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Biochimica et Biophysica Acta 1237 (1995) 169–175

BBA Biochimica et Biophysica Acta

Increased permeability of phase-separated liposomal membranes with mixtures of ethanol-induced interdigitated and non-interdigitated structures

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Received 19 December 1994; revised 13 March 1995; accepted 30 March 1995

Abstract

It has been suggested by many workers using model membranes that the interdigitated structure formation, in which the acyl chains fully interpenetrate the hydrocarbon chains of the opposing monolayer, plays an important role in regulating many functions of biomembranes. In the present study the control of permeability was focused on as one of the biomembrane functions, and the effects of ethanol on the permeability of large unilamellar vesicles made by the extrusion technique (LUVET) (average diameter: about 250 nm), composed of dipalmitoyl or egg yolk phosphatidylcholines, were studied by monitoring the leakage of fluorescent dye, calcein, entrapped in the inner aqueous phase of the LUVET. The permeability was estimated from the apparent rate constant of calcein leakage at 25°C. Large permeabilities were observed in the region of 0.6 M to 1.3 M ethanol, with a concentration dependence. In this range of ethanol concentrations the normal bilayer and interdigitated structures coexist and the membrane is in a phase-separated state. The large permeability is due to the instability of the boundary regions, the interdigitated membrane being characterized by a thinner structure and more rigid hydrocarbon regions in the layer than its non-interdigitated counter part. These results suggest the possibility of biomembrane-permeability regulation by interdigitated membrane formation.

Keywords: Interdigitation; Ethanol; Permeability; Liposome; Phase separation; Vesicle

1. Introduction

It was now well established that saturated-chain phosphatidylcholines can form interdigitated membranes, in which the terminal methyl group of the acyl chains extend beyond the bilayer midplane, effectively interpenetrating into the opposing monolayer, in the presence of short chain alcohols 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 [3–16]. In our previous studies [12,17], the effects of unilamellar liposomal sizes (curvature of liposomes) on the ethanol-induced interdigitation in dipalmitoyl phosphatidylcholine (DPPC) were investigated and it was clearly demonstrated that the microscopically planar membranes can form interdigitated structures in the presence of ethanol.

It has been suggested by many studies using model membranes that the interdigitated structure formation plays an important role in regulating many functions of biomembranes [1,2]. In our previous study [18,19], we demonstrated the participation of interdigitated structure formation in ethanol-induced liposomal aggregation and fusion. In the present study, in order to investigate whether membrane permeability is one of the biomembrane functions which can be controlled by the interdigitated structure formation of membranes, the effects of ethanol on the permeability of large unilamellar vesicles made by the extrusion technique (LUVETs), composed of DPPC or egg yolk phosphatidylcholine (eggPC), were studied by monitoring the leakage of fluorescent dye, calcein, entrapped in the inner aqueous phase of the LUVET.

Abbreviations: MLV, multilamellar vesicle; LUVET, unilamellar vesicle made by the extrusion technique; DPPC, dipalmitoyl-L-α-phosphatidylcholine; eggPC, L-α-phosphatidylcholine egg yolk; L'_β, tilted-chain bilayer gel phase; $L_\beta I$, interdigitated gel phase; PCS, photon correlation spectroscopy.

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SSDI 0005-2736(95)00098-4

2. Materials and methods

2.1. Chemicals

Dipalmitoyl L-α-phosphatidylcholine (DPPC) (99 + %, Crystalline, P-6267, lot #62H8350) and L-α-phosphatidylcholine egg yolk (eggPC) (99%, Type V-E, P-5763, lot #11H8407) were obtained from Sigma (St. Louis, MO, USA) and no further purifications were performed. Ethanol was purchased from Wako Pure Chemical Industries, 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 analytical grade.

2.2. Preparation

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

Multilamellar vesicles (MLVs) were prepared as follows. A sample of the stock solutions of lipids were dried in a rotary evaporator under reduced pressure to form a lipid film on the wall of a round-bottomed flask. This film was left in vacuo for at least 12 h to ensure complete removal of the solvent. 4 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 the thin film containing 0.5 mmol lipid. Nitrogen gas was bubbled to remove any dissolved oxygen and the lipid was hydrated at about 55°C (at room temperature, ca. 20°C in the case of eggPC). During this incubation, the sample was vortexed periodically.

Calcein-entrapped LUVETs were obtained using freezing-thawing and extrusion methods [20,21] as follows: frozen and thawed MLVs were obtained by freezing in liquid nitrogen and thawing in a 55°C water bath (at room temperature, ca. 20°C in the case of eggPC), where the freeze-thaw cycle was repeated five times. Extrusion of the frozen and thawed MLV through two (stacked) polycarbonate filters of 400 nm pore size was performed at 55°C (in the case of eggPC at room temperature, ca. 20°C) employing a nitrogen pressure of 5 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 20°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 at pH 7.0 using HCl solution. Average diameters of the DPPC and eggPC LUVETs evaluated by the photon correlation spectroscopy (PCS) method (details described in the next section) were about 250 nm in the peak analysis by weight and the size distributions were homodispersed. The estimated average sizes agreed well with the reported value of Mayer and co-workers, of about 243 nm [21]. The entrapped volumes of the LUVETs evaluated using calcein as a marker were also comparable with reported values [22].

2.3. Calcein-leakage measurements

The leakage measurements were started by addition of 20 μl of calcein-entrapped liposomal solution to 3 ml of the 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 an excitation at 490.0 nm at 25.0 ± 0.1°C using a Hitachi F-650 spectrofluorometer equipped with a thermostated cuvette holder. The bandpass was 2 nm for both excitation and emission monochrometers. The sample was magnetically stirred well during the measurements. The concentrations of DPPC and eggPC were determined as those of P_i by a modified version of the procedure of Bartlett [23]. The final concentration of lipids was 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 liposome solution is diluted, since the concentration of the entrapped liposomal contents remains unchanged. On the other hand, calcein, which has leaked out of the liposomes into the extra-liposomal medium, will display enhancement of fluorescence upon dilution of 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 [24,25].

Fig. 1 shows fluorescence intensities of calcein at a variety of concentrations of calcein in the absence and presence of a detergent, Triton X-100, and/or ethanol. A liner relationship was established between fluorescence intensities and calcein concentrations without and with 0.33% Triton X-100 and/or a high concentration of ethanol under our experimental conditions. For subsequent measurements, the concentrations of calcein-entrapped liposomes were controlled in this region, where a linear relationship between the fluorescence intensities and the calcein concentrations could be obtained even if all calcein entrapped into the inner aqueous phase of liposomes will leak out.

2.4. Estimation of average size of liposomes

A PCS technique was used to estimate the liposomal average-size, and it was carried out with a Zetasizer 4

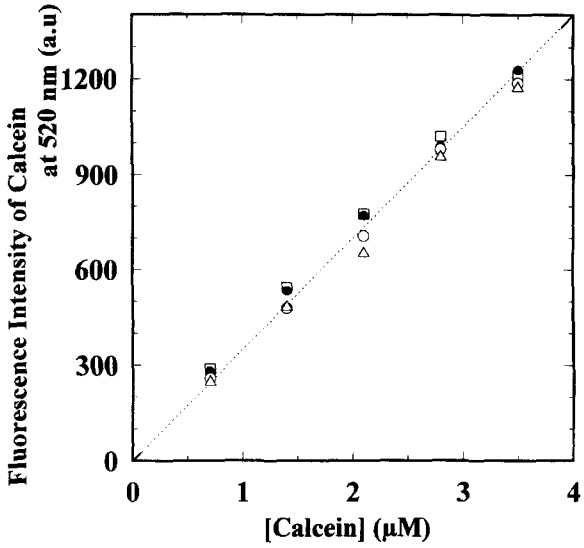

Fig. 1. Fluorescence intensities of calcein at a variety of concentrations in the absence and presence of ethanol and/or Triton X-100. Filled circles, without additives; open squares, with 0.33% Triton X-100; open circles, with 2.2 M ethanol; and open triangles, with 0.33% Triton X-100 and 2.2 M ethanol. The excitation wavelength is 490 nm.

photon-correlation spectrometer of Malvern Instruments (Worcs., UK) with temperature control by a Peltier heating/cooling unit. It uses a 632 nm He-Ne laser at a power of 5 mW and the light scattering was measured at 90°. The size was calculated with an NEC Power Mate SX/16i computer (Boxborough, MA, USA) connected with the photometer. The analysis of average size and distribution was performed using software ver. 1.1 supplied by Malvern Instruments, and it contained some automatic correction of the refractive index for layered particles. Average diameters and size distributions were evaluated as a Z-average using monomodal method (a cumulant analysis) and as a weight-weighted distribution in diameter using a multimodal method (exponential sampling algorithm), respectively [26,27]. An analysis range in a multimodal method was set from 5 nm to 3 μm in diameter. Further details are described in a previous paper [28].

Effects of ethanol and other chemicals containing the buffer solutions on refractive index were very small (blow 0.6%) and they were not taken into consideration. However, influences of ethanol on the viscosity of the sample solutions are significantly large and the corrections were performed using the reported values [29].

3. Results and discussion

3.1. Leakage rate constants

It is known that the leakage of entrapped substances from the inner aqueous phase of liposomes follows first-order kinetics [30]. The reduction rate of calcein concentration in the inner aqueous phases can be written as

$$dC_i/dt = k(C_i - C_e) \quad (1)$$

where C_i and C_e are calcein concentrations in the inner and external aqueous phases of liposomes, and t and k are the incubation time and rate constant, respectively. If the calcein concentration in the external aqueous phase in the equilibrium state of leakage reaction, that is the infinite time, is $C_{e,inf}$ and the initial concentration in the external aqueous phase is $C_{e,init}$, (see equation 1) becomes

$$\ln(C_{e,inf} - C_e) = \ln(C_{e,inf} - C_{e,init}) - kt \quad (2)$$

Here, $C_{e,inf}$ was determined from the calcein concentration after lysis of liposomes using a detergent, Triton X-100. As depicted in the experimental section, since the addition of ethanol and/or Triton X-100 to the liposomal solution exerted scarcely an influence on the fluorescence intensity of calcein, the results are in proportion to the calcein concentration. (see equation 2), therefore, leads to (see equation 3).

$$\ln(1 - I/I_{inf}) = A - kt \quad (3)$$

where I_{inf} is the fluorescence intensity after the lysis of liposomes. Here, $A = \ln(1 - I_{init}/I_{inf})$ and I_{init} is the initial fluorescence intensity.

Typical plots of $\ln(1 - I/I_{inf})$ vs. t according to (see equation 3) for the DPPC LUVET are shown in Fig. 2. At 0.34 M ethanol a linear relationship could be established but at the high concentrations of ethanol this was not possible. In order to reach equilibrium, lipid membranes must be exposed to identical solutions on both sides. In the

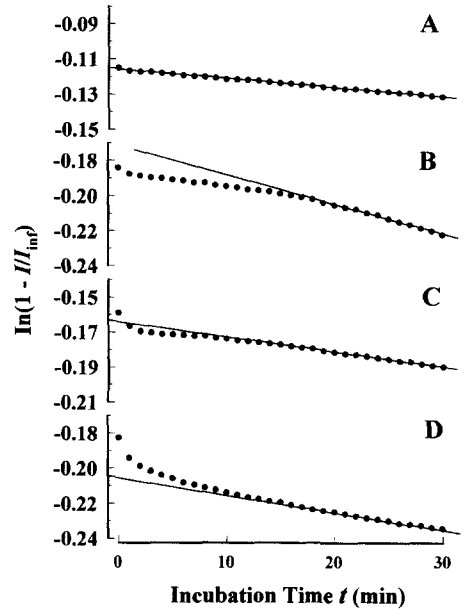

Fig. 2. Plots of $\ln(1 - I/I_{inf})$ vs. t according to Eq. (3). Ethanol concentrations: A, 0.34 M; B, 1.11 M; C, 1.26 M; and D, 1.83 M. The concentration of DPPC is 33 μM.

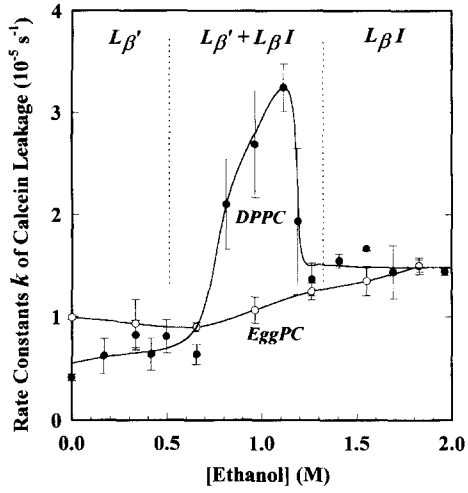

Fig. 3. The rate constants of calcein leakage from DPPC and eggPC LUVETs as a function of ethanol concentrations. Filled circles, DPPC; and open circles, eggPC. Error bars denote standard errors evaluated from three to six individual runs.

period required to achieve the 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. [31] 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. Judging from the plots in Fig. 2, in the presence of a high concentration of ethanol it may take at least 20 min to achieve equilibrium of the whole system after the addition of ethanol in our case. In this study, therefore, the linear portions after about 20 min were used to estimate rate constants k at high concentrations of ethanol, where the fluorescence intensities, from which the rate constants k were evaluated, were below about 15% of the I_{inf} values.

Fig. 3 shows the evaluated rate constants k in the DPPC LUVET as a function of ethanol concentration, a clear dependence being exhibited. Below 0.6 M ethanol, the leakage moderately increased with increasing ethanol concentration, with large leakage observed in the region of 0.6 M to 1.3 M ethanol and a maximum value at about 1.2 M ethanol. Above 1.3 M ethanol, the values were constant.

It has already been demonstrated that in the case of DPPC LUVETs (> 100 nm), the membranes are in a normal structure, for example, in the tilted-chain bilayer gel ($L_{\beta'}$) phase below 0.5 M ethanol and in the interdigitated gel ($L_{\beta}I$) phase above 1.3 M ethanol [31]. It was also indicated that $L_{\beta'}$ and $L_{\beta}I$ phases coexist and the membrane is in a phase-separated state in the range between 0.6 M and 1.3 M ethanol [31,32]. This concentration region coincided well with ethanol concentrations for which the large leakages were observed.

In order to confirm that the phenomenon depends on interdigitated structure formation, leakage measurements were performed with eggPC LUVET, which do not form interdigitated structures even in the presence of a high concentration of ethanol [1,2,36]. Fig. 3 also depicts rate constants k evaluated from measurements of eggPC LUVET, indicating the lack of any dependence upon the ethanol concentration. This result clearly suggests that the large leakage of calcein is related to the interdigitated structure formation and also to the coexistence of $L_{\beta'}$ and $L_{\beta}I$ phases, that is phase-separation of the membrane.

3.2. Membrane permeation coefficients

In general, when the permeability of substances through various membranes is discussed the differences in the concentrations of substances between inner and external aqueous phases of liposomes and the liposomal sizes, that is, surface areas and inner volumes of liposomes, should be taken into consideration [33]. Therefore, the membrane permeation coefficients, which are defined using differences in liposomal size, were calculated from the average diameters observed at a variety of ethanol concentrations.

It has been already suggested that when membranes of the large unilamellar vesicles form interdigitated structures the vesicle size changes; the size is reduced in the mixed $L_{\beta'}$ and $L_{\beta}I$ phases, and in the $L_{\beta}I$ phase the diameter increases and becomes about the square root of 2 in comparison with their original size [1,2,31]. Fig. 4 shows data for apparent average-diameters of DPPC LUVETs as a function of the ethanol concentration. The observed changes in diameters as a function of ethanol concentration agreed with the reported phenomena except above 1.5 M ethanol, where DPPC liposomes, as suggested by many workers, tend to aggregate and/or fuse with each other [18,19,35–40]. At concentrations from 1.25 M to 1.5 M

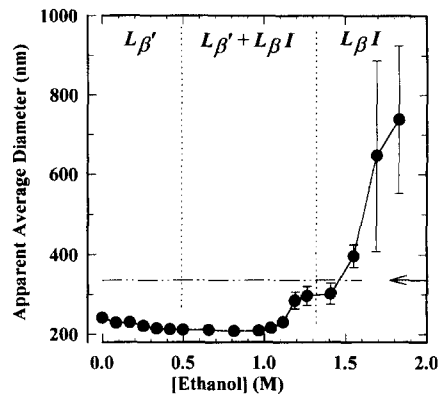

Fig. 4. Apparent average-diameter of DPPC LUVET as a function of ethanol concentrations. An arrow presents 1.41-times (the square root of 2) of the original size. In order to avoid the aggregation of liposomes, the solution contained 10 mM calcium chloride [34].

ethanol, the average diameter was near to 1.41-times (the square root of 2) larger than the original size, in line with reported findings [31].

The leakage rate constant k is related to both the surface area A' and inner volume V_i of liposomes as in the following equation [33].

$$k = A'P'/V_i \quad (4)$$

Here, P' is a permeation coefficient defined using the differences in the concentrations of calcein between inner and external aqueous phases of liposomes, $(C_i - C_e)$. The flux J may be written as [33]

$$J = P'(C_i - C_e) = P(C_i - C_e)/L \quad (5)$$

and therefore, a permeation coefficient P defined using the concentration gradient between inner and external aqueous phases of liposomes can be described as

$$P = P'L \quad (6)$$

Here, L is the layer thickness of the liposomal membranes. Permeation coefficients P' and P can be calculated by Eqs. (4) and (6) using A and V_i values estimated from the liposomal size at the various concentrations of ethanol and the membrane thickness L. In this study, for the calculation, the membrane thicknesses L for the $L_{\beta'}$ and $L_\beta I$ phases were assumed to be 4.2 nm and 3.0 nm, respectively, which are reported values estimated by the X-ray diffraction method [13] (Here, these values are the thickness determined from the phosphate to the phosphate moiety in the bilayer. For permeability, however, it is the hydrocarbon thickness of the bilayer. Consequently, the thickness of the head groups should be subtracted from the total thickness. However, it is essentially negligible small as compared with the thickness determined from the width of the polar group layer [41].) Fig. 5 shows calculated permeation coefficients as a function of the ethanol concentration. Here, it was assumed that the thickness linearly decreases from 4.2 nm to 3.0 nm with the increase in ethanol concentrations in the mixed $L_{\beta'}$ and $L_\beta I$ phases because of the linear relationship between the area of the $L_\beta I$ phase and the ethanol concentration [31].

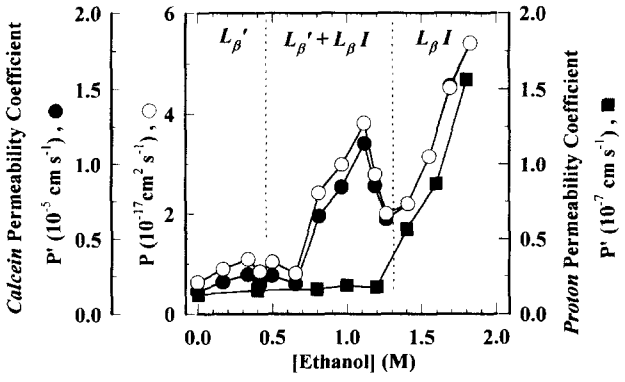

Fig. 5. Calculated permeation coefficients of calcein and proton as a function of ethanol concentrations. The permeation coefficients, P' and P, are defined using the differences in the concentrations of calcein and the concentration gradient between inner and external aqueous phases of liposomes, respectively. The permeation coefficient P of proton is quoted from the data reported by Zeng et al. [42].

The calculated permeation coefficients clearly depend on the ethanol concentrations and the largest permeabilities were observed in the region of 0.6 M to 1.3 M ethanol. Fig. 5 also illustrate the permeation coefficients P' for proton, reported by Zeng et al. [42]. The permeation coefficients of proton are almost constant below 1.3 M ethanol and thus differs markedly from our results. This difference is contrary to expectation from the size difference of trapped substances; the size of the proton molecule is considerably smaller than that of the calcein molecule.

It has previously been suggested that interdigitated membrane formation depends on the curvature of liposomal membranes or the size in the case of unilamellar vesicles [12,17,35,36]. It was also demonstrated that in the presence of high concentrations of ethanol, the entire membrane can form interdigitated structure in those above about 200 nm in diameter but with the liposomal sizes below this figure the interdigitation is induced in only part, with $L_\beta I$ phase proportion being dependent upon the liposomal size [12,17,35,36]. In this study, LUVETs having about 250 nm in diameter were used while Zeng et al. applied DPPC unilamellar vesicles sized about 100 nm for their proton leakage measurements, with a small portion of $L_\beta I$ phases in the membrane as opposed to the entire in the case of large unilamellar vesicles. Therefore, the observed differences in the permeabilities between calcein and proton in the region of 0.5 M to 1.3 M ethanol can be concluded to be due to the liposomal sizes used.

Abrupt increases in the permeability in both entrapped substances, proton and calcein, were evident above 1.3 M ethanol, where liposomal aggregation/fusion is induced dramatically, as suggested by many workers [18,19,35–40]. Therefore, the correction for permeation coefficient values could not obtained for this region of ethanol concentrations ($>$ 1.3 M ethanol) because of the uncertain liposomal sizes (sizes of aggregated/fused liposomes).

3.3. Leakage mechanisms

The interdigitated membrane is characterized by a thinner layer with more rigid hydrocarbon regions than its non-interdigitated counter part [1,2]. For example, the thickness reduced about 70% in the transition from a non-interdigitated to interdigitated structure phases [13]. Also the closer packing of hydrocarbon chains is established in the interdigitated membranes. That is, the $L_{\beta'}$ phase has a double wide-angle reflection at 4.21 Å and 4.10 Å (meaning each hydrocarbon chain has four neighbors at a distance of 4.73 Å); on the contrary, the $L_\beta I$ phase has a single wide-angle reflection at 4.09 Å (meaning each hydrocarbon chain has six nearest neighbors at a

distance of 4.72 Å) in X-ray diffraction patterns [13]. The difference in their membrane characters is enough to lead to instabilities and structure defects in the boundary regions of membranes with mixed $L_{\beta'}$ and $L_{\beta}I$ phases. Such structure defects would be expected to cause increased permeability. This is very similar to the phenomena of leakage induced in the phase-separate state at the gel/liquid-crystalline transition temperature of phospholipids such as dimyristoylphosphatidylcholine or DPPC, or in mixed membranes composed of different species of lipids [43–46].

Our conclusion is that with mixed phases of $L_{\beta'}$ and $L_{\beta}I$, the membrane permeability is changed in proportion to the amount of the $L_{\beta}I$ phase in the membrane. We can, therefore, speculate the possibility of biomembrane-permeability regulation by the interdigitated membrane formation. Such formation in biological membranes has not yet been observed, in spite of many efforts, but the possibility has been suggested by many studies which have been carried out using model membranes [1,2].

In summary, large permeabilities of DPPC LUVETs were observed in the presence of 0.6 M to 1.3 M ethanol in the present study and the permeabilities depended on the ethanol concentration within this range. With these ethanol concentrations, normal bilayer and interdigitated phases coexist and the membrane is in a phase-separated state. Instability of the boundary regions in the mixed phases could be playing an important role because the interdigitated membrane is characterized a thinner layer with more rigid hydrocarbon regions than the normal non-interdigitated bilayer.

Acknowledgements

A part of this work was supported by grant for the Encouragement of Basic Research and by the Special Coordinate Funds for Promoting Science and Technology from the Science and Technology Agency of Japan. Also, this work was partly supported by a Grant-in-Aid for Science Research from the Ministry of Health and Welfare of Japan. The authors thank Miss Maki Sugimoto, Osaka College of Pharmacy, for her technical assistance. We are grateful to Dr. Malcolm A. Moore for helpful comments on the manuscript.

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