Interactions between Bacteriohopane-32,33,34,35-tetrol and Liposomal Membranes Composed of Dipalmitoylphosphatidylcholine

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The effects of bacteriohopane-32,33,34,35-tetrol (Tetrol), on liposomal membranes composed of dipalmitoylphosphatidylcholine (DPPC) were examined and compared with those of bacteriohopane-32-ol (Monol) and cholesterol (Chol) by means of ESR and NMR techniques. ^{1}H-NMR spectra of Tetrol-incorporated DPPC membranes showed splitting of the signals of the choline N-methyl resonance, whereas Monol- and Chol-incorporated membranes showed no splitting of signals above the transition temperature of DPPC. It was suggested that the incorporation of Tetrol affected only the fluidity near the polar head groups of the DPPC membranes. The characteristics of the interactions between Tetrol and membranes are due to the fact that in DPPC bilayers Tetrol and Monol have an inverted orientation contrary to Chol, and that the hydroxy groups of Tetrol suppress the hydrophobic interaction between DPPC molecules whereas the methyl groups of the hopanoid ring promote this.

Key words hopanoid; cholesterol; membrane fluidity; ESR spectrum; NMR spectrum

Our interest in hopanoids stems from their role in the membranes of *Prokaryote* and also their utility as membrane components. Previously, we reported the effects of bacteriohopane-32-ol (Monol), a semi-artificial hopanoid derived from common hopanepolyols, on the stability and fluidity of liposomal membranes composed of dipalmitoylphosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (egg PC).[1,2] It was found that the effect of Monol on the membranes differed from that of cholesterol (Chol), although Monol seems to be equivalent to sterols in many respects. Furthermore, we observed that Monol is cytotoxic to several kinds of leukemia cells.[3] Thus, it seems that hopanoids have some characteristic effects on membrane properties such as fluidity, phase transitions and stability. In this study, we examined the effects of bacteriohopane-32,33,34,35-tetrol (Tetrol), a natural hopanoid found universally in bacteria, on the physicochemical properties of a DPPC membrane.

Thomson *et al.*[4] reported that, in the absence of sterols, *Tetrahymena pyriformis*, a eukaryotic ciliate, synthesized a quasi-hopanoid, tetrahymanol, as a membrane component. In the presence of sterols, however, the synthesis of tetrahymanol is completely inhibited, and these sterols are incorporated into membranes.[5,6] The growth of *Mycoplasma mycoides* is dependent on Chol but is also maintained in the presence of a hopanoid, diplopterol.[7] The content of Tetrol in *Bacillus acidocaldarius*, a thermoacidophilic bacterium, increases in a temperature-dependent manner.[8] Furthermore, the synthesis of hopanoids in *Zymomonas mobilis*, an ethanol-producing bacterium, increases with increasing ethanol concentration in the culture medium.[9,10] Therefore, it seems that under these conditions, some membrane reinforcers are required to counterbalance the destabilizing effect induced by temperature or ethanol.[11] This may be correlated with the fact that Tetrol regulates fluidity and phase transitions.[12–16] Similar results have also been found for interactions between Tetrol and PC containing branched chain[17] or cyclohexyl fatty acids.[18]

In the present study, in order to investigate the influence of hopanoids on membrane fluidity, the effects of Tetrol on fatty acyl chain motion in DPPC membranes were investigated by means of ESR and NMR.

MATERIALS AND METHODS

Materials DPPC was purchased from Nippon Oil & Fats Co., Ltd. Stearic acid spin labels, 5-doxyl stearic acid (5-SASL) and 16-doxyl stearic acid (16-SASL), were obtained from Aldrich Chemical Co., Ltd. Chol was obtained from Wako Pure Chemical Industries, Ltd. and was used after recrystallization from C_2H_5OH–H_2O (2 : 1, v/v). Hopanoids were prepared from freeze-dried cells of *A. aceti* which were cultivated by submerged fermentation and were stored under −80 °C before use. Monol was prepared according to the method of Rohmer and Ourisson.[19] Tetrol was prepared from the unsaponifiable fraction by preparative TLC on silica-gel or argentation silica-gel with hexane–diethyl ether–acetic acid (90 : 10 : 1, v/v) as the development solvent.[20]

Preparation of Liposomes Liposomes were prepared by the following method: DPPC (10 μmol/phosphorus), various amounts of Chol, Monol or Tetrol and spin probes (0.7 mol% of phospholipid) were dissolved in 2 ml $CHCl_3$ and the solvent evaporated under reduced pressure to give a homogeneous thin film in a tube. Phosphate-buffered saline (pH 7.4, 0.5 ml) was added and the mixture was vigorously vortexed for 1 min and then sonicated for about 20 min or more at 50 °C using a bath-type sonicator.

Spectral Measurements ESR spectra were recorded using a JEOL JES-FE spectrometer, at a field intensity of 3290 G (100 G field sweep), equipped with a temperature-control accessory. The microwave was kept at 4 mW and at this setting no power saturation effect was observed.

^{1}H-NMR spectra were obtained from the liposome samples in D_2O using a JEOL JNM-GX 500 spectrometer with a temperature-control accessory. Measurements of chemical shift were made at a concentration of 10 μmol

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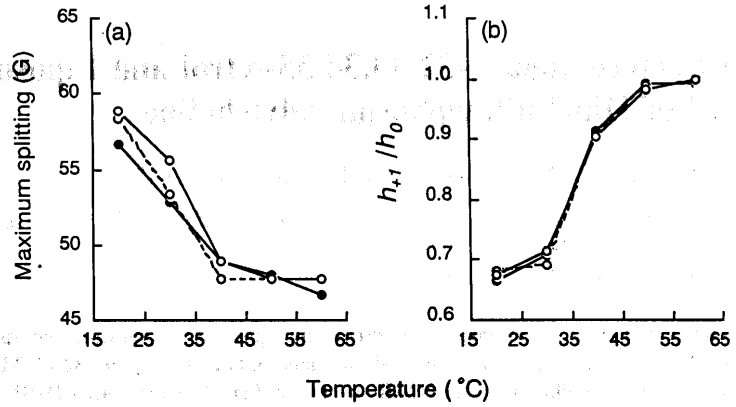

Fig. 1. Changes in the Maximum Splitting Values in ESR Spectra of 5-SASL (a) and the Peak Height Ratio of 16-SASL (b) Incorporated into DPPC Liposomes Containing Tetrol as a Function of Temperature

Content of Tetrol: —●—, 0 mol%; ---○---, 10.0 mol%; —○—, 20.0 mol%.

phospholipid phosphorus in 1.0 ml D_2O using tetramethylsilane as an internal standard.

RESULTS

Studies Involving ESR Spectra The effect of Tetrol on liposomal membrane fluidity was examined using 5-SASL and 16-SASL. Since a spectrum of 5-SASL incorporated into DPPC liposomes is anisotropic, as previously reported, a maximum splitting value in the spectrum can be used as a simple parameter to monitor the rotational motional freedom of the phospholipids close to the polar head groups in the liposomal membranes.[2] The maximum splitting value decreases as the motional freedom increases. On the other hand, the spectrum of 16-SASL in the membranes reflects an isotropic motion of the acyl chain of phospholipids.[2] The ratio of the height of the low-field peak to the central one, h_{+1}/h_0, can be used as an empirical parameter to monitor the fluidity of the bilayer near the hydrophobic end. This parameter is more sensitive to small changes in fluidity than other order parameters in spite of its simplicity.[21]

Figure 1a shows the changes in the maximum splitting value of 5-SASL incorporated into the DPPC membranes as a function of temperature in the presence of different concentrations of Tetrol. The maximum splitting values progressively decreased with increasing temperature. A phase transition could be observed at about 40 °C. The transition temperature (T_m) was not influenced by Tetrol incorporation. It seems that the maximum splitting values are increased by Tetrol addition below T_m, but are not affected above T_m. This indicates that the membrane fluidity near the polar groups was decreased by Tetrol below T_m (condensing effect).

Figure 1b represents the change in the ratio of the peak height of 16-SASL incorporated into the DPPC membranes containing Tetrol as a function of temperature. The spectrum of 16-SASL incorporated into the DPPC membranes was not affected by the addition of Tetrol.

Studies Involving NMR Spectra ^{1}H-NMR spectra were measured at different temperatures to compare the effects of hopanoids with those of Chol on the DPPC membranes. Figures 2 and 3 show the high-resolution NMR spectra

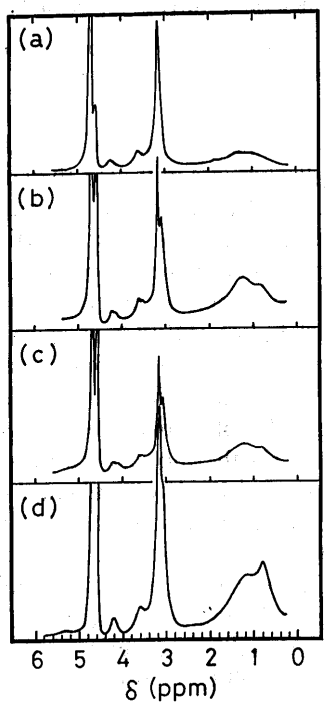

Fig. 2. 500 MHz ^{1}H-NMR Spectra of DPPC Liposomes Containing Tetrol, Monol or Chol in D_2O at 23 °C

(a) DPPC liposomes, (b) DPPC liposomes containing Tetrol, (c) DPPC liposomes containing Monol, (d) DPPC liposomes containing Chol. The concentration of the additives was of 33.3 mol% content.

of the DPPC membranes in the presence and absence of 33.3 mol% of hopanoids or Chol at 23 °C (below T_m) and at 45 °C (above T_m), respectively. It is well known that the DPPC membranes exhibit no high resolution features below T_m and resolved signals in the liquid-crystalline state above T_m.[22-25] Although the chemical shifts of each signal at 45 °C (Fig. 3a) differ slightly from those at 23 °C (Fig. 2a), these signals are assigned as follows: a strong and sharp peak at 3.1 ppm is from the choline methyl $-N(CH_3)_3$ protons, an intense peak at 1.2 ppm is from the chain methylene $(CH_2)_n$ (bulk methylene) protons and a peak at 0.7 ppm is from the chain terminal $-CH_3$ protons.[25,26] At 2.2 ppm, a signal from the first methylenes (α-methylenes) of the hydrocarbon chains can be observed. Signals from the $-CH_2-$ (choline PO-

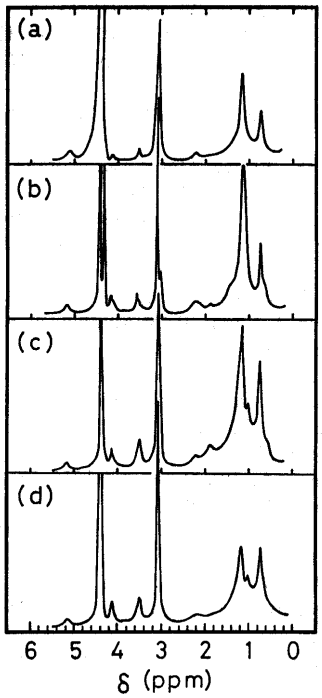

Fig. 3. 500 MHz ^{1}H-NMR Spectra of DPPC Liposomes Containing Tetrol, Monol or Chol in D_2O at 45 °C

(a) DPPC liposomes, (b) DPPC liposomes containing Tetrol, (c) DPPC liposomes containing Monol, (d) DPPC liposomes containing Chol. The concentration of the additives was of 33.3 mol% content.

methylene) and $-CH_2N-$ (choline N-methylene) of the choline group are observed at 4.2 and 3.5 ppm, respectively (Fig. 3a). The addition of Tetrol, Monol and Chol altered the line width and intensity of each signal. These results are summaried as follows.

a) Effects of Tetrol Addition: The spectrum of the DPPC-Tetrol system below T_m is shown in Fig. 2b. The spectrum is characterized by a splitting of the choline N-methyl resonance at 3.1 and 3.2 ppm. These are derived from head groups on the inner and outer surfaces of the bilayer.[25,27] A similar splitting could be observed at the 4.2 and 3.5 ppm signals. These results demonstrate that Tetrol causes the two monolayers to exist in different motional states. The bulk methylene and terminal methyl signals at 1.2 and 0.7 ppm were slightly better resolved, indicating a slight increase in the fluidity of the hydrocarbon chains.

Above T_m, the peak due to allylic protons is seen at 1.9 ppm.[28] A broad signal at 1.5 ppm was observed, which originates from the methylenes second to the carbonyl (β-methylenes). Furthermore, a splitting of the methyl resonance was observed at 0.6 ppm.[25] These changes correspond to the increased motion of the hydrocarbon chains produced by Tetrol.

b) Effects of Monol Addition: Below T_m, the signals from the hydrocarbon chains at 0.8 and 1.2 ppm slightly increased in intensity, indicating that the motion of the hydrocarbon chains is increased by Monol (Fig. 2c). Overall, however, the spectrum of the DPPC-Monol system was similar to that of the DPPC-Tetrol system.

Above T_m, the splitting and β-methylene signals were diminished (Fig. 3c). The bulk methylenes were solved at 1.1 ppm. These results suggest that Monol has a fluidizing effect on the membranes near the hydrophobic end.

c) Effects of Chol Addition: Below T_m, the signals from the terminal methyls and bulk methylenes at 0.8 and 1.2 ppm became clear (Fig. 2d). The relative intensity of the terminal methyl signal was greater than that of the bulk methylene signal. These spectral changes imply that Chol has not the same effect on all the protons along the fatty acyl chains.[22] The increase in the peaks arising from the protons associated with the hydrocarbon chains of DPPC implies that the molecular motion of the hydrocarbon chains is increased by Chol addition. The relative intensity of the signals from the head groups at 3.2, 3.6 and 4.2 ppm was little affected.

On the other hand, above T_m, the splitting and β-methylene signals were diminished, indicating that the motion of the hydrocarbon chains of the phospholipids is reduced by Chol (Fig. 3d). No resolution of the 0.7 ppm signal was observed.

Thus, the appearance of the split signals at 3.1 and 3.2 ppm is the most characteristic effect of Tetrol or Monol. This indicates that the molecular packing in the DPPC-Tetrol and -Monol systems is different from that in the DPPC-Chol system. The packing difference should be due to the fact that hopanoids are oriented parallel to the hydrocarbon chains of DPPC, in an inverted manner compared with the orientation of Chol.[18]

DISCUSSION

In the present study, the effect of Tetrol on the fluidity of DPPC membranes was examined and compared with that of Monol and Chol. As can be seen in Fig. 1, Tetrol had a condensing effect on the DPPC membranes of the phospholipids near the polar head groups below T_m. It has been observed previously that Monol has a condensing effect near the polar groups above T_m and a fluidizing effect close to the hydrophobic end of the phospholipids below T_m. Chol showed a fluidizing effect close to the hydrophobic end below T_m and a condensing effect above T_m. Near the polar groups of the phospholipid, Chol has the condensing effect regardless of temperature. Thus, the incorporation of Tetrol affected the fluidity of the DPPC membrane depending on both the phase transition temperature and the distance from the membrane surface, similarly to Monol or Chol.[2] On the other hand, Kannenberg *et al.*[12-14,17,18] reported that hopanoids have Chol-like effects on DPPC membranes. Bissert *et al.*[29] also obtained a similar result. Tetrol, however, exhibits unique effects on the membranes, as shown in Figs. 2 and 3. Such effects of Tetrol on the phospholipid membranes are interpreted as follows:

Since hopanoids have an inverted orientation contrary to Chol in bilayers, the planar hopanoid ring extends to the acyl chain ends of DPPC. Such an orientation should result in hopanoids and Chol having similar effect. However, it seems that the contribution of the methyl groups of the hopanoid ring is important. They are on both sides of the plane, whereas sterol methyl groups are directed only to one side of the ring system. Such orientation of methyl groups may result in a fluidizing effect close to the hydrophobic end below T_m. Above T_m, the

hydrophobic interaction between the fatty acyl chains and the side-chain of hopanoids is predominant, which produces condensing effects near the head groups. Furthermore, hydrogen bonds are formed between the hydroxyl groups on the side-chain of Tetrol and Monol and the ester carbonyl groups of DPPC. The interactions of the four hydroxy groups of Tetrol with the carbonyl groups of DPPC and/or the aqueous phase are presumably important for the molecular packing of Tetrol with DPPC. This is revealed by the splitting signals in Figs. 2 and 3. The bulkiness of the Tetrol side-chain may suppress the hydrophobic interactions above T_m.

These effects of the methyl and hydroxy groups of Tetrol on the phospholipid chains are visible in the NMR spectra. Therefore, although the effects of hopanoids are similar, though smaller, as a whole, their effects on the localized detailed regions of the phospholipids differ from those of Chol. This is consistent with the results of the thermodynamic study of the interactions of hopanoids with DPPC and bacterial phospholipids. This will be reported in a later paper.

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