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Effect of soybean-derived sterol and its glucoside mixtures on the stability of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine/cholesterol liposomes

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Abstract

The stability of liposomes is important when using them as a reservoir for drugs. A soybean-derived sterol mixture (S) and a sterol glucoside mixture (SG) were used to stabilize liposomes prepared from dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Ch). Four kinds of liposomes were prepared: liposomes consisting only of DPPC (DPPC-liposomes); DPPC-liposomes containing Ch (DPPC/Ch-liposomes); and DPPC-liposomes or DPPC/Ch-liposomes containing S or SG, respectively. In the present study, the effect of S and SG on the stability of liposomes entrapping calcein was investigated by measuring the leakage of calcein, by differential scanning calorimetry (DSC) and fluorescence anisotropy. The stable liposomes on heating showed that the initial leakage temperatures of calcein and the transition temperatures were high, the heats large, and the fluidity of the liposomal layer low. The results indicated that the stability of the liposomes depended on the concentration of S and SG in the DPPC- and DPPC/Ch-liposomes. The effect of S and SG on the stability of DPPC- and DPPC/Ch-liposomes in the gel state was greater in the following order: S > Ch > SG above about 10 mol% S or SG in the DPPC-liposomes and 5 mol% in the DPPC/Ch-liposomes.

Key words: Dipalmitoylphosphatidylcholine; Cholesterol; Liposome; DSC; Fluorescence anisotropy; Stability

1. Introduction

Liposomes formed by a bilayer membrane of phospholipids can contain or entrap various drugs. Since the liposome is biodegradable and considered to be scarcely poisonous (Rustum et al., 1979), it is potentially useful as a drug delivery system that would deliver desired drugs to a specific site (Kikuchi and Inoue, 1983, 1985; Gregoriadis, 1984). However, the stability of liposomes is still a problem. Many researchers have investigated the stability of liposomes using Ch. Animal cells mainly contain Ch while plants contain sterols and their glycosides. Sterols in plants are present as complex mixtures; β-sitosterol, campesterol, stigmasterol, etc. Soybeans contain S in the oil and SG that remains in the residue after the oil has been extracted. The structure of

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S is similar to that of Ch so that it may be able to regulate the fluidity of DPPC liposomes. In addition, since SG contains a glucose group, the interaction of SG with DPPC liposomes is therefore of special interest.

Sterol glucosides have been reported to exhibit an inhibitory effect on vascular permeability (Nomura et al., 1978) and to have antiulcerogenic and hemostatic effects (Okuyama and Yamazaki, 1983). It has also been reported that vesicles made of sterol glucoside and plasma lipoproteins are useful as drug carriers for intravenous administration (Seki et al., 1985). Recently, SG was used as an enhancer for nasal administration of insulin (Yamamoto et al., 1993). On the other hand, sterols were reported to show a function similar to that of Ch in membranes (Demel and Kruyff, 1976). There are few reports on the use of S and SG for stabilization of liposomes.

We intended to make DPPC-liposomes stable by adding S or SG; S is a mixture of β-sitosterol, campesterol, stigmasterol, and brassicasterol; SG is the glucoside of S. In this report, the effect of S or SG on the stability of liposomes entrapping calcein was investigated by measuring the leakage of calcein, by DSC and fluorescence anisotropy.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), cholesterol (Ch) and diphenylhexatriene (DPH) were purchased from Sigma Chemical Co. (St. Louis, MO). Calcein was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and cholesteryl anthracene-9-carboxylate (CA9C) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). S and SG were kindly supplied by Ryukakusan Co., Ltd. SG is a mixture of glucosides of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (6.2%) as shown in Fig. 1. S is obtained by the hydrolysis of the glucoside bond of SG, i.e., S is the aglycon of SG. The ratio of each sterol in S was determined using gas chromatography (Oku et al., 1972).

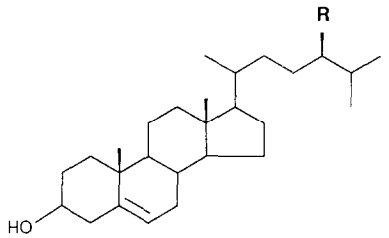

Fig. 1. Chemical structures of soybean-derived sterols (S). R = CH_3, campesterol; R = C_2H_5, sitosterol; R = C_2H_5 and Δ^{22}, stigmasterol; R = CH_3 and Δ^{22}, brassicasterol.

2.2. Preparation of liposomes

Liposomes were prepared according to a standard method (Bangham et al., 1974) as described in previous studies (Maitani et al., 1990, 1992). A mixed solution of DPPC (70 μmol), Ch (10 μmol), S or SG (2 μmol) in $CHCl_3$ was dried in an evaporator. After 15 h in vacuo, the dried lipid film was swollen in 3 ml of phosphate-buffered saline (PBS) (1 : 10 dilution of PBS in distilled water; 137 mM NaCl/2.6 mM KCl/6.4 mM Na_2HPO_4/1.4 mM KH_2PO_4; pH 7.4). PBS contained either 0 or 70 mM calcein. This solution containing swollen lipids was allowed to stand for 1 h to hydrate the liposomes. It was mixed by vortexing for 5 min, followed by sonication for 30 min at 45°C in a bath-type sonicator (Honda Electronics W220R) and centrifugation at 9500 × *g* for 5 min. The average size of the liposomes was determined to be about 100 nm using a Sub-Micron Particle Analyzer (Coulter model N4, Coulter Co.). The liposomes entrapping calcein were separated by passing 500 μl of the liposome suspension over a column (1.9 × 32 cm) of Sephadex G-50 (Pharmacia) with PBS in all fractions. Each fraction contained 4.5 ml.

2.3. DSC measurement

DSC was performed on a Thermoflex DSC 8230 (Rigaku Denki Co., Tokyo). 10 μl of the liposomal suspension containing an average of 0.019 mg of DPPC was sealed in a 20 μl aluminum pan. The samples were analyzed by heating at a scanning rate of 4°C/min over the temperature range 25–100°C, using PBS as a refer-

ence. The transition temperature (T_m) was determined as the highest temperature of the peak.

2.4. Measurement of the amount of calcein released from liposomes

The amount of calcein in the liposomes was determined using a fluorophotometer as reported previously (Oku et al., 1982). In the preparation of the liposomes, the dried lipid film (DPPC 70 μmol) was swollen in 3 ml of PBS containing 70 mM calcein. The percentage of calcein released was obtained according to the following equation:

% of calcein release

$$= 100(F_t - F_R)/(F_\infty - F_R) \quad (1)$$

where F_R is the fluorescence intensity of the suspension of liposomes entrapping calcein, F_∞ denotes the fluorescence intensity of calcein when the liposomes entrapping calcein were completely disrupted by Triton X-100 and F_t is the fluorescence intensity of calcein at time t.

2.5. Temperature dependence of calcein leakage from liposomes

The effect of temperature on the amount of calcein released from the liposomes was measured at a heating rate of 1.25°C/min over the range 25–35°C and 0.83°C/min over the range 35–50°C, respectively. The percentage of calcein released was obtained using Eq. 1. Here, F_∞ is the fluorescence intensity of calcein when the liposomes entrapping calcein were completely disrupted by Triton X-100 over 50°C, where the liposomes changed to a liquid crystalline state.

2.6. Measurement of fluorescence anisotropy

The fluorescence anisotropy of DPH (Shinitzky and Barenholz, 1978) or CA9C (Waggoner and Stryer, 1970), which is a marker for the fluidity of the lipid bilayer, was measured by the reported methods. A solution of DPH in tetrahydrofuran or CA9C in hexane was added to the liposome suspension at a final concentration of 0.25 or 2 mol%, respectively. The samples were measured at the same heating rate as in the case of calcein leakage. The excitation and emission wavelengths used for DPH were 357 and 430 nm, and for CA9C 310 and 425 nm, respectively.

3. Results

3.1. Phase transition temperature

Our system produces multilamellar liposomes composed of DPPC and S or SG, and DPPC/Ch and S or SG. The particle size and entrapped volume of calcein in DPPC/Ch-liposomes (mol, DPPC : Ch = 7 : 1) were 100.2 nm and 0.45 l/mol, respectively. On adding 0.2 mol S, Ch or SG to DPPC/Ch-liposomes (mol, DPPC : Ch = 7 : 1), the particle size of the liposomes increased to 108.7, 126.6 and 113 nm, respectively.

Fig. 2 shows DSC curves (heating mode) of DPPC-liposomes containing S, Ch or SG (mol, DPPC : X = 7 : 1.2; X = S, Ch, SG). Differences in the thermotropic behavior of DPPC-liposomes caused by the presence of S, Ch and SG are evident. S, Ch and SG produce a shift of the T_m

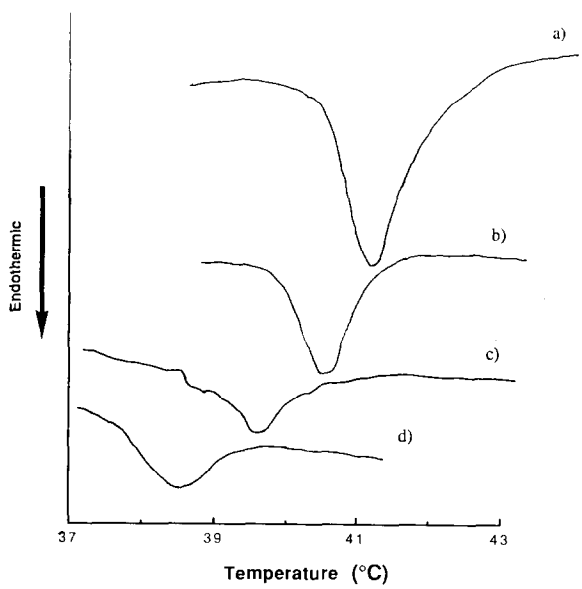

Fig. 2. DSC curves (heating mode) of DPPC-liposome suspension (mol, DPPC : X = 7 : 1.2; X = S, Ch, SG). DPPC-liposomes (a); DPPC-liposomes containing S (b), Ch (c), and SG (d).

Table 1
Transition temperature, enthalpy and initial calcein releasing temperature of DPPC-liposomes and DPPC/Ch-liposomes

X	T_m (°C) [a]	ΔH (kJ/mol) [b]	T_c (°C) [c]
DPPC: X = 7:1.2			
O	41.2	40.2	45.0
S	40.7	32.9	42.5
Ch	39.7	18.3	35.0
SG	38.6	14.6	30.0
DPPC:Ch: X = 7:1:0.2			
S	40.0	25.6	37.5
Ch	39.7	18.3	35.0
SG	39.1	10.9	37.5

[a,b] Transition temperature (T_m) and enthalpy (ΔH) were obtained by DSC in heating scans.
[c] Initial calcein releasing temperature (T_c) was obtained from Fig. 4 and 5.

value with variations in the enthalpy values (ΔH) associated with the liquid crystalline phase transition (see Table 1).

The T_m and ΔH values of the DPPC-liposomes and DPPC/Ch-liposomes containing S, Ch or SG are summarized in Table 1. The effect of S or SG on DPPC-liposomes was investigated and compared with that of Ch in two ways. One involved adding S or SG to DPPC-liposomes (mol, DPPC: X = 7:1.2, X = S, Ch, SG), the other consisting of the addition of S or SG to DPPC/Ch-liposomes (mol, DPPC : Ch : X = 7:1:0.2). In DPPC-liposomes, the magnitude of the T_m and ΔH values followed the ascending order of DPPC > S > Ch > SG. In DPPC/Ch-liposomes, the corresponding was S > Ch > SG.

3.2. Change in fluorescence anisotropy due to S, Ch or SG at 37.5°C

The effects of S, Ch or SG on the fluorescence anisotropy of DPH embedded in DPPC-liposomes were investigated at 37.5°C. Fig. 3a shows the effects of S or SG on the anisotropy parameter of DPH embedded in DPPC-liposomes. Below 10 mol% S, the fluorescence anisotropy decreased. Below 14.6 mol% SG (mol, DPPC:SG = 7:1.2), the fluorescence anisotropy did not change significantly, however, at 22.2 mol% (mol, DPPC:SG = 7:2), SG led to an increase in

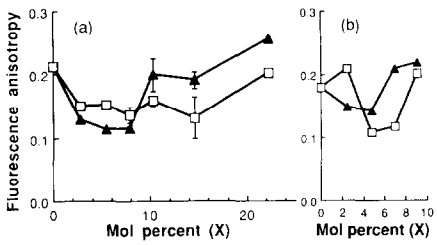

Fig. 3. Comparison of fluorescence anisotropy between S and SG in DPPC-liposomes (a) (mol, DPPC: X = 7:0–2; X = S, SG) and DPPC/Ch-liposomes (b) (mol, DPPC:Ch: X = 7:1:0–2; X = S, SG) containing S or SG at 37.5°C. S (▲), SG (□). Error bars represent ±S.D. (n = 3, panel a).

anisotropy. The enhancement of the fluorescence anisotropy of S and SG was reversed at about 10 mol% in DPPC-liposomes.

Fig. 3b demonstrates the effects of S or SG on DPPC/Ch-liposomes. Up to 4.8 mol% S, the fluorescence anisotropy did not change greatly, whereas above 7.0 mol% an increase in fluorescence anisotropy was evident. On the other hand, 4.8 mol% SG (mol, DPPC:Ch:SG = 7:1:0.4) decreased the anisotropy, whilst above 7.0 mol%, it resulted in an increase in anisotropy. The enhancement of fluorescence anisotropy of S and SG was reversed at about 5 mol% in DPPC/Ch-liposomes.

3.3. Leakage of calcein from liposomes

In Fig. 4a and b, the leakage of calcein from DPPC-liposomes and DPPC/Ch-liposomes con-

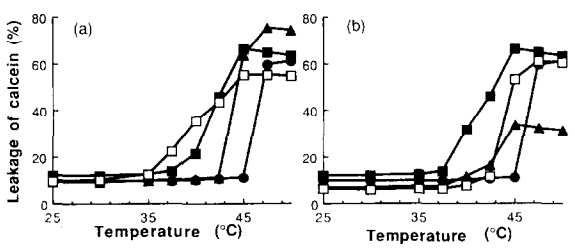

Fig. 4. Temperature dependence of leakage of calcein from DPPC-liposomes (a) (mol, DPPC: X = 7:1.2; X = S, Ch, SG) and DPPC/Ch-liposomes (b) (mol, DPPC:Ch: X = 7:1:0.2; X = S, Ch, SG) containing S, Ch or SG incubated in PBS. DPPC-liposomes (●); DPPC- and DPPC/Ch-liposomes containing S (▲), Ch (■) and SG (□).

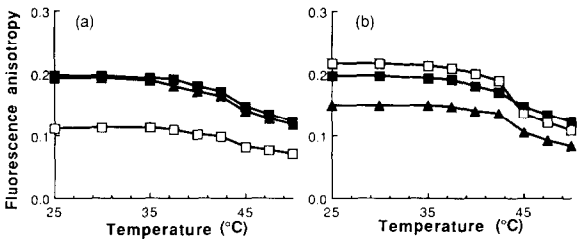

Fig. 5. Temperature dependence of fluorescence anisotropy of DPH in DPPC-liposomes (a) (mol, DPPC: X = 7:1.2; X = S, Ch, SG) and DPPC/Ch-liposomes (b) (mol, DPPC:Ch: X = 7:1:0.2; X = S, Ch, SG) containing S, Ch or SG. S (▲), Ch (■) and SG (□).

taining S, Ch or SG is plotted vs temperature, respectively. Each plot contains the cumulative data for DPPC and DPPC/Ch-liposomes incubated in PBS. The initial calcein releasing temperatures (T_c) of DPPC-liposomes containing S, Ch or SG were in the following order: S(42.5°C) > Ch(35°C) > SG(30°C).

The T_c values of DPPC/Ch-liposomes containing S, Ch or SG were in the following order: S = SG(37.5°C) > Ch(35°C), as summarized in Table 1.

3.4. Temperature dependence of fluorescence anisotropy

To determine the effect of S, Ch or SG on the fluidity of DPPC-liposomes and DPPC/Ch-liposomes, the fluorescence anisotropies of DPH in DPPC- and DPPC/Ch-liposomes were determined, the results being shown in Fig. 5a and b, respectively. Based on the fluorescence anisotropies of DPH in DPPC-liposomes, SG decreased the anisotropy more than Ch and S (Fig. 5a). In contrast, SG increased the anisotropy, whereas S decreased it in DPPC/Ch-liposomes (Fig. 5b).

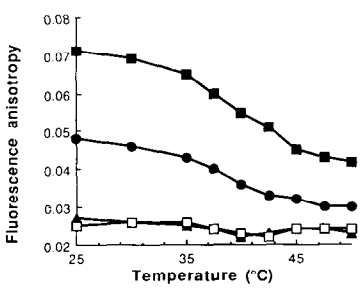

Fig. 6. Temperature dependence of fluorescence anisotropy of CA9C in DPPC- and DPPC/Ch-liposomes (mol, DPPC:Ch: X = 7:1:0.2; X = S, Ch, SG) containing S, Ch or SG. DPPC-liposomes (●); (▲) DPPC/Ch-liposomes containing S, Ch (■) and SG (□).

Fig. 6 depicts the temperature dependence of the fluorescence anisotropy of CA9C in liposomes. The fluorescence anisotropy of CA9C in DPPC- and DPPC/Ch-liposomes decreased on the addition of S and SG. No change in fluorescence anisotropy was found on the addition of 2.4 mol% S or SG in DPPC/Ch-liposomes (mol, DPPC:Ch:S or SG = 7:1:0.2).

4. Discussion

4.1. T_m and ΔH values

In general, the phase of the liposomal layer is a gel and/or liquid crystalline state. Below the T_m, lipids in the bilayer are in a highly ordered gel state, the hydrocarbon chains being in an all-*trans* configuration. Above the T_m, the lipids become more fluid as a consequence of *trans-gauche* rotational isomerization along the chains, resulting in lateral expansion and a decrease in the thickness of the bilayer, and they revert to one-dimensional arrangements (Mabrey Gaud, 1981; Fendler, 1982). This so-called gel-to-liquid crystalline phase transition of multilamellar liposomes is specific to the phospholipid structure and occurs for DPPC-liposomes at 41.2°C (Table 1). Since the ascending order of the T_m and ΔH values of DPPC-liposomes containing S, Ch or SG is S > Ch > SG, S increased the T_m and ΔH values of DPPC-liposomes. This means that the ability of S to stabilize DPPC-liposomes is greater than that of Ch and that the ability of SG is less than that of Ch at 14.5 mol% S or SG (mol, DPPC: X = 7:1.2, X = S, Ch, SG).

4.2. Change in fluorescence anisotropy as a function of composition of S and SG at 37.5°C

The fluidity change in liposomes at 37.5°C was monitored by the fluorescence anisotropy of DPH

in the liposomes. DPH provides information on the acyl chain orientational order of the DPPC bilayers. Since the movement of DPH is restricted in the gel state of the lipid, DPH shows high fluorescence anisotropy. As the fluidity of the lipid layer increases, the anisotropy decreases.

In DPPC-liposomes, the fluorescence anisotropy did not change greatly on the addition of SG (Fig. 3a). On the other hand, below about 10 mol% S, the anisotropy was low but increased on the addition of more than 10 mol% S. The solidifying effect of S on DPPC-liposomes was stronger than that of SG above 10 mol% S or SG. In DPPC/Ch-liposomes, above about 5 mol% S solidified DPPC/Ch-liposomes more than SG (Fig. 3b), which was also observed in DPPC-liposomes above about 10 mol% of S (Fig. 3b). However, the enhancement effect of S and SG on the fluorescence anisotropy changed at 5 or 10 mol% in DPPC- or DPPC/Ch-liposomes, respectively. The difference in concentration of S and SG in the liposomes was discussed with respect to the temperature dependence of fluorescence anisotropy.

Schuler et al. (1990) reported that less than 20 mol% sitosterol and campesterol, which are the main components in S, appear to be the most suitable sterols in ordering the acyl chains of soybean lecithin bilayers; they are even more efficient than Ch at 20°C. Soybean lecithin consists of unsaturated fatty acids. Therefore, S stabilized the liposomes of the unsaturated fatty acids. It is known that the opposite effect of Ch on the stabilization of liposomes depends on the phase state and the kind of fatty acids; Ch has a condensing effect on the lipid membrane of liposomes of a saturated fatty acid such as DPPC in the liquid crystalline state and a fluidizing effect on it in the gel state (Demel and De Kruyff, 1976). S may behave in DPPC-liposomes in the same manner as Ch in soybean lecithin. Therefore, it is supposed that S may fluidize the DPPC bilayers in the gel state and stabilize them in the liquid crystalline state. This hypothesis is inconsistent with the experimental result that S stabilized DPPC- and DPPC/Ch-liposomes in the gel state above about 10 mol% S in DPPC-liposomes and 5 mol% S in DPPC/Ch-liposomes. This difference might be due to the fact that S, a mixture of four kinds of sterols, was used in our experiment.

4.3. T_c values

The fact that the T_c value of the liposomes is low (Fig. 4a) corresponds with the result that the T_m and ΔH values of the liposomes are low. The T_c and T_m values in DPPC-liposomes show a good linear relationship ($r = 0.994$, Table 1). The T_c values of DPPC-liposomes with added S were greater than those with added SG. It is supposed that the glucose group of SG increased liposomal fluidity through microscopic regions of disorder, however, S increased the liposomal stability since S was closely packed in the DPPC lipid layer in the gel state. In the gel state, the packing state of the acyl group of the lipid may be important for the stabilization of liposomes.

In the gel state of DPPC/Ch-liposomes (mol, DPPC : Ch : $X = 7 : 1 : 0.2$, X = Ch, S, SG), the leakage of calcein was greater in the order Ch > S = SG, whereas, in the liquid crystalline state, the order was Ch > SG > > S (Fig. 4b). DPPC/Ch-liposomes contained 2.4 mol% X, where SG solidified the liposomes in the gel state, since this concentration was less than 5 mol% as shown in Fig. 3b. This result shows that 2.4 mol% SG fluidized DPPC/Ch-liposomes in the liquid crystalline state (Fig. 4b), the opposite of the reaction in the gel state (Fig. 3b). The difference between S and SG in the leakage of calcein in the liquid crystalline state might be due to the interaction of the glucose groups of SG with DPPC.

4.4. Temperature dependence of fluorescence anisotropy

Based on the results of the temperature dependence of fluorescence anisotropy, SG decreased the fluorescence anisotropy and increased the liposomal fluidity of DPPC-liposomes (Fig. 5a) whereas it decreased the liposomal fluidity of DPPC/Ch-liposomes in the gel state (stabilization, Fig. 5b). This result corresponds with the observation that SG decreased the T_m and T_c

values (fluidization), whilst S increased these values of DPPC-liposomes (stabilization).

The stabilizing effect of S and SG depended on the concentration of S and SG, i.e., about 10 mol% S and SG in DPPC-liposomes (Fig. 3a) and about 5 mol% in DPPC/Ch-liposomes (Fig. 3b). Below this concentration of S or SG, SG stabilized DPPC- and DPPC/Ch-liposomes. Above this level, S resulted in stabilization. The concentration of S or SG which reversed stabilization was lower in DPPC/Ch-liposomes than in DPPC-liposomes. This difference might be due to the fact that S has the same effect on DPPC-liposomes as Ch and interacts with Ch. The distribution of S in DPPC-liposomes might change above about 10 mol%. Less than 5 or 10 mol% S might aggregate heterogeneously in DPPC- or DPPC/Ch-liposomes, and above this, S might distribute in the liposomes homogeneously.

The fluorescence anisotropy of DPH embedded in a phospholipid is directly related to the viscosity of the phospholipid core.

In general, the expression which describes the general relation between the fluorescence anisotropy of DPH and the hydrodynamic properties of the surrounding medium is known as the Perrin equation (Shinitzky and Barenholtz, 1978):

$$\eta = 2.4r/(0.362 - r) \quad (2)$$

where r is the fluorescence anisotropy of DPH and η denotes the microviscosity of the lipid membrane. On the other hand, the viscosity of solvent decreased exponentially with increasing temperature according to:

$$\eta = Ae^{\Delta E/RT} \quad (3)$$

where ΔE is the energy of the flow activation, T represents the absolute temperature, R is the gas constant and A denotes the frequency factor.

Therefore, Fig. 7 shows that Arrhenius plots of ln η vs $1/T$ of DPPC-liposomes (DPPC: X = 7:1.2, X = S, Ch, SG) conform to a linear relationship (r = 0.919). We can calculate ΔE of liposomal fluidity from the slope of Fig. 7. According to the data derived from the fluorescence anisotropy of DPH, the ΔE values of DPPC-liposomes containing S, Ch or SG were in the following order: S (31.4 kJ/mol) > Ch (32.3 kJ/mol) > SG

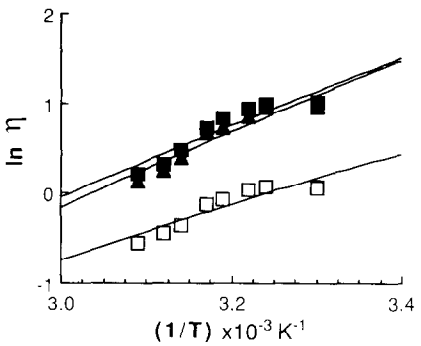

Fig. 7. Arrhenius plot of ln η vs $1/T$ in DPPC-liposomes (mol, DPPC: X = 7:1.2; X = S, Ch, SG) containing S, Ch or SG. S (▲), Ch (■) and SG (□). Each line was determined by the linear least-squares method using experimental mean values.

(24.7 kJ/mol). It is known that a ΔE value and a ΔH value determined via DSC measurement lead to a linear relationship. Actually, the ΔH (Table 1) and ΔE values show a good linear relationship (r = 0.924). This result shows that the lipid phase transition from the fluidity of the liposome membrane is consistent with the calorimetric measurement. The data on T_m, ΔH and ΔE values show that the stabilization effect of S derives from the stabilization of the gel state of the liposomes.

The fluorescence anisotropy of CA9C provides information on the fluidity of Ch in the liposomes. To investigate the interaction of S or SG with Ch, the fluorescence anisotropy of CA9C in DPPC-liposomes and DPPC/Ch-liposomes (mol, DPPC: Ch: X = 7:1:0.2, X = S, Ch, SG) was measured (Fig. 6). DPPC/Ch-liposomes containing S or SG showed almost equal fluorescence anisotropies. The difference in the interaction of S or SG with Ch was not clearly detected.

5. Conclusions

The effect of S and SG on the stability of liposomes depends on the concentration of S and SG in the liposomes. Above about 10 mol% in DPPC-liposomes or about 5 mol% in DPPC/Ch-liposomes, S stabilized the liposomes, whereas SG fluidized them in the gel state. The stabilizing effect of S or SG on DPPC-and DPPC/Ch-lipo-

somes in the gel state was greater in the following order: S > Ch > SG. This result suggests that S was closely packed in the DPPC lipid layer, whereas the glucose group of SG increased liposomal fluidity through microscopic regions of disorder in the gel state.

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