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A ^{2}H-NMR study on the glycerol backbone of phospholipids extracted from *Escherichia coli* grown under high osmotic pressure: evidence for multiconformations of phosphatidylethanolamine

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A glycerol-requiring auxotroph was isolated from mutagenized *Escherichia coli* K-12 UFA^{ts} cells. This auxotroph was used for the specific deuteration of *E. coli* phospholipids. The cells were grown under high osmotic pressure (in the presence of 2.0% KCl). The membrane had a highly saturated fatty acid composition (76% phosphatidylethanolamine, 20% cardiolipin and 4% phosphatidylglycerol). The deuterium magnetic resonance spectra of coarse liposomes of the extracted phospholipids with perdeuterated glycerol incorporated into them were measured. To obtain well characterized information, phospholipid mixtures reconstituted from the deuterated and nondeuterated components at the same ratios as in the case of the total extract were used. On the analysis of the spectra, the following conclusions were drawn. (1) The whole polar region of cardiolipin is dynamically symmetric and quite rigid in the presence of phosphatidylethanolamine. (2) Although the quadrupole splittings of the deuterons at the C-2 and C-3 positions of the glycerol backbone were similar to each other, those at the C-1 position for phosphtidylethanolamine and cardiolipin are different, even in the same bilayer. (3) Furthermore, each C-1 deuteron of phosphatidylethanolamine gave rise to a doublet, suggesting the presence of two backbone conformations, between which there is slow exchange. (4) The polar head group of phosphatidylethanolamine interacts with cardiolipin and phosphatidylglycerol in different ways, which could be responsible for the different osmotic properties of the vesicles composed of them.

1. Introduction

A temperature-sensitive auxotroph requiring unsaturated fatty acids (*E. coli* K-12 UFA^{ts}) can grow without a supply of unsaturated fatty acids under high osmotic pressure [1,2]. The membrane phospholipids of the cells have a unique chemical composition. Namely, the fatty acid composition is highly saturated and the membrane accumulates a high amount of cardiopolin instead of phosphatidylglycerol. The phase transition profile of this membrane system has been extensively examined [3,4]. It was shown that the membrane is in a state of phase separation even at the growth temperature, 42°C. The contribution of polar head groups to the osmotic stability was investigated using multilamellar liposomes [5]. It was suggested

Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

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that phosphatidylglycerol plays an essential role in osmotically stabilizing the membrane and that the high amount of cardiolipin must be responsible for the instability of the cells grown at 42°C under hypertonic conditions. To clarify the role of polar groups in the osmotic properties of the membrane, more detailed investigation on the dynamic structure of the polar groups is necessary. Deuterium magnetic resonance (^{2}H-NMR) is one of the powerful methods for such an investigation [6,7].

A glycerol-requiring mutant is a good tool for labelling of the polar region of *E. coli* phospholipids for ^{2}H-NMR investigations [8–11]. The properties of the glycerol backbones and head groups of phosphatidylethanolamine and phosphatidylglycerol were investigated extensively using the *E. coli* T131GP strain [8,9]. This mutant lacks the ability to synthesize cardiolipin and its membrane is simply composed of phosphatidylethanolamine (approx. 80%) and phosphatidylglycerol (approx. 20%). The properties of the polar group of cardiolipin were previously investigated in detail in the absence and presence of phosphatidylcholine [11]. An investigation on a system mainly containing phosphatidylethanolamine and cardiolipin would provide more complete information on the polar group interactions in the *E. coli* membrane together with the earlier results. This was carried out in this work using our mutant, which contains a high amount of cardiolipin (20 mol%). First of all, a glycerol-requiring auxotroph was isolated from the UFA^{ts} mutant for the incorporation of deuterated glycerol into the membrane phospholipids. Then the structure and dynamics of the polar region of the membrane system were investigated by ^{2}H-NMR. We previously reported a preliminary study on the phospholipids of this *E. coli* strain [10].

Materials and Methods

Isolation of a glycerol-requiring mutant

The parental strain, K-12 UFA^{ts}, was treated with *N*-methyl-*N'*-nitrosoguanidine [12]. The survivors were grown overnight in the basal medium supplemented with 0.4% glycerol. The basal medium had the following composition: K_2HPO_4, 7 g; KH_2PO_4, 2 g; Na_3citrate, 0.5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and vitamin B, 1 mg/l. The cells were transferred to the basal medium containing 1.0% casamino acids, with which glycerol-requiring auxotrophs cannot grow. The growing cells were selectively killed by the addition of 200 units/ml of penicillin [13]. Glycerol-requiring auxotrophs were obtained from the penicillin survivors at a frequency of approx. 5×10^{-3}. Spontaneous revertants were obtained at a frequency of less than 10^{-6} by plating on basal medium–casamino acids agar. A mutant obtained, designated as UFA^{ts} GRA, was used in this work. Its defect possibly corresponds to *gps* A, as judged from the results of biochemical analysis.

Incorporation of [$^2H_5$]glycerol into E. coli phospholipids

The mutant obtained was grown at 42°C in the basal medium containing 1% casamino acids, 2% KCl and 0.002% perdeuterated glycerol. Glucose cannot be used as a carbon source because of the feedback inhibition to the glycerol-3-phosphate synthase [14]. The perdeuterated glycerol ([$^2H_5$] glycerol) was purchased from the Commissariat à l'Energie Atomique (CEA) and [2-^{2}H]glycerol was obtained by reduction of dihydroxyacetone with NaB^2H_4 in ^2H_2O. The percentage of deuteration was estimated from a ^{1}H-NMR spectrum for phosphatidylethanolamine. From the relative intensity of the glycerol signals to that of the β-methylene signal of the ethanolamine group, the extent of deuteration was estimated to be 65–70%.

Phospholipids

Phospholipids were extracted from the cells according to the method of Bligh and Dyer [15] and then further purified by silicic acid column chromatography. The total phospholipid extract was loaded on a silicic acid thin layer plate, which was then developed with chloroform/methanol/water (45 : 25 : 4, v/v). Each phospholipid component was recovered from the respective spot on the plate with chloroform/methanol (2 : 1, v/v). The mole fraction of each phospholipid species was determined by a combination of thin layer chromatography and the phosphorus assay [16]. In order to remove polyvalent metal ions, cardiolipin and phosphatidylglycerol were dissolved in chloroform/methanol (1 : 1, v/v) and then washed with

a 0.5 vol. of 1 0.5 M Na_2SO_4, 2.0 mM EDTA solution (pH 7.2). Phosphatidylglycerol was obtained by transphosphatidylation of purified phosphatidylethanolamine using cabbage phospholipase D [17].

^2H and ^{31}P-NMR measurements

Phospholipids were dispersed at 50°C in a solution of 0.1M Pipes buffer (pH 7.2) containing 2 mM EDTA, and then centrifuged to obtain a pellet. Deuterium-depleted water (less than 0.2 ppm, purchased from CEA) was used for all sample preparations to eliminate the naturally abundant deuterium signal of water. ^2H-NMR spectra of phospholipid dispersions (coarse liposomes) were measured with a Bruker WM360 wb spectrometer at 55.3 MHz with a spectral width of 100 kHz and a recycle time of 0.15 s, employing the quadrupole echo technique [7]. The pulse sequence of $90_x - \tau_1 - 90_y - \tau_2$ was used with $\tau_1 = \tau_2 = 120$ μs. The 90° pulse width was 24 μs. ^{31}P-NMR spectra were recorded on a JEOL FX-100 spectrometer equipped with a solid-state NMR system at 40.3 MHz under broad-band proton decoupling, with a 60° pulse (3.0 μs) and recycle time of 2.0 s.

Results

The ^2H-NMR spectrum of an aqueous dispersion of the total phospholipid fraction was obtained at various temperatures. The results of thermal analysis indicated that the gel to liquid-crystalline phase transition finished at around 45°C with an asymmetric thermogram, which was similar to that obtained for UFA^{18} phospholipids [4]. The ^2H-NMR powder pattern showed a gradual collapse with decreasing temperature from 40°C, at which the gel and liquid-crystalline phases coexist. Fig. 1a shows the spectrum for the total phospholipid fraction carrying [$^2H_5$]glycerol moieties at 50°C. In the powder-pattern spectrum, a single deuteron gives rise to a couple of peaks separated by so-called quadrupole splitting with shoulders. Since we used a conventional high-resolution NMR spectrometer, the obtained powder pattern lacked the shoulders and was not a typical one. However, the quadrupole splittings can be read even from such a spectrum. Actually,

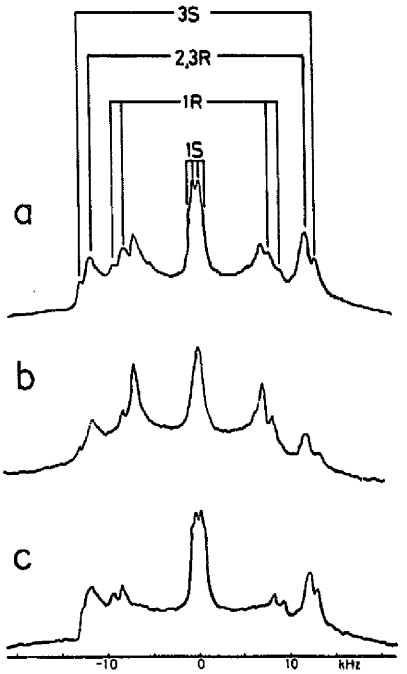

Fig. 1. ^2H-NMR spectra for aqueous dispersions of the total phospholipid fraction (a), deuterated cardiolipin with the other nondeuterated components (b) and deuterated phosphatidylethanolamine with the other nondeuterated components (c) at 50°C. The phospholipid compositions of (b) and (c) are the same as that of (a) (see text). [$^2H_5$]Glycerol was specifically incorporated into the phospholipids. The assignments for the glycerol backbone are given on the top. The *S* and *R* nomenclature means that when a proton of interest is replaced by a deuteron, the system takes on the *S* and *R* configurations, respectively. The quadrupole echo sequence ($90_x - \tau_1 - 90_y - \tau_2$) was used with $\tau_1 = \tau_2 = 120$ μs.

the spectrum in Fig. 1a shows distinct quadrupole doublets. A singlet at the center was also observed, depending on the temperature. The ^{31}P-NMR spectrum of the same sample showed that it took on a pure bilayer structure at below 75°C, and the isotropic phase became predominant at above 80°C. Consequently, the central signal should be due to the lamellar lipids as well. All the glycerol moieties of phosphatidylethanolamine, cardiolipin and phosphatidylglycerol contribute to the spectrum. The proportions of these phospholipids were 76%, 20% and 4%, respectively [10].

Such complexity makes difficult the assignment of these quadrupole splittings to specific deuterons of specific phospholipids.

To simplify the spectrum and to make the assignment easier, ^{2}H-NMR spectra of individual phospholipid species in this phospholipid mixture were obtained using reconstituted bilayers. The bilayers were reconstituted as follows. Each component of the total lipids was purified as described under Materials and Methods. A purified deuterated phospholipid species was remixed with the other nondeuterated ones at the same ratio as in the case of the total phospholipid fraction. Typical spectra for deuterated cardiolipin and phosphatidylethanolamine obtained with such systems at 50 °C are presented in Fig. 1b and c, respectively. The phosphatidylglycerol content was too small (4.0%) to obtain a spectrum with a sufficient S/N ratio. Since the phosphatidylglycerol content is very small, the spectral pattern of the total phospholipid fraction (Fig. 1a) can be explained in terms of the spectra for cardiolipin (1b) and phosphatidylethanolamine (1c).

As to the glycerol backbone of phosphatidylethanolamine, the assignment of the quadrupole splittings was performed unambiguously, with stereospecifically monodeuterated samples [8]. Following their assignment, we could discern the corresponding splittings of phosphatidylethanolamine in our spectrum. The outermost signals were assigned to the 3*S* deuteron and the broad signals showing the second-largest quadrupole splitting were assigned to the 2- and 3*R* deuterons. Nomenclatures of *S* and *R* mean that when a proton of interest is replaced by a deuteron, the system takes on the *S* and *R* configurations, respectively. The quadrupole splittings of around 15 and 0 kHz should be ascribed to the 1*R* and 1*S* deuterons, respectively. However, a doublet appeared at each position, showing that each deuteron at the 1*R* and 1*S* positions gives rise to two different quadrupole splittings. The assignments are given in Fig. 1.

Cardiolipin has three glycerol moieties, namely, two glycerol backbones and one head segment bridging two phosphate groups, as shown in Fig. 2. Despite the fact that signals of all the glycerol moieties contribute to the spectrum, the powder pattern is quite simple. The assignment of the

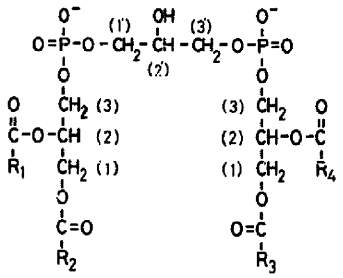

Fig. 2. Structure of and nomenclature for the polar region of cardiolipin.

cardiolipin signals was carried out partly by Yoshikawa et al. [10] and thoroughly by Allegrini et al. [11]. As to the glycerol backbone, common features of the ^{2}H-NMR spectra are known for various phospholipid species (for phosphatidylcholine [18], phosphatidylethanolamine and phosphatidylglycerol [8], phosphatidylserine [19]). That is, all three deuterons at the C-3 and C-2 positions give rise to similar quadrupole splittings, in the range of 22–28 kHz, and the two deuterons at C-1 show two different quadrupole splittings, of around 0 and 17 kHz, respectively. This is also the case with cardiolipin. Quadrupole splittings of 25, 22, 16 and 0 kHz were observed in the spectrum for cardiolipin in the reconstituted system at 50 °C. In particular, the two outermost signals overlapped those of phosphatidylethanolamine in the spectrum for the total phospholipids. Since just one set of signals was observed for the glycerol backbones of cardiolipin, it can be concluded that the two glycerol backbones take on dynamically equivalent structures in the lipid bilayer, as already indicated [10,11]. Besides the quadrupole splittings assigned to the glycerol backbones, a couple of strong signals were observed at around 14 kHz. These signals are readily assigned to the deuterons of the polar head. The strong intensity indicates that the majority of the five deuterons in the bridge segment give rise to the same or similar quadrupole splittings.

To check the quadrupole splittings of the methine deuteron, [2-^{2}H]glycerol was incorporated into the phospholipids and then the ^{2}H-NMR spectrum was measured for the total phospholipid fraction. Fig. 3 shows the spectrum ob-

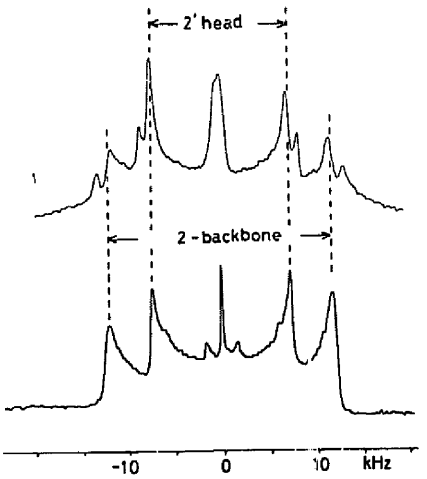

Fig. 3. Comparison of the ^{2}H-NMR spectra for the total phospholipid fraction from cells with incorporated [2-^{2}H]glycerol (lower) and perdeuterated glycerol (upper) at 50 °C. The innermost doublet in the lower spectrum is attributed to the head group of phosphatidylglycerol.

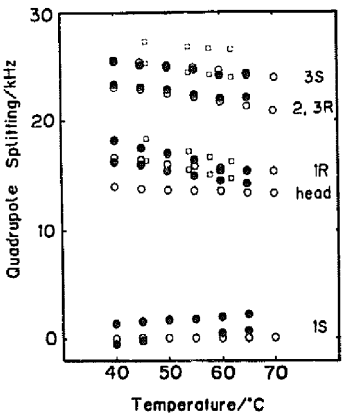

Fig. 4. Temperature dependence of the quadrupole splittings for cardiolipin (○) and phosphatidylethanolamine (●) in reconstituted phospholipid liposomes and for pure phosphatidylethanolamine (□) bilayers. The assignments are also given in the figure.

tained at 50 °C. Three well discerned splittings were observed. The outermost splitting of 23 kHz can be assigned to the glycerol backbones of all three phospholipid species. From the intensities, the inner two splittings also can be assigned unambiguously. Namely, the strong signal showing the splitting of 14 kHz can be assigned to the polar head of cardiolipin, and the weak one with the splitting of 3 kHz can be assigned to that of phosphatidylglycerol. These results led us to the conclusion that all five deuterons of the cardiolipin head group accidentally take on the same quadrupole splitting. Allegrini et al. [11] showed that the quadrupole splittings of the four deuterons at C-1′ and C-3′ are slightly different in the cardiolipin bilayer but that they become similar to each other in the presence of dioleoylphosphatidylcholine.

The quadrupole splittings and the assignments are summarized in Fig. 4. As can be seen, the quadrupole splittings of the C-2 and C-3 deuterons of the glycerol backbone are practically identical for cardiolipin and phosphatidylethanolamine. However, the quadrupole splittings of their C-1 deuterons are different. Both the 1*R*

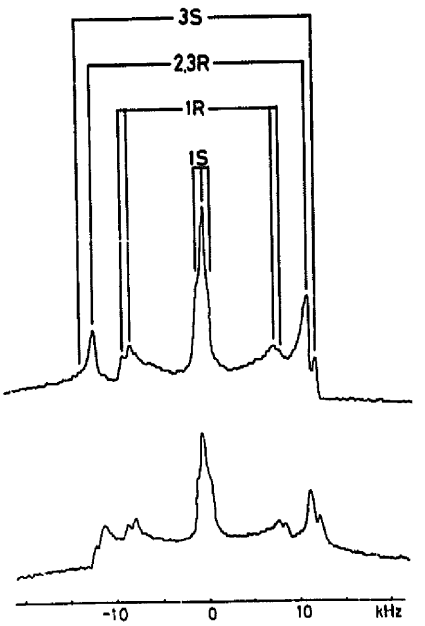

Fig. 5. ^{2}H-NMR spectra for phosphatidylethanolamine with incorporated [$^2H_5$]glycerol in the presence of nondeuterated phosphatidylglycerol (upper) and mainly nondeuterated cardiolipin (lower) at 45 °C.

and 1*S* deuterons of phosphatidylethanolamine gave rise to two sets of quadrupole splittings. Furthermore, the temperature dependence of the splitting of cardiolipin is much lower than that of phosphatidylethanolamine, suggesting that cardiolipin has a more rigid structure. There is a possibility that the two quadrupole splittings at the C-1 position of the glycerol backbone are due to the interaction between phosphatidylethanolamine and cardiolipin. To check this possibility, the purified phosphatidylethanolamine was mixed with nondeuterated phosphatidylglycerol (ratio, 4:1). The ^{2}H-NMR spectrum of the mixture at 45°C is presented in Fig. 5. Although the quality of the spectrum is rather poor, it is clear that the signals of the 1*R* and 1*S* deuterons gave rise to two sets of quadrupole splittings. A doublet was also detected for the pure phosphatidylethanolamine bilayer. The temperature dependence of the quadrupole splittings is shown in Fig. 4. These results indicate that the phospholipid composition does not affect the appearance of the two quadrupole splittings at the C-1 position of the glycerol backbone. It should also be noted that the effect of cariolipin on the quadrupole splittings of the glycerol backbone of phosphatidylethanolamine is great at the C-2 and C-3 positions but small at the C-1 position, as can be seen in Fig. 4.

Discussion

The combination of deuterium labelling and ^{2}H-NMR measurement is very useful for studying biological membranes, because accurate information can be obtained with even a complicated system [6,8]. In the present study, each deuterated phospholipid component was purified and then remixed with the other nondeuterated components. This procedure enables us to measure the ^{2}H-NMR spectrum for only a single component in the *E. coli* phospholipid system. The results showed the interesting fact that the dynamic properties of the glycerol backbone are not identical for each phospholipid species. The major component in the *E. coli* membrane is phosphatidylethanolamine, minor components being acidic phospholipids, phosphatidylglycerol and cardiolipin. The membrane of *E. coli* T131GP contains only phosphatidylethanolamine and phosphatidylglycerol [20]. A ^{2}H-NMR study on its phospholipids showed that the glycerol backbone is practically the same for phosphatidylethanolamine and phosphatidylglycerol [8]. Our present work entailed the analysis of the *E. coli* membrane with a different phospholipid combination (mostly phosphatidylethanolamine and cardiolipin). While the quadrupole splittings of the C-2 and C-3 deuterons are indistinguishable not only between phosphatidylethanolamine and phosphatidylglycerol, but also between phosphatidylethanolamine and cardiolipin, the C-1 deuterons of the latter behave in a different manner. That is, the temperature dependence of the quadrupole splitting is lower for cardiolipin than for phosphatidylethanolamine, and two sets of quadrupole splittings were observed for single deuterons of phosphatidylethanolamine. Such a difference was not observed for the two phospholipid components of the T131GP mutant. The two quadrupole splittings show the presence of two motionally different states at the C-1 position of the glycerol backbone of phosphatidylethanolamine, the exchange rate of which is in slow. The two states can be ascribed to a difference either in conformation or fluctuation. Since doublets were not observed for the deuterons at the C-2 and C-3 positions in both the presence and absence of acidic phospholipids, fluctuation is unlikely to be responsible for the appearance of the doublets. It thus may be attributed to different conformations. The two conformations of the glycerol backbone were conserved even in a mixture of phosphatidylethanolamine and phosphatidylglycerol. Therefore, the two conformations cannot be explained by the interaction of the polar head groups between phosphatidylethanolamine and cardiolipin. Considering the compositional difference between the T131GP mutant and ours, the appearance of the two sets of quadrupole splittings should be ascribed to the highly saturated fatty acids of our mutant. It is likely that the exchange rate between the two conformations is faster than the NMR time-scale in the presence of a high amount of unsaturated fatty acids.

Since the C-1 position of the glycerol backbone is farthest from the polar head group, it was unexpected that the polar head group had an effect on the backbone conformation at this posi-

tion. This must take place through the hydrocarbon chain packing which is affected by the polar head group interaction. Since the appearance of the doublet for the C-1 deuterons was influenced by neither cardiolipin nor phosphatidylglycerol, the doublet must originate from the phosphatidylethanolamine–phosphatidylethanolamine interaction. Thus, it can be said that the phosphatidylethanolamine self-interaction is still preferential even in a mixed lipid bilayer. It was also suggested by the results of a calorimetric study that phosphatidylethanolamine molecules exhibit a net tendency to self-associate preferentially in a mixture with phosphatidylcholine [21]. Therefore, such preferential self-interaction must be a general feature of phosphatidylethanolamine in a lipid mixture. Of course, it does not directly indicate the immiscibility of these lipids. In the case of a lipid system with heterogeneous fatty acids such as that used in this work, there could be multidomains in the bilayer. Thus, the two conformations could originate from either different domains or the same domain. In view of the quite small amount of unsaturated fatty acids (less than 10%), the two conformations are likely to occur in the same domain.

As to the conformation of the glycerol backbone of phosphatidylethanolamine, Blume et al. [22] suggested that the conformation in the gel phase must be different from that in the liquid-crystalline phase. Allegrini et al. [11] also indicated that the quadrupole splittings of the 1*S* and 1*R* deuterons (about 0 and 17 kHz, respectively) cannot be explained by a single conformation. Actually, two conformations were found for the crystal structure of 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine [23], although a single conformation was reported for 1,2-dilauroyl-*rac*-glycero-3-phosphorylethanolamine [24]. The correlation between these conformations and our observations is not clear yet.

The quadrupole splittings of the cardiolipin polar group have been thoroughly investigated [10,11]. It was reported that the quadrupole splitting of the C-2′ deuteron increased on addition of phosphatidylcholine (from about 17 kHz to 22–20 kHz). However, this was not the case in the presence of phosphatidylethanolamine. This must be another piece of evidence for the weak head group interaction between phosphatidylethanolamine and cardiolipin. In addition to this weak polar head group interaction, the difference in the temperature dependence of the quadrupole splittings of the C-1 deuterons of the backbone shows that the polar groups do not behave like a part of the homogeneous bilayer in a mixture of phosphatidylethanolamine and cardiolipin. In contrast, the quadrupole splittings of the head groups affected each other in a mixture of phosphatidylethanolamine and phosphatidylglycerol [25]. Therefore, it can be said that the polar group interaction between phosphatidylethanolamine and cardiolipin is clearly different from that between phosphatidylethanolamine and phosphatidylglycerol. It was shown that phosphatidylglycerol osmotically stabilizes *E. coli* phospholipid vesicles but that cariolipin does not [5]. The difference in the polar group interaction mentioned above could be responsible for the different roles of the two acidic phospholipids in the formation of osmotically stable *E. coli* bilayers, through either elastic properties or the vesicle shapes. In view of the observed change in the surface area of a unilamellar vesicle under osmotic pressure, the latter is more plausible [26,27].

Acknowledgement

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