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Biochimica et Biophysica Acta 1283 (1996) 73–79

BBA Biochimica et Biophysica Acta

Ethanol-enhanced permeation of phosphatidylcholine/phosphatidylethanolamine mixed liposomal membranes due to ethanol-induced lateral phase separation

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Received 18 December 1995; revised 25 April 1996; accepted 26 April 1996

Abstract

Effects of ethanol on permeability of large unilamellar vesicles (ca. 160 nm in diameter), composed of dipalmitoyl phosphatidylcholine/dilauroyl phosphatidylethanolamine (DLPE) mixture, were studied by monitoring leakage of the fluorescent dye, calcein, entrapped in the inner aqueous phase of the vesicles. In the presence of 2.1 M ethanol, permeabilities of membranes in various phases were *G* (bilayer gel) phase > *L* (bilayer liquid-crystalline) phase with a high mole fraction of DLPE and (*I* (ethanol-induced interdigitated gel phase) + *G*) phase > (*I* + *L*) at 20 mol %DLPE. Arrhenius plots of the leakage rate constants demonstrated that the permeability was greater with 2.1 M ethanol than without ethanol, especially in the temperature above 33°C, suggesting that the presence of ethanol can induce lateral phase separation of liposomal membranes and cause them to have a high permeability even if they are stable and have low permeability in its absence.

Keywords: Ethyl alcohol; Interdigitation; Permeability; Liposome; Sterilization

1. Introduction

It is now well established that saturated-chain phosphatidylcholines can form interdigitated membranes, in which the terminal methyl groups of the acyl chains extend beyond the bilayer midplane, effectively interpenetrating into the opposing monolayer, in the presence of short chain alcohols, some polyoles such as ethylene glycol and glycerin, anesthetics such as chlorpromazine and tetracaine, and some other amphiphilic ligands [1,2]. The induction of the interdigitated phase by many kinds of alcohols, especially ethanol, has been studied in detail in our laboratories and others [3–23].

It has been suggested by numerous studies that formation of the interdigitated structure as well as the inverted hexagonal phase (H_{II}) [24] can play an important role in regulating many functions of biomembranes [1,2]. In our recent paper [23], we documented that the formation of interdigitated structure domains in membranes could be one of the triggers causing membrane adhesion and/or fusion. Our previous studies [25–27] focused on control of permeability as one of the biomembrane functions, and the effects of ethanol on the permeability of large unilamellar vesicles, composed of dipalmitoyl phosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (eggPC), were studied by monitoring leakage of the fluorescent dye, calcein, entrapped in their inner aqueous phase. Large permeabilities were observed in the range of 0.6 M to 1.3 M ethanol, where the interdigitated structure domain could be formed in the liposomal membrane: Normal bilayer and interdigitated structures coexist, and the membrane is in a phase-separated state. The high permeability was due to instability of boundary regions around the interdigitated structure domain, the interdigitated membrane being characterized by a thinner structure and more rigid hydrocarbon regions than its non-interdigitated counterpart [25–27].

Phosphatidylethanolamines are a major constituent of most mammalian and bacterial cell membranes. These

Abbreviations: *G*, regular bilayer gel phase; *I*, interdigitated gel phase; *L*, bilayer liquid-crystalline phase; DPPC, dipalmitoyl phosphatidylcholine; eggPC, egg yolk phosphatidylcholine; LUVET, unilamellar vesicle made by the extrusion technique; H_{II}, inverted hexagonal phase; DLPE, dilauroyl phosphatidylethanolamine; MLV multilamellar vesicle

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PII S0005-2736(96)00082-X

lipids have attracted attention because of the formation of non-bilayer configurations such as the H_{II} phase at a physiological temperature and pH, seeming to be important in functional processes such as membrane fusion, cell division and transbilayer movement of lipids and proteins [28–31]. Rowe [5] have studied phase structures of binary dilauroyl phosphatidylethanolamine (DLPE) and DPPC mixture systems, and clearly shown phase diagrams in the presence and absence of ethanol. It was demonstrated that in the presence of ethanol the phase involves the coexistence of a regular bilayer gel and the fully interdigitated gel phase, and that in the absence of ethanol these two lipids are miscible in both the gel and liquid-crystalline states [5]. It can be, therefore, expected that in the presence of ethanol lateral phase separations, with mixtures of non-interdigitated and interdigitated phases, would be associated with high membrane permeability. In the present study, mixed membranes, composed of DPPC and DLPE were chosen to assess effects of ethanol on membrane permeability. For this purpose, leakage of calcein, entrapped in the inner aqueous phase of large unilamellar vesicles made by the extrusion technique (LUVETs), was examined for a variety of lipid ratios and at various temperatures.

2. Materials and methods

2.1. Chemicals

Dipalmitoyl L-α-phosphatidylcholine (DPPC)(99 + %, Crystalline, P-6267, lot#62H8350) and dilauroyl L-α-phosphatidylethanolamine (DLPE)(99%, P-6270, lot#92H8371) were obtained from Sigma (St. Louis, MO) and no further purifications were performed. Ethanol was purchased from Wako Pure Chemicals, Osaka, Japan. Calcein was obtained from Dojindo Laboratories, Kumamoto, Japan. De-ionized and reverse-osmotic treated water was distilled with a quartz still. All other agents were of analytical grade.

2.2. Preparation

Stock solutions of DPPC and DLPE were prepared in chloroform/ethanol (99:1, v/v) and (50:50, v/v), respectively, and kept in a freezer under a nitrogen gas atmosphere in the dark until used.

Multilamellar vesicles (MLVs) were prepared as follows. Various mixtures of the stock solutions of lipids were dried in a rotary evaporator under reduced pressure to form lipid films on the walls of round-bottomed flasks. These films were left in vacuo for at least 12 h to ensure complete removal of the solvent. 1 ml of calcein-containing buffer solution (277 mosM), composed of 70 mM calcein, 30.5 mM NaCl and 1 mM Tris, adjusted at pH 7.0 using NaOH solution, was added to each thin film containing 0.12 mmol lipids. Nitrogen gas was bubbled to remove any dissolved oxygen and the lipid was hydrated at about 50°C. During this incubation, the sample was vortexed periodically.

Calcein-entrapped LUVETs were obtained using freezing-thawing and extrusion methods [32,33] as follows: Frozen and thawed vesicles were obtained by freezing in liquid nitrogen and thawing in a 50°C water bath, with the freeze-thaw cycle being repeated five times. Extrusion of the frozen and thawed vesicles through two (stacked) polycarbonate filters of 0.2 mm pore size was performed at 50°C employing a nitrogen-gas pressure of 15 kg/cm^2. In order to eliminate calcein which was not trapped into the inner aqueous phase of the LUVETs, 0.5 ml of liposomal solution, diluted 4 times with buffer solution without calcein, was gel-filtered through a Sephadex G-50 column (15 mm I.D. × 29 cm) using the buffer solution as an eluent at about 25°C, and orange-colored fractions in the eluted solution were collected. Here, the buffer solution (277 mosM) was composed of 0.15 M NaCl and 1 mM Tris, adjusted to pH 7.0 using HCl solution.

Average diameters of the DLPE/DPPC LUVETs, evaluated using the dynamic light scattering technique (details described in our previous report [26]), were independent of the content of DLPE, and their average was about 160 nm on cumulate analysis with a range of DLPE mole fractions from 0 to 0.7. The estimated average sizes of DPPC LUVETs were comparable with the value reported by Mayor and co-workers, of about 180 nm, estimated for eggPC LUVET [33]. Measurements of size distribution using a multimodal method (exponential sampling algorithm) [34,35] suggested that the size distributions of the LUVETs are homo-dispersed. The narrow size distribution was also confirmed by freeze-fracture electron micrography. The entrapped volumes of the DPPC LUVETs evaluated using calcein as a marker were also comparable with reported values [36]. In the cases of liposomes above a 0.8 DLPE mole fraction, gel-filtration could not be performed because the column was clogged: The formation of LUVETs was uncertain.

2.3. Calcein-leakage measurements

Leakage measurements were started by addition of 20 μl of calcein-entrapped liposomal solution to 3 ml of buffer solution, containing the desired concentration of ethanol, in a fluorescence cuvette. This procedure was required in order to avoid high local ethanol concentrations in the LUVET before the sample could be completely mixed. After the sample addition, fluorescence intensities at 520.0 nm were continuously measured with excitation at 490.0 nm at various temperatures with an accuracy of ±0.1°C using a Hitachi F-650 spectrofluorometer (Tokyo, Japan) equipped with a thermostated cuvette holder. The bandpass was 2.0 nm for both excitation and emission monochrometers. All samples were thoroughly magneti-

cally stirred during the measurements. The concentrations of DPPC and DLPE were determined as those of P_i by a modified version of the procedure of Bartlett [37]. The final concentration of lipids was about 33 μM.

At high concentrations above 70 mM, calcein fluoresces only weakly because of self-quenching. Reducing the concentration of calcein by diluting calcein solutions results in increased fluorescence as this quenching is reduced. Calcein which is entrapped inside liposomes at high concentration will give no increase in fluorescence, however, even when the liposomal solution is diluted, since the concentration of the entrapped liposomal contents remains unchanged. On the other hand, calcein, which has leaked out of liposomes into the extra-liposomal medium, displays enhancement of its fluorescence upon dilution in the suspension. This may also be accomplished by lysis of diluted liposomes with a detergent such as Triton X-100. This technique is described in detail elsewhere [38,39].

In our previous studies, it was demonstrated that a linear relationship exists between fluorescence intensity and calcein concentration with or without 0.33% Triton X-100 and/or a high concentration of ethanol under our experimental conditions [25–27]. For the present measurements, the concentrations of calcein-entrapped liposomes were controlled in the region where a linear relationship between the fluorescence intensity and the calcein concentration could be obtained even if all calcein entrapped in the inner aqueous phase of liposomes leaked out.

3. Results and discussion

3.1. Leakage rate constants

The leakage from the inner aqueous phase of various LUVETs showed two stages; fast leakage within 30 sec, followed by slow leakage, comparable with our previous findings for DPPC LUVETs about 250 nm in diameter [25–27]. The fast stage occurred rapidly when aliquots of calcein-entrapped liposomal solution were added to the buffer solution, containing the desired concentration of ethanol, and the solution was mixed in a fluorescence cuvette. In our measurements, therefore, the fast leakage stage could not be followed using our experimental apparatus.

The changes in fluorescence intensity in the initial stage were dependent upon the ethanol concentration but they were below about 20% of the I_{inf} values. Here, the symbol I_{inf} represents the fluorescence intensity after lysis of liposomes using a detergent, Triton X-100. At the first step of the phase change process, dynamic changes in liposomal structure would be induced: Particularly in the phase transition from normal bilayer to interdigitated structures, changes in liposomal size and surface area (and as a result, inner volume) become apparent [40]. In this step, phase separation occurs frequently and can cause formation of small holes, of heterogeneous size, in the liposomal membranes. Thus, the fast leakage observed within 30 sec could be ascribed to such leakage in the initial process of the phase-changes. Our present interest, however, was not focused on the stages of change in phase but on the leakage from phase-separated liposomal membranes, which are in almost at equilibrium in the presence of ethanol.

In the previous studies [12,23,41,42], it was demonstrated that the presence of high concentration of ethanol can lead to fusion of small DPPC-liposomes. The fusion can cause the liposome to leak its contents. Although it is still unknown what effects DLPE may exert such fusion, the fusion of small liposomes, contaminating the LUVETs, may be also one of the mechanisms responsible for the fast leakage component. Details of the mechanism remain to be established and therefore in the present study, permeability was estimated only for the slow leakage stage.

It is known that the leakage of entrapped substances from the inner aqueous phase of liposomes follows first-order kinetics [43]. The fluorescence intensity I of calcein at the incubation time t can be written as [26]

$$\ln(1 - I/I_{inf}) = A - kt \quad (1)$$

where k is the rate constant. Here, $A = \ln(1 - I_{init}/I_{inf})$ and I_{init} is the initial fluorescence intensity. The detailed derivation of Eq. (1) was described in our previous paper [26].

Typical plots of $\ln(1 - I/I_{inf})$ *vs.* t according to Eq. (1) for the DLPE/DPPC-mixed LUVETs are shown in Fig. 1. In the present study, a linear relationship could be obtained when both aliquots of calcein-entrapped liposomal solution and the buffer solution in a fluorescence cuvette were kept at the same temperature during the mixing of samples at the first step of incubation in the absence of ethanol, as

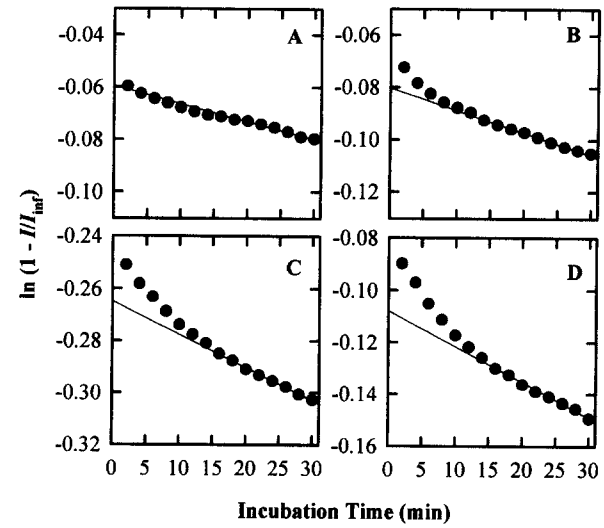

Fig. 1. Plots of $\ln(1 - I/I_{inf})$ vs. t according to Eq. (1). A: 20 mol% DLPE without ethanol at 25°C; B: 20 mol% DLPE without ethanol at 31°C; C: 20 mol% DLPE with 2.1 M ethanol at 32°C; and D: 70 mol% DLPE with 2.1 M ethanol at 22°C.

shown in Fig. 1A. In other cases, as shown in Fig. 1B,C and D, however, the plot of $\ln(1 - I/I_{inf})$ vs. t was not found to be linear. In the case of DPPC LUVETs with or without ethanol at low concentrations at 25°C in our previous studies, a linear relationship could be established but at high concentrations of ethanol at 25°C this was not possible [25–27].

In order to reach equilibrium, membranes of vesicles must be exposed to identical solutions in both the interior and exterior vesicles. During the period required to achieve equilibrium the apparent leakage is not necessarily a simple process due to various complications. It may take time after the addition of ethanol to the liposomal exterior before perfectly equilibrated membranes are generated. Nagel et al. [40] pointed out the lack of any evidence of interdigitation immediately after the addition of alcohol and suggested that depending on the bulk ethanol concentration and initial liposomal size, it took several minutes to hours for the intra-liposomal concentration to reach the critical level at which massive membrane reorganization sets in.

In the absence of ethanol, a linear relationship could not be obtained in spite of the same osmosis between the calcein-entrapped liposomal solution and the buffer solution in the mixing of samples at the first step of incubation, except in the case of incubation at room temperature. Change in the liposomal structure would be expected because the equilibrium of the liposomal system must shift to that at the temperature of the buffer solution.

Judging from the plots in Fig. 1, it may take at least 15 min to achieve equilibrium of the whole system after mixing of the liposomal and the buffer solutions. In this study, therefore, the linear portions after about 15 min were used to estimate the k rate constants. The fluorescence intensities, from which the rate constants k were evaluated, were below 20% of the I_{inf} values, and therefore the contents of the liposomes remained sufficient to make a comfortable fit of the slow component of leakage with the equation.

In general, when the permeability of substances through various membranes is discussed, differences in the concentrations of substances between inner and external aqueous phases of liposomes and in the liposomal sizes, that is, surface areas and inner volumes of liposome, should be taken into consideration [44]. In our previous study, membrane permeation coefficients were evaluated from the liposomal sizes at a variety of ethanol concentrations and from the membrane thickness in the interdigitated gel (I) and regular bilayer gel (G) phases, with certain assumptions [26]. It has been suggested that when liposomal membranes form interdigitated structures the liposomal size changes: The size reduces in the mixtures of G with I phases, and in the I phase the diameter increases and becomes about the square root of 2 times its original diameter [1,2,26,40]. With binary lipid membranes, it is difficult to estimate the liposomal size and the membrane

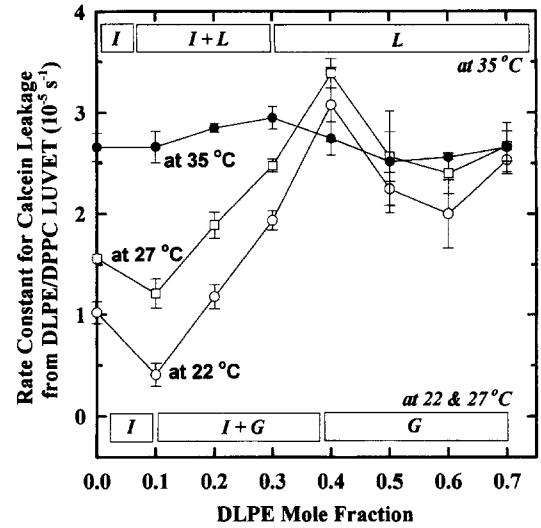

Fig. 2. The rate constants for calcein leakage at various temperatures as a function of the DLPE mole fraction in the presence of 2.1 M ethanol. Rectangles at the top and bottom of the figure show the phase diagram of DLPE/DPPC systems in the presence of 2.1 M ethanol at each temperature [5]. Italic letters in the rectangles, I, L and G, refer to interdigitated gel, bilayer liquid-crystalline, and normal bilayer gel phases, respectively. Error bars denote standard errors evaluated from three to six individual runs.

thickness in the intricate phase-separated state. For instance, it is necessary to assume the membrane thickness in the ($I + G$) or (I + bilayer liquid-crystalline (L)) phase for calculations of permeation coefficients [25–27]. In our previous study, we could discuss the permeability of ethanol-induced phase-separated membranes using the leakage rate constant k because both the rate constant and the permeation coefficient indicated similar profiles [25–27]. Therefore, in the present study, the permeability is discussed in terms of the rate constant.

3.2. Effects of mole fraction of DLPE on the rate constant

Fig. 2 shows the evaluated k rate constants for the DLPE/DPPC LUVETs at 22, 27 and 35°C as a function of mole fraction of DLPE in the presence of 2.1 M (100 mg/ml) ethanol. Rowe [5] earlier constructed a phase diagram of DLPE/DPPC systems in the presence of 2.1 M ethanol. Phase structures, based on the reported phase diagram of MLVs, at each temperature in the presence of 2.1 M ethanol, are shown as italic letters in rectangles at the top and bottom of the figure.

The rate constants were found to be almost invariable at 35°C but they proved dependent upon mole fraction of DLPE at 22 and 27°C. In the latter cases, similar changes in the rate constants were indicated in the DLPE mole fraction range of 0 to 0.7. Differences were due to the dependence of the rate constant on temperature. At high mole fraction of DLPE the same rate constants were observed. Therefore, when the temperature is taken into account it could suggest that the permeability is greater for

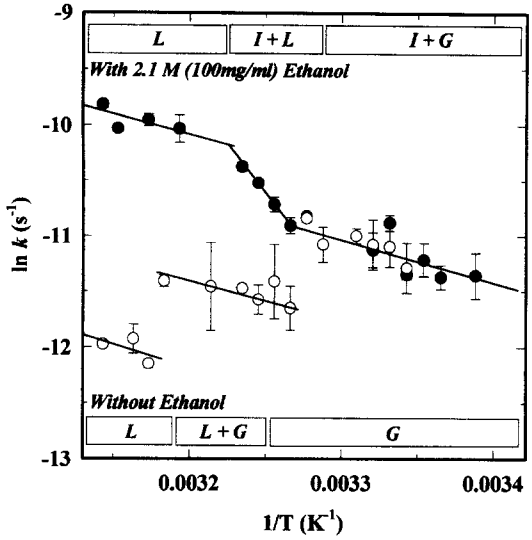

Fig. 3. Arrhenius plots of the leakage rate constant k for 20 mol% DLPE LUVET with and without 2.1 M ethanol. Filled circles, with 2.1 M ethanol; and open circles, without ethanol. Error bars denote standard errors evaluated from three to six individual runs.

the G phase than the L phases in the presence of 2.1 M ethanol.

3.3. Effects of temperature on rate constants in the DLPE / DPPC (20:80) liposomal membranes

In the previous section, effects of the DLPE mole fraction on the rate constants were observed at constant temperature. In this section, the effect of temperature on the rate constants, when the lipid component is constant, is covered. The 20:80 ratio was chosen as the molar ration of DLPE/DPPC because various phase structures, containing phase separations composed of two different phases, are then available in the presence of 2.1 M ethanol.

Arrhenius plots of the estimated rate constants k in the binary DLPE/DPPC (20:80) LUVETs with and without 2.1 M ethanol are presented in Fig. 3. Phase structures, based on the reported phase diagram [5], are shown as italic letters in rectangles at the top and bottom of the figure. In the absence of ethanol, three structure phases, G, $L + G$ and L phases, form under our experimental conditions. The plots in the figure resulted in three lines having a similar slope, leading to a conclusion of an activation energy of about 30 kJ. On the other hand, in the presence of 2.1 M ethanol, the plots showed three consecutive lines with different slopes for each of the three phases. In the L and $I + G$ phases, the plots demonstrated the same slope, coinciding with those in the absence of ethanol. However, the slope of the line in the $I + L$ phase was much greater. The activation energy evaluated from the slope was about 120 kJ, indicating that the large activation energy is necessary for the leakage reaction to occur in this structure phase. On the base of the activation energy, it clearly indicates that the leakage proceeds more easily in the $I + G$ phase than in the $I + L$ phase.

The temperature ranges of the three lines correspond with those of the three phases except the $L + G$ phase without ethanol and the $I + G$ phase with ethanol, in which there are slight differences in the temperature ranges between the lines and the phases. In the present study, LUVETs were used for the measurements but the phase diagram was quoted from the MLVs' one. In recent reports, it was demonstrated that LUVETs and MLVs may have different interdigitation behavior [12,41]. Therefore, the difference in the temperature ranges could be due to different interdigitated behavior between them.

In Fig. 3, the rates are all the same without and with ethanol below 33°C for the right hand part of the plots, i.e., the $I + G$ phase with ethanol and the G phase without ethanol. On the other hand, above about 33°C, comparison of results with and without ethanol, revealed a high permeability of liposomal membranes in its presence. This suggests the important conclusion that ethanol can cause high permeability of membranes even if they are stable and have low permeability in its absence.

3.4. Leakage mechanisms

In our previous study, large permeabilities were observed in the range of ethanol concentrations, where regular bilayer and interdigitated structures coexist, and the membrane is in a phase-separated state. This was considered due to instability of the boundary regions, the interdigitated membrane being characterized by a thinner structure and more rigid hydrocarbon regions than its non-interdigitated counterpart [1,2]. Thus, the thickness was reduced by about 30% in the transition from a non-interdigitated to an interdigitated structure phase [13]. Also closer packing of hydrocarbon chains has been established for interdigitated membranes [1,2,13]. Fig. 4A shows a schematic

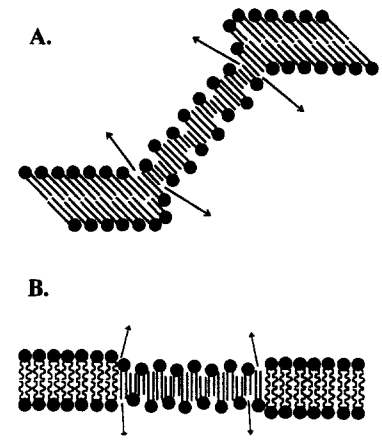

Fig. 4. The proposed mechanisms of ethanol-enhanced leakage of DLPE/DPPC liposomal membranes in lateral phase-separation. A: the $I + G$ phase; and B: the $I + L$ phase.

description of phase-separated membranes, resulting from mixtures of normal bilayer G and interdigitated I phases. The difference in their membrane character is enough to lead to instabilities and structure defects in the boundary regions which would be expected to cause increased permeability.

As described in Section 3.3, permeability in the $I + G$ phase is higher than in the $I + L$ phase. The hydrocarbon chains of lipids in the L phase have a high mobility and are much more flexible than those in the G phase, contributing to a relative stability of the boundary regions between the I and G phases in the phase-separated membrane. It is also well known that membranes in the L phase are thicker than in the G phase. For example, the anhydrous bilayer thickness of dimyristoyl phosphatidylcholine liposomal membrane is about 5 nm at 10 and 15°C, when the membrane is in the G phase [45], whereas in the L phase at 25 and 30°C it is reduced to about 3 nm [45]. Thus the difference in the thickness of the membranes between in the I and L phases is smaller than that between the I and G phases, as illustrated in Fig. 4B, so that the boundary regions of phase-separated membranes in the $I + L$ phase are more stable.

As shown in Fig. 2, the liposomal membranes in the presence of 2.1 M ethanol were leaky in spite of being in the G phase. It is noteworthy that the G phase structures of membranes is formed in the presence of ethanol with a high mole fraction of DLPE. In the present study, lipid-dispersed colloids, composed of binary DLPE and DPPC, had no retention of calcein with a mole fraction of DLPE above 0.8. Therefore, the high leakage of liposomal membranes might not arise from the addition of ethanol but from the inherent instability of original liposomes. Further studies are necessary, however, to establish details of the leakage mechanisms further.

3.5. Biological considerations

The interdigitated structure formation has not yet been observed for biological membranes in vivo, in spite of many efforts. However, Boggs and Thmmler recently demonstrated that polymyxin B as well as its nonapeptide can form a small domain of interdigitated structure in a lipid extract from the gram-negative bacteria *Pseudomonas aeruginosa* although the major lipid is phosphatidylglycerol [46]. It was well known that polymixin B, being an antibiotic, can induce interdigitated structure formation in liposomal membranes, composed of phosphatidylgycerols [47]. These are strongly indicative the possibility for the interdigitated structure formation in biological membranes. In this study, it was found that the presence of ethanol can lead to high permeability in DLPE/DPPC liposomal mixture-membranes. The ethanol-enhanced permeation of the mixture membranes is of interest with regard to the relation of the ethanol-induced phase separation of membrane with mechanisms of ethanol-sterilization of bacteria such as *Esherichia coli*. In wild type *E. coli* cells, the major constituent of cytoplasmic membrane is phosphatidylethanolamine (about 75%) although the minor components are not phosphatidylcholine but phosphatidylglycerol and cardiolipin [48]. The mechanism of the sterilization of *E. coli* with ethanol has been ascribed to destabilization of nucleic acids and proteins associated with the cell membranes, solubilization of hydrophobic components, and increase in the membrane permeability, causing osmotic cellular lysis [49–51]. The present study speculates that an increment in the permeability of the cell membrane in *E. coli* could be due to lateral phase separations induced by ethanol.

To summarize the present findings, in cases of mixed liposomal membranes of DLPE and DPPC, especially at high temperature above about 33°C, large permeabilities were observed in the presence of high concentration of ethanol in spite of low permeabilities in the absence of ethanol. The presence of ethanol can lead to high permeability even if the membranes are stable and have a low permeability in its absence. These results speculated that an increment of permeability in cellular membranes, attributed to lateral phase separations induced by ethanol, could be one of the mechanisms of ethanol-sterilization of bacteria.

Acknowledgements

A part of this work was supported by a Grant-in-Aid for Science Research from the Ministry of Health and Welfare of Japan. This work was also partly supported by a grant from the Foundation for Encouragement of Pharmaceutical Research of Japan.

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