[13b] It is obvious that the addition of Monol or Chol decreased the values of E_a and activation entropy change, ΔS^*; the addition of Monol or Chol decreased the value of E_a, corresponding to a decrease in rigidity, and decreased also the value of ΔS^*, corresponding to an increase in order.

Effects of Hopanoids on BPL Membranes To further investigate the effects of hopanoids on phospholipid membranes, similar experiments and analysis for Monol–, Tetrol– and Chol–BPL membranes were carried out. The addition of Tetrol or Chol increased the values of $2T_{//}$ in the ESR spectra of 5-SASL incorporated into BPL membranes, indicating a condensing effect of Tetrol or Chol on the membranes near the polar head groups (data not shown). The BPL membranes did not show any phase transition at the temperatures examined (20—60 °C). On the other hand, Monol decreased the $2T_{//}$ value. Thus, Monol has a fluidizing effect on BPL membranes. Figure 3 shows changes in the ratio of the peak height in the ESR spectra of 16-SASL incorporated into the BPL membranes as a function of temperature. A decrease in the value of h_{+1}/h_0 suggests a condensing effect on the membranes. The results shown in Fig. 3 were consistent with those observed in the ESR spectra of 5-SASL. The values of the effective correlation time were calculated also from Eq. 1 under various conditions. From the Arrhenius display of these data, the values of the thermodynamic parameters

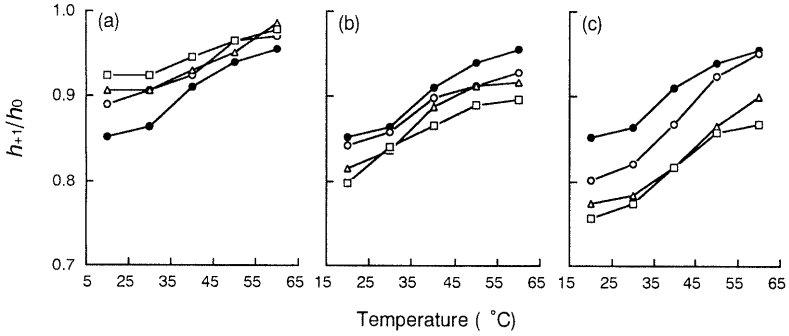

Fig. 3. Changes in the Peak Height Ratio in the ESR Spectra of 16-SASL Incorporated into the BPL Membranes Containing Monol (a), Tetrol (b) or Chol (c) as a Function of Temperature

Content of hopanoids or Chol: (●), 0 mol%; (○), 20 mol%; (△), 33.3 mol%; (□), 42.8 mol%.

Table 2. Thermodynamic Parameters for the Rotational Motion of 16-SASL Embedded in Various BPL Membranes

Membranes		E_a (kcal/mol)	ΔS^* (cal/mol·degree)	ΔF^* (kcal/mol)
BPL		5.8	0.7	5.6
BPL–Monol	4:1	6.0	0.9	5.7
	4:2	5.9	0.7	5.7
	4:3	6.3	2.2	5.6
BPL–Tetrol	4:1	4.9	−2.6	5.8
	4:2	4.9	−2.6	5.8
	4:3	4.4	−4.2	5.8
BPL–Chol	4:1	5.6	−0.6	5.7
	4:2	4.3	−5.2	5.7
	4:3	3.2	−8.9	5.7

were calculated for the BPL membranes containing 0—42.8 mol% of hopanoids or Chol. The results are collected in Table 2. The effects of the addition of Tetrol or Chol on BPL membranes are similar to those of Monol or Chol addition on DMPC membranes.

Discussion

Influences of Hopanoids on the DMPC or BPL Membranes In the present study, it was observed that the effects of Monol and Tetrol on DMPC and BPL membranes are similar to those of Chol, respectively. Previously, we reported that Monol and Chol enhanced the stability of the DPPC membranes,[10a,b] and that Tetrol showed a decrease in membrane fluidity near the polar head groups below T_m.[10e] Near the hydrophobic end of the bilayer, Monol incorporated into the DPPC membranes showed a fluidizing effect below T_m (40 °C), but no effect above T_m. Near the phospholipid head groups, Monol had a condensing effect above T_m but no effect below T_m. On the other hand, the effects of Chol on the fluidity of the DPPC membranes were similar to those on the DMPC membranes, consistent with the many previous reports.[13,19,20] Further, when about 20 mol% of Monol was incorporated into the DPPC membranes, the stability of the liposomal membranes was reduced, and the fluidity of the membranes increased drastically.[10a] Such a peculiar effect of Monol was not observed in the DMPC and Tetrol systems. The influences of Monol and Tetrol on the fluidity of the DPPC membranes were strongly dependent on both the temperature and the distance from the membrane surface. The association between hopanoids and phospholipids was unusually influenced by the acyl-chain length of the phospholipids.

Monol and Tetrol are highly similar to Chol in molecular dimensions.[3a,9] The pentacyclic ring system and hydroxyl group at the side chain makes the molecule amphiphilic in character. The hopanoid ring system should extend to the acyl-chain ends of phospholipids, with the hydroxyl group at the side chain forming a hydrogen bond with the phospholipids polar head group. Thus, hopanoids have an inverted orientation in the phospholipid bilayers compared to Chol.[5d] The hopanoid ring system, however, has six perpendicularly oriented methyl groups on both sides of the pentacyclic ring plane. These methyl groups may disturb optimum molecular packing with acyl-chains, resulting in the fact that the influence of Monol and Tetrol on membrane fluidity depends on the acyl-chain length. On the other hand, two methyl groups of sterol are directed to only one side of its ring system. Consequently, the molecular packing of the sterol ring with acyl-chains is not highly influenced by the acyl-chain length. The results shown in Table 2 support such a consideration, because the BPL used in this study consists mainly of vaccenic acid, 12-methyl-2-hydroxytridecanoic acid, palmitic acid, and myristic acid, with phosphatidylglycerol, phosphatidylethanolamine and cardiolipin as polar groups (unpublished data).

Thermodynamic Effects of Hopanoids on the Phospholipid Membranes It is assumed that the segmental motion of carbon atoms near the hydrophobic end is approximately isotropic. The motions under the various conditions are well reflected in the 16-SASL signal.[13b] Thus, we can consider qualitatively the effects of additives on the basis of the values of the thermodynamic parameters estimated from the ESR spectra of 16-SASL. The data shown in Tables 1 and 2 are apparently not self-consistent. Such somewhat surprising results were also reported for carotenoid-DMPC and Chol–dioleoylphosphatidylcholine systems by Subczynski *et al.*[13b] and Kusumi and Pasenkiewicz-Gierula,[21] respectively. These results reflect that fluidity, in the normal sense, refers to the rate of motion but not to the ordering of the molecular system.[22] The relations between the two quantities can be interpreted as follows:

There are two types of molecular contributions of phospholipids to the liquid phase structure. One is an

intermolecular contribution and the other is intramolecular. The overall changes in the rigidity of the phospholipid membranes result from the complex formation between phospholipids and Monol, Tetrol or Chol. The complex may cause a reduction in the degree of freedom of rotation of carbon–carbon bonds in the acyl-chains. The activation energy, E_a, is correlated to a wobbling diffusion of the phospholipids in the membranes. Therefore, the decrease in the E_a value indicates that the addition of Monol (in the DMPC system), Tetrol (in the BPL system) or Chol does not induce a concomitant decrease in molecular mobility near the hydrophobic end.[23] In other words, Monol, Tetrol or Chol have a very small effect on the lateral or rotational diffusion motion of a phospholipid. This is the intermolecular contribution to the thermodynamic properties.

On the other hand, the decrease in the value of the activation entropy change, ΔS^*, results from an increase in the acyl-chain conformational order.[20] It seems that the order of acyl-chain conformation depends on the position in the chain. That is, the rotational motion of 16-SASL is strongly influenced by the segmental motion which comes from *gauch-trans* isomerization of the acyl-chain. Monol (in the DMPC system) and Chol suppress the *gauch-trans* isomerization, resulting in the small value of ΔS^*. This is the intramolecular contribution. Thus, it can be said that Monol (in the DMPC system), Tetrol (in the BPL system) and Chol have an ordering influence on the phospholipids in the bilayer, but have little effect on the dynamics in the membrane.[23] As seen from the results of Monol in the BPL system, the above mentioned effects are strongly dependent on alkyl-chain length, unsaturation, and the mole fraction of additives.[21]

These special effects of hopanoids and Chol are embodied by the appearance of a β-phase. According to Vist and Davis,[24] three distinct phases can be identified in the Chol–DPPC system. They are the L_α- or liquid crystalline, gel- and β-phases. The L_α-phase is characterized by highly flexible phospholipid chains with a rapid axially symmetric reorientation. The β-phase is a high-Chol content phase in which lipid chains are highly ordered, but in which there is a rapid translocational and axial rotational motion of the lipid molecules comparable to those of the L_α-phase. The appearance of the β-phase can also be expected in the DMPC–Monol, DMPC–Chol, BPL–Tetrol and BPL–Chol systems.[15-17,25] The rapid axially symmetric reorientation of lipid chains in the β-phase and L_α-phase is reflected in the small values of E_a. Such a consideration is consistent with the thermodynamic and microscopic interaction model proposed by Ipsen *et al.*[26]

Comparing the results obtained in this study with those in the previous study, it becomes apparent that hopanoids have both a Chol-like function in membranes, but a very different effect from Chol on the membranes. Although the influences of hopanoids are strongly dependent on the kind of phospholipids involved, the thermodynamic effects observed in this study should approximate hopanoid–phospholipid interactions in the membranes of prokaryotes, because Tetrol and BPL are considered to be natural products.

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