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An essential role of phosphatidylglycerol in the formation of the osmotically stable liposomes of *Escherichia coli* phospholipids

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A temperature sensitive auxotroph of *Escherichia coli* K-12 requiring unsaturated fatty acids can grow normally at 28°C, but requires an osmotic stabilizer such as a high amount of salt or sugar in the medium for the growth at 42°C. Namely, the apparent osmotic stability of the cells at 28°C and 42°C is quite different. The osmotic properties of liposomes of the phospholipids extracted from these cells were investigated. The osmotically induced volume change of the multilamellar liposomes was examined by the turbidimetric method. The liposomes prepared from cells grown at 28°C can swell and shrink under a wide range of hypo- and hypertonic conditions. However, those from cells grown at 42°C could not swell under hypotonic conditions. These results exhibit a good correlation between the apparent osmotic stability of *E. coli* cells and the osmotic properties of the liposomes prepared from the extracted total phospholipids. To clarify the role of each phospholipid component, the osmotic properties of the liposomes reconstituted from the purified phospholipid species were further investigated. The results clearly showed that phosphatidylglycerol is the key factor that stabilizes the membranes of *E. coli* phospholipids against osmotic pressure.

Introduction

Unsaturated fatty acid auxotrophs of *Escherichia coli* are good systems to investigate membrane properties and have been extensively used over the last decades. Although they basically cannot grow in the absence of unsaturated fatty acids, some of them were found to be able to survive in the presence of osmotic protectors such as salts and sugars even under non-permissive conditions [1,2]. For example, *E. coli* K-12 UFA^{ts} can grow normally at around 30°C but not at above 40°C without supplementation of unsaturated fatty acids. In the presence of a high concentration of salt, sugar or glycerol, however, the auxotroph can grow at 42°C even in the absence of unsaturated fatty acids and accumulates an abnormally high amount of saturated fatty acids in the membrane. The concentrations of these substances sufficient to protect the auxotroph fall in the same osmolarity range [2]. Therefore these substances were supposed to osmotically stabilize the cells and were designated as 'osmotic stabilizers'. The reason for the apparent osmotic instability of cells grown at higher temperature was taken to be the abnormally high content of saturated fatty acids of the membrane lipids, since the membrane was ex-

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Abbreviations: PL28, total phospholipid fraction extracted from cells cultured at 28°C; PL42(KCl), PL42(sucrose) and PL42(glycerol), total phospholipid fractions extracted from cells cultured at 42°C in the presence of 2.0% KCl, 15.0% sucrose and 9.5% glycerol, respectively.

pected to be fragile under such conditions [2]. The properties of the phase transition of the cell membrane of the auxotroph were investigated extensively [3,4]. It was shown that the membrane is in a state of phase separation even at 42°C.

As an approach to clarify the mechanism of stabilization of the cells against osmotic pressure, the osmotic properties of liposomes of the phospholipids extracted from the cell membranes were investigated in this work. The osmotic behavior of the multilamellar liposomes was examined by the turbidimetric method developed by Bangham et al. [5]. As shown in our previous paper [6], the relationship between the volume of the liposomes (V) and the absorbance (A) is given by

$$V \propto (1/A)^{3/2} \quad (1)$$

The results have shown that there is a good correlation between the apparent osmotic stability of *E. coli* cells and the osmotic properties of liposomes of the extracted phospholipids, and that phosphatidylglycerol plays a key role in stabilizing the *E. coli* phospholipid membranes against osmotic pressure.

Materials and Methods

Media and growth conditions. An auxotroph (*E. coli* K-12 UFA^{ts}) was grown at 28°C in a basal medium containing the following (per liter); K_2HPO_4, 7 g; KH_2PO_4, 2 g; trisodium citrate, 0.5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; vitamin B_1, 1 mg; glycerol, 2.5 g; and casamino acids (Difco, vitamin free), 10 g. The auxotroph was also grown at 42°C in the basal medium containing 2.0% KCl, 15.0% sucrose or 9.5% glycerol without supplementation of unsaturated fatty acids. The cells were cultured overnight in a jar fermenter (100 litre) and then harvested with a Sharples centrifuge.

Phospholipids. The total phospholipids were extracted from the cells with chloroform/methanol according to the method of Bligh and Dyer [7], and further purified by silicic acid column chromatography with elution with a chloroform/methanol (3 : 2, v/v) mixture. Phosphatidylethanolamine and cardiolipin were purified from the total phospholipids by DEAE-cellulose column chromatography [8] followed by silicic acid column chromatography with stepwise elution with chloroform/methanol. Phosphatidylglycerol was synthesized from the purified phosphatidylethanolamine by one-step transphosphatidylation catalyzed by phospholipase D in the presence of excess glycerol [9]. In order to remove polyvalent metals and displace the gegen ion with a sodium ion, cardiolipin and phosphatidylglycerol dissolved in chloroform/methanol (1 : 1, v/v) were washed with a half volume of a 0.5 M Na_2SO_4, 2.0 mM EDTA solution.

Phospholipid and fatty acid compositions. The total phospholipids were subjected to silica gel thin-layer chromatography. The thin-layer plate was developed with chloroform/methanol/water (65 : 25 : 4, v/v/v), and the spots were detected by exposure to iodine vapour. The mole fractions of the separated phospholipids were determined by a phosphorus assay [10]. The fatty acid composition was determined with a Shimadzu GC-4APT gas chromatograph equipped with an integrator, model ITG-2A. Fatty acid methyl esters were obtained by transesterification with HCl/methanol, and the methyl esters were applied on a column (0.3 × 300 cm) packed with 15% diethylene glycol succinate on Chromosorb W, with methyl palmitate as the internal standard.

Preparation of multilamellar liposomes. The phospholipid solution in chloroform was put into a round-bottom flask. The solvent was removed in a rotary evaporator to dryness and further under high vacuum overnight. The lipid on the surface of the flask was suspended in an aqueous solution containing 50 mM glucose, 2 mM EDTA and 10 mM Tris (pH 7.5) with a vortex mixer. This was used as the stock dispersion.

Optical measurements. 0.2 ml of the stock dispersion was diluted with 3.0 ml of glucose solutions. Before the optical measurements, each mixture was incubated for 6 h at 25°C for the phospholipids in the liquid-crystalline state at this temperature. Otherwise, each was incubated for 24 h to ensure that the equilibrium was achieved. The liposomes were stable during the incubation and measurement.

The absorbance of the dispersions was measured at 450 nm with a Hitachi 124 spectrophotometer, equipped with thermospacers on both

sides of the cell compartment, through which water from a thermostatted bath was circulated to keep the temperature of the cell compartment constant.

Results

Chemical compositions of E. coli phospholipids

Listed in Tables I and II are the fatty acid and phospholipid compositions of the total phospholipids, respectively, which were extracted from cells cultured under various conditions. The total phospholipids extracted from cells cultured at 42°C in the presence of 15.0% sucrose, 2.0% KCl and 9.5% glycerol, and those cultured at 28°C are designated as PL42(sucrose), PL42(KCl), PL42 (glycerol) and PL28, respectively. The data for PL28 and PL42(KCl) in Table II are taken from our earlier report [3]. A large amount of saturated fatty acids is accumulated at 42°C. Apparently myristic acid is introduced instead of unsaturated fatty acids. The total percentage of unsaturated fatty acids is suppressed to 8–23%, which is much lower than that of cells cultured at 28°C (PL28) and that of cells cultured at 42°C with supplementation of oleic acid (about 30% unsaturated fatty acids; Ref. 2).

As to the phospholipid composition, phosphatidylethanolamine is the major component in all cases. Especially, it comprises 90% in the case of PL42(sucrose) while it is about 80% for the other phospholipids. In contrast to the fatty acid composition, the acidic phospholipid composition of PL42(glycerol) is similar to that of PL28 and different from those of PL42(KCl) and PL42(sucrose). Phosphatidylglycerol is the major component in the former, while cardiolipin is major in the latter.

TABLE I

THE FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS

Abbreviations: PL28, total phospholipid fraction extracted from cells grown at 28°C; PL42(KCl), PL42(sucrose) and PL42(glycerol), total phospholipid fractions from cells grown at 42°C in the presence of 2.0% KCl, 15.0% sucrose and 9.5% glycerol, respectively; 12:0, lauric acid; 14:0, myristic acid; 14:1, myristoleic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:1, *cis*-vaccenic acid; 17Δ and 19Δ, cyclopropane ring-containing fatty acids with the indicated carbon numbers.

	Mol %			
	PL28	PL42 (glycerol)	PL42 (KCl)	PL42 (sucrose)
12:0	1.4	1.3	1.7	–
14:0	3.0	33.1	37.6	18.1
14:1	1.5	1.6	3.6	–
16:0	41.1	53.2	52.0	59.3
16:1	24.8	2.8	–	6.4
17Δ	6.0	2.8	3.0	3.8
18:1	18.4	–	–	4.7
19Δ	3.9	4.9	2.0	7.7
Total unsatd. [a] fatty acids	54.6	12.1	8.6	22.6

[a] Including cyclopropane fatty acids.

TABLE II

THE PHOSPHOLIPID COMPOSITIONS OF PL28 AND PL42 FRACTIONS

Abbreviations: PE, phosphatidylethanolamine; CL, cardiolipin; PG, phosphatidylglycerol.

	Mol %			
	PL28	PL42 (glycerol)	PL42 (KCl)	PL42 (sucrose)
PE	78.3	80.1	79.8	90.2
CL	4.1	6.5	15.7	8.5
PG	11.3	13.4	4.5	1.3
Lyso-PE	6.2	–	–	–

Osmotic behavior of liposomes of extracted total phospholipids

The osmotic behavior of multilamellar liposomes was examined by the turbidimetric method. The ability of the liposomes to keep solute was confirmed using PL42(glycerol) and [^{14}C]glucose in such a way as reported for egg yolk phosphatidylcholine and phosphatidic acid in our previous paper [6]. An osmotic gradient across the liposomal membrane was formed by the use of glucose as described in Materials and Methods. The osmotic gradient would induce a volume change of liposomes due to the uptake or release of water. When multilamellar liposomes act as perfect osmometers, the total volume of liposomes (V_{total}) changes according to the following equation as

shown in the Appendix.

$$V_{total} = K(C_{in}/C_{out}) + V_{dead} \quad (2)$$

where C_{in} and C_{out} are the original concentrations of the solute inside and outside liposomes, respectively. V_{dead} is the osmotically inactive volume of the liposomes and K is a constant. Using Eqn. 1, one is led to

$$(1/A)^{3/2} = K'(C_{in}/C_{out}) + K'' \quad (3)$$

where A is absorbance and K' and K'' are constants [6].

Osmotically induced turbidity changes are presented in Fig. 1 as a function of the osmotic gradient for the multilamellar liposomes of PL28 at 0°C and 25°C. The absorbance at 450 nm was measured 24 and 6 h after the formation of the osmotic gradients at 0°C and 25°C, respectively, since the phase transition from the gel to the liquid-crystalline state was completed at around 19°C [3]. The osmotic gradient was generated by changing C_{out}. The turbidity of each suspension changes with the osmotic gradient in a wide range both under hypertonic ($C_{in}/C_{out} < 1.0$) and hypotonic conditions ($C_{in}/C_{out} > 1.0$). The observed linear relationship between $(1/A)^{3/2}$ and C_{in}/C_{out} indicates that the liposomes change in volume in accordance with Eqn. 3. This ideal osmotic behavior shows that the liposomal membranes are actually working as barriers for the solute, namely, they are osmotically stable. The ideal osmotic behavior observed both at 0°C and 25°C suggests that multilamellar liposomes of PL28 are osmotically active not only in the liquid-crystalline state but also in the gel state. This coincides with the results for phosphatidylcholine liposomes [11]. The difference in turbidity between Figs. 1a and 1b comes from the different refractive indices of liposomal membranes in the gel and liquid-crystalline states.

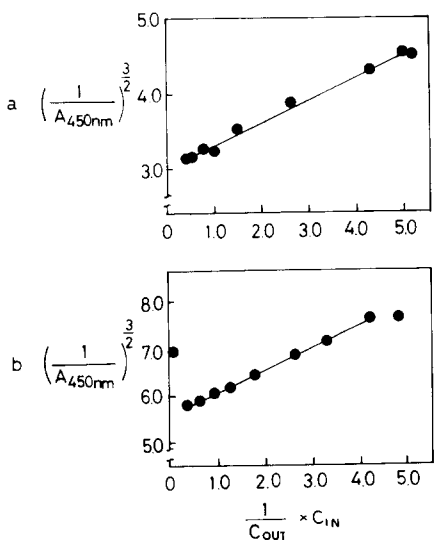

Fig. 1. The osmotically induced turbidity changes of multilamellar liposomes of PL28. PL28 is the total phospholipid fraction extracted from cells grown at 28°C in the basal medium. 0.2 ml of the stock dispersion including 50 mM glucose was diluted with 3.0 ml of glucose solutions of various concentrations. After equilibrium was achieved, the absorbance at 450 nm was measured. Experiments were conducted at 0°C (a) and 25°C (b). $(1/A_{450})^{3/2}$ is proportional to the total volume of multilamellar liposomes.

The turbidities of the multilamellar liposomes of PL42(KCl), PL42(sucrose) and PL42(glycerol) were measured at 25°C under different osmotic gradients. Thermal analysis of them showed broad phase transitions ending at around 45°C (Ref. 4 for PL42(KCl)). At 25°C the phospholipid bilayers are in the state where the gel and liquid-crystalline phases coexist. As shown in Fig. 2, these multilamellar liposomes also act as ideal osmometers in certain ranges. The slope (K' in Eqn. 3) depends on the average size of liposomes and the distribution of their sizes [6]. The range in which they are osmotically active, however, is very limited compared with that in the case of PL28. Especially, the multilamellar liposomes of PL42 (sucrose) and PL42(KCl) can hardly swell. They act as osmometers only under hypertonic conditions. As was shown in our previous paper [6], the inflexion point under the hypotonic conditions means that the bilayer becomes leaky to the solute. The osmotic behavior shown in Fig. 2 suggests that the liposomal membranes of PL42(sucrose) and PL42(KCl) are less stable under hypotonic conditions and that they are stabilized only under hypertonic conditions. This appears to well correspond to the properties of the original cells.

In contrast, the multilamellar liposomes of PL42(glycerol) retain the stability in a wider range ($C_{in}/C_{out} \leqslant 3.0$). Since glycerol is known to penetrate egg phosphatidylcholine membranes and

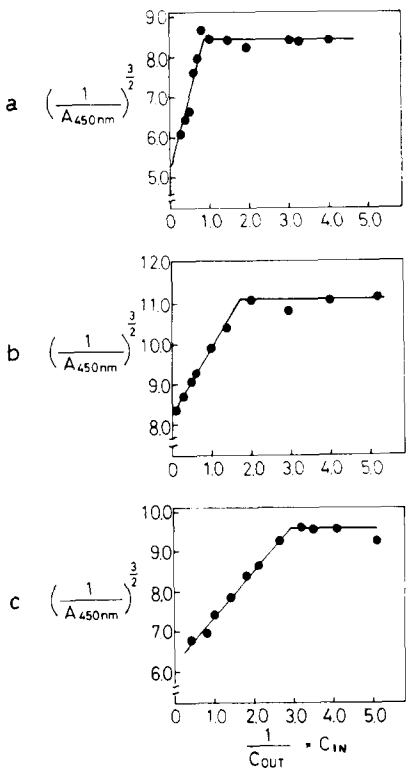

Fig. 2. The osmotic behavior of multilamellar liposomes of PL42(sucrose), PL42(KCl) and PL42(glycerol). The phospholipid fractions were derived from cells grown at 42°C in the presence of 15.0% sucrose (a), 2.0% KCl (b) and 9.5% glycerol (c). All the experiments were conducted at 25°C.

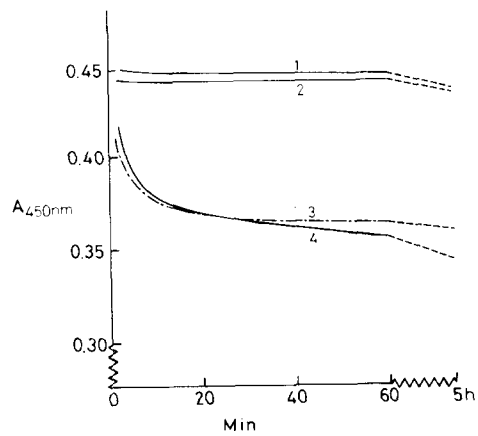

Fig. 3. The time-course of the absorbance at 450 nm of PL42(glycerol) liposomes. The absorbance at 450 nm was monitored at 25°C after 0.2 ml of the stock dispersion of PL42(glycerol) (prepared in 25 mM KCl) was diluted with 3.0 ml of the following solutions: 1, 25 mM KCl; 2, 50 mM glucose; 3, 50 mM glycerol; 4, 12.5 mM KCl.

erythrocyte membranes [5,12,13], the permeability of glycerol through the liposomal membrane of PL42(glycerol) was examined. Fig. 3 shows the time-courses of absorbance at 450 nm at 25°C, after 0.2 ml of the stock dispersion of PL42(glycerol) (prepared in 25 mM KCl solution) was diluted with isotonic solutions of KCl, glucose and glycerol, as well as with a 12.5 mM KCl solution. The change represents the relative rate of permeation of the solutes and water [11,14,5]. The rapid change of the absorbance in the presence of isotonic glycerol indicates that glycerol permeates the liposomal membrane quite easily compared with KCl and glucose. Therefore, glycerol cannot work as an osmotic stabilizer in this system. This would be the case in vivo as well when the cells do not have an active transport system for glycerol. The results of Fig. 3 provide additional evidence for the sealing of the liposomes of *E. coli* phospholipids and for the assumption that glucose cannot permeate their membranes.

Osmotic behavior of reconstituted liposomes

What is the essential factor in the stabilization of the *E. coli* phospholipid bilayers against osmotic pressure? Originally it was assumed to be the unsaturated fatty acids. On comparison of Figs. 2a, 2b and 2c with the fatty acid composition (Table I), however, no correlation can be found between the osmotically stable range and the amount of unsaturated fatty acids. On the other hand, there is a simple correlation between the range and the acidic phospholipid composition (Table II). That is, the range becomes wider with an increase in phosphatidylglycerol. To confirm the correlation mentioned above, the phospholipid species were purified individually from PL28 and then they were mixed with each other in different ratios to prepare liposomes. The osmotic behavior was examined at 25°C for the multilamellar liposomes of (phosphatidylethanolamine + phosphatidylglycerol) (phosphorus ratio, 8 : 2) and (phosphatidylethanolamine + cardiolipin) (phosphorus ratio, 8 : 2), and the results are presented in Fig. 4. The difference between the two systems is remarkable in the hypotonic region. The liposomes of (phosphatidylethanolamine + phosphatidyl-

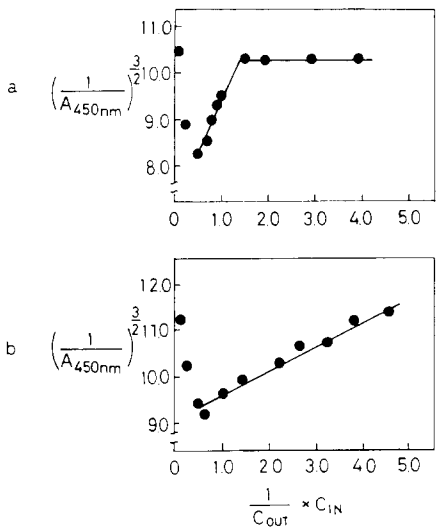

Fig. 4. The osmotic behavior of multilamellar liposomes prepared from purified phospholipids at 25°C. (a) Phosphatidylethanolamine + cardiolipin, (b) phosphatidylethanolamine + phosphatidylglycerol. The phosphorus ratio of the two phospholipids is 8 : 2 in both cases. Phosphatidylethanolamine and cardiolipin were purified from PL28. Phosphatidylglycerol was synthesized from phosphatidylethanolamine of PL28.

glycerol) provide a wide osmotic gradient range in which the liposomes are osmotically active. In contrast, the liposomes of (phosphatidylethanolamine + cardiolipin) can only provide a limited range as observed for PL42(KCl) and PL42(sucrose). Similar results were also obtained for (phosphatidylethanolamine + cardiolipin) (phosphorus ratio, 6 : 4).

The contribution of fatty acid composition to the results of Fig. 4 can be neglected at first approximation because of the following reasons. In the first place, the fatty acid composition of *E. coli* K-12 cells is not significantly different among phosphatidylethanolamine, phosphatidylglycerol and cardiolipin [16,17]. Therefore such difference would become very minor when the two kinds of acidic phospholipids were diluted 5-fold with the same phosphatidylethanolamine. In the second, even if the difference is still not negligible, the amount of saturated fatty acids is usually higher in phosphatidylethanolamine than in phosphatidylglycerol and cardiolipin [16,17]. Phosphatidylglycerol in Fig. 4 was synthesized from phosphatidylethanolamine. If the fatty acid composition is the major factor to determine the osmotic properties, the liposomes of (phosphatidylethanolamine + cardiolipin) would be expected osmotically more stable than those of (phosphatidylethanolamine + phosphatidylglycerol), judging from the results of PL28 and PL42(KCl) liposomes. The results observed in Fig. 4 were just opposite, indicating that the fatty acid composition cannot be the major factor. Now it can be concluded that the phospholipid composition is the major factor determining the osmotic behavior of liposomes and that phosphatidylglycerol plays a key role in stabilizing the membranes of *E. coli* phospholipids against osmotic pressure.

There is a possibility that the morphology of liposomes is quite different for (phosphatidylethanolamine + phosphatidylglycerol) and (phosphatidylethanolamine + cardiolipin), and the difference is responsible for the different osmotic properties. There could be a common critical ratio of surface area to trapped volume at which a liposome bursts. When the shapes of liposomes with different phospholipid compositions are different under isotonic conditions the osmotic concentrations at which the swelling or shrinking structure reaches the critical condition would be different. Such a contribution of the morphology of liposomes cannot be ruled out. It is likely, however, not to be a major factor which determines the osmotic properties in Fig. 4. If the morphology is the essential factor, both of the hypotonic and hypertonic limits of the osmotically stable range are expected to shift to the same direction when the phospholipid composition is changed. Any shift cannot be seen, however, in the hypertonic region of Fig. 4. Therefore, we may infer that the ratio of surface area to trapped volume of the critical structure depends upon the phospholipid composition even if the morphology contributes to the results of Fig. 4.

The plots of $(1/A)^{3/2}$ deviate greatly from the linear relationship under extremely hypertonic conditions ($C_{in}/C_{out} < 0.5$) in Fig. 4. Such an abrupt decrease in absorbance was also observed for the multilamellar liposomes of PL28 (Fig. 1). Furthermore, despite that PL42(glycerol) has an acidic phospholipid composition similar to that of PL28, liposomes of the former are more osmotically unstable in the hypotonic region. These facts

indicate that the fatty acid composition is an additional factor that modifies the limits of the osmotically stable range. The results of PL28 suggests that unsaturated fatty acids stabilize the phospholipid membrane in the hypotonic region and those of PL42's (Fig. 2) imply that saturated fatty acids contribute to the stabilization in the hypertonic region.

Discussion

Two subcomponents of phospholipids, namely, fatty acids and polar groups, are known to work in different manners in determining the nature of the phospholipid bilayers. In the phase transition between the gel and liquid-crystalline states, the fatty acids play an essential role. In contrast, the polar groups turned out to be the essential factor determining the osmotic properties of the phospholipid bilayers in this work. Phosphatidylglycerol was identified as the key phospholipid stabilizing the liposomes of *E. coli* phospholipids against osmotic pressure. At this stage, however, the role of phosphatidylglycerol should not be considered to be unique, because Bangham et al. [5] already showed that acidic lipid molecules are indispensable to prepare osmotically active liposomes from egg yolk phosphatidylcholine. It is very interesting that two major acidic phospholipids of *E. coli*, phosphatidylglycerol and cardiolipin, play completely different roles in the osmotic stability of the lipid bilayers. Phosphatidylethanolamine cannot form sealed liposomes by itself under the physiological conditions. The introduction of cardiolipin enable phosphatidylethanolamine to form such liposomes as can be seen in Fig. 4. However, the fact that liposomes of (phosphatidylethanolamine + cardiolipin) in (8 : 2) and (6 : 4) showed similar osmotic properties suggests that cardiolipin cannot play an active role in osmotic properties of the liposomes. Actually, cardiolipin is known to be dispensable for the growth of *E. coli* cells [18]. In contrast, cells lacking phosphatidylglycerol are nonpermissive [19]. However, no evidence of the particular vital roles of phosphatidylglycerol has ever been presented. Our results, therefore, are the first evidence of a vital role of phosphatidylglycerol. In addition, it was shown in this work that there is a good correlation between the apparent osmotic stability of the cells and the osmotic properties of the liposomes of the extracted phospholipids. It would strongly suggest that the osmotic properties of phospholipid bilayers are mainly responsible for the apparent osmotic stability of the original cells. As a whole one may infer that phosphatidylglycerol also plays an essential role in stabilizing the plasma membrane of *E. coli* cells against osmotic pressure.

The mechanism by which phosphatidylglycerol plays such a role can be speculated in some ways. Phosphatidylethanolamine prefers the hexagonal phase (H_{II}) at high temperatures. Cardiolipin also undergoes the transition from the lamellar to the hexagonal phase in the presence of divalent cations [20,21]. In contrast, phosphatidylglycerol prefers the lamellar structure and suppress the transition to the hexagonal phase when it is introduced into the phosphatidylethanolamine bilayers [22]. The tendency taking the hexagonal phase of phosphatidylethanolamine and cardiolipin is attributed to the small size of their polar head groups [21]. The difference in osmotic nature between phosphatidylglycerol and cardiolipin becomes evident under hypotonic conditions as can be seen in Fig. 4. Under such conditions the membrane would experience lateral expansion which increases the intermolecular distance. Therefore, it is probable that the intermolecular interaction through the polar head groups of phosphatidylethanolamine and cardiolipin cannot be maintained on lateral expansion of the membrane because of their small size.

It was pointed out in this work that glycerol might not be an osmotic stabilizer because it can permeate through the liposomal membranes. The possibility if permeable organic molecules can protect cells under non-permissive conditions was examined. Ethyleneglycol, urea and erythritol also permeate through phospholipid bilayers [5]. We checked the stabilizing effects of these materials, but the cells could not grow under high concentrations of these materials. Therefore, it can be said that these permeable materials cannot protect the cells, with the exception of glycerol. The different osmotic behavior of PL42(glycerol) from that of PL42(KCl) and PL42(sucrose) strongly suggests that the mechanism involved in the survival of the

auxotroph in the presence of 9.5% glycerol is associated with the maintenance of high content of phosphatidylglycerol. The presence of a high concentration of glycerol in the medium would suppress the synthesis of cardiolipin and glycerol from two moles of phosphatidylglycerol (for biosynthesis scheme, see Ref. 23), which would result in a higher content of phosphatidylglycerol than that of cardiolipin in the cells. This could be the reason why PL42(glycerol) contains a larger amount of phosphatidylglycerol than that of cardiolipin in contrast to PL42(KCl) and PL42(sucrose).

Appendix

As indicated by Bangham et al. [5], the total volume of liposomes changes linearly with the inverse concentration of the solute outside the liposomes, provided that the liposomes act as perfect osmometers. We would like to describe the relationship as a function of the osmotic gradient (C_{in}/C_{out}) explicitly. Here, C_{in} and C_{out} are the concentrations of solute inside and outside the liposomes, respectively. Imagine an ideal osmometer which is divided into two compartments by a freely movable semipermeable-membrane. The volumes and the solute concentrations of the two compartments in the initial state are represented by V_1 and V_2, and C_1 and C_2, respectively. If C_1 and C_2 are not equal, a volume change will take place at equilibrium. The change in volume in compartment 1, dV ($-dV$ for compartment 2), can be given by

$$dV = [V_1V_2(C_1-C_2)]/(V_1C_1+V_2C_2) \quad \text{(A-1)}$$

In case of

$$V_1C_1 \gg V_2C_2 \quad \text{(A-2)}$$

we get

$$dV = V_2 - V_2(C_2/C_1) \quad \text{(A-3)}$$

or

$$V_2' = V_2(C_2/C_1) \quad \text{(A-4)}$$

where V_2' is the volume of compartment 2 at equilibrium. If we take compartments 1 and 2 for the outside and inside of liposomes in the dispersion, condition A-2 is fulfilled except when under extremely hypotonic conditions. Taking the osmotically inactive volume of the liposomes (V_{dead}) into account, Eqn. A-4 can be rewritten as

$$V_{total} = V_0(C_{in}/C_{out}) + V_{dead} \quad \text{(A-5)}$$

where V_{total} is the total volume of the liposomes and V_0 is the osmotically active volume of the liposomes when they are formed under $C_{out} = C_{in}$.

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