Direct Evidence of Induction of Interdigitated Gel Structure in Large Unilamellar Vesicles of Dipalmitoylphosphatidylcholine by Ethanol: Studies by Excimer Method and High-Resolution Electron Cryomicroscopy

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ABSTRACT Interaction of large unilamellar vesicle (LUV) of dipalmitoylphosphatidylcholine (DPPC) with ethanol was investigated by the excimer method developed by Yamazaki et al. (Yamazaki, M., M. Miyazu, and T. Asano. 1992. *Biochim. Biophys. Acta.* 1106:94–98) and the high-resolution electron cryomicroscope with a new cryostage (top-entry superfluid stage) (HiRECM) developed by Fujiyoshi, Y. et al. (Fujiyoshi, Y., T. Mizusaki, K. Morikawa, H. Yamagishi, Y. Aoki, H. Kihara, and Y. Harada. 1991. *Ultramicroscopy.* 38:241–251). The excimer method is based on the fact that the ratio of excimer to monomer fluorescence intensity (*E/M*) of pyrene PC is lowered in the membrane in the interdigitated gel structure ($L_{\beta I}$), because structural restriction of $L_{\beta I}$ structure largely decreases collisions of pyrene rings of the pyrene PCs in the membrane. *E/M* of pyrene PC in DPPC LUV decreased largely at high concentrations of ethanol, which indicated the induction of $L_{\beta I}$ structures in DPPC LUV. Frozen-hydrated DPPC LUVs in a vitreous ice were observed at 4K with HiRECM, and these images were characterized by a pair of concentric circles. The membrane thickness of DPPC LUV which was estimated from the distance between the two concentric lines decreased largely at high concentration of ethanol. The mean value of membrane thickness of the LUV in the absence of ethanol was 3.8 nm, while at 15% (w/v) ethanol was 3.0 nm. These values were almost same as those obtained from the electron density profile of DPPC MLV by the x-ray diffraction analysis in each structures, $L_{\beta'}$ and $L_{\beta I}$ structures, respectively. These results indicated directly the induction of $L_{\beta I}$ structure in DPPC LUV at high concentration of ethanol.

INTRODUCTION

Recently, interdigitated gel ($L_{\beta I}$) structures in phospholipid membranes have attracted much attention (Slater and Huang, 1988; Ohki, 1991). In these structures, lipid molecules from opposing monolayers are interpenetrated or interdigitated and the terminal of the alkyl chain faces aqueous phase. In the presence of various small molecules such as ethanol (Rowe, 1983; Simons and McIntosh, 1984; Ohki et al., 1990), ethylene glycol (EG) (Yamazaki et al., 1992a), oligomers of EG (Yamazaki et al., 1992b), Tris buffer (Wilkinson et al., 1987) and anions (Cunningham et al., 1989), or under high pressure (Braganza and Worcester, 1986), multilamellar vesicles (MLV) of phosphatidylcholine (PC) or phosphatidylglycerol can form $L_{\beta I}$ phase. About the interaction of water-soluble low molecular weight substances with biomembranes, we proposed a new hypothesis that, in the absence of specific interaction of the head group of the lipid with substances, smaller (amphipathic) substances than the head group region could induce the $L_{\beta I}$ structure and larger substances than the head group region could increase the phase transition temperature of the membrane (Yamazaki et al., 1992b). The latter phenomena may be explained by the osmoelastic coupling theory which was proposed by Yamazaki et al. (1989).

In order to detect and study $L_{\beta I}$ structures on the MLVs, x-ray diffraction and neutron diffraction have been used extensively. However, these methods can not be applied to the detection of $L_{\beta I}$ structure in unilamellar vesicles owing to their low sensitivity. Several spectroscopic methods also have been applied to the study the $L_{\beta I}$ structure (for example, Nambi et al., 1988; Boggs et al., 1989). However, their interpretations were not straightforward.

It is very important to investigate functions of $L_{\beta I}$ structures (Slater and Huang, 1988; Ohki, 1991), for example, effects of $L_{\beta I}$ structures on association and membrane fusion of unilamellar vesicles and on functions and structures of membrane proteins. However, they have not been investigated, because there were no good methods for detecting $L_{\beta I}$ structure in the unilamellar vesicles and reconstituted vesicles containing membrane proteins.

Yamazaki et al. have recently developed a new, highly sensitive spectroscopic method (excimer method) in order to detect the $L_{\beta I}$ structure in phospholipid vesicles and studied the transition between $L_{\beta I}$ and $L_{\beta'}$ structure (1992c). This method is based on the fact that the ratio of excimer to monomer fluorescence intensity (*E/M*) of pyrene PC is lowered in the membrane in $L_{\beta I}$ structure, because the structural restriction of $L_{\beta I}$ structure largely decreases collisions of pyrene rings of the pyrene PCs in the membrane. In the interaction of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) MLV with ethanol or oligomers of EG, the *E/M*

Received for publication 9 July 1993 and in final form 21 December 1993.

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Abbreviations used: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DMPC, 1,2-dimiristoyl-*sn*-glycero-3-phosphatidylcholine; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; $L_{\beta'}$, tilted chain bilayer gel; $L_{\beta I}$, interdigitated gel; *E/M*, the ratio of excimer to monomer fluorescence intensity; pyrene-PC, 1-palmitoyl-*sn*-glycero-3-phosphatidylcholine.

0006-3495/94/03/729/05 $2.00

value showed a sharp decrease at the same concentration as that at which the transition from $L_{\beta'}$ to $L_{\beta I}$ structure was observed, determined by the scanning densitometry (Ohki et al., 1990) and x-ray diffraction method (Yamazaki et al., 1992b; 1992c).

On the other hand, recent developments of cryoelectron microscopy (Taylor and Glaesar, 1974; Adrian et al., 1984; Dietrich et al., 1979; Zemlin et al., 1985; Dubochet et al., 1988; Fujiyoshi et al., 1991) have enabled us to observe structures of biological molecules at high resolution. The cryoelectron microscope, together with the rapid freezing and cryofixation techniques, has made it possible to observe chemically unfixed and unstained specimens in a vitreous ice (glassy state of water) under a transmission electron microscope (Dubochet et al., 1988). The application of these techniques to the analysis of two-dimensional crystals of membrane proteins has extended a resolution of their structures to near-atomic scale (for example, Sass et al., 1989; Henderson et al., 1990; Kühlbrandt and Wang, 1991; Jap et al., 1991; Kühlbrandt, 1992). Fujiyoshi et al. developed a new cryostage (top-entry superfluid helium stage) in order to reduce irradiation damage upon biological specimens by cooling them to around 4K with liquid helium (Fujiyoshi et al., 1991), whose cooling method is very different from that of the cryoelectron microscope developed by Dietrich et al. (1979). This stage was also designed to eliminate both the mechanical vibration caused by the boiling of the coolant, and specimen drift induced by temperature change of the specimen stage as much as possible by the sophisticated mounting and the refined cooling mechanism of the stage. This stage has achieved higher resolution of images than a conventional cryostage cooled by liquid nitrogen. Kume et al. showed that this high-resolution electron cryomicroscope (HiRECM) was very useful for studying structures of biomembranes (Kume et al., 1990).

In this brief communication, we investigated the interaction of large unilamellar vesicles (LUV) of DPPC with ethanol by means of the excimer method and observed the induction of the $L_{\beta I}$ structure in these LUVs. Further, we examined structures of these LUVs by the HiRECM, in order to confirm the induction of $L_{\beta I}$ structures. This research was presented at the 29th Annual Meeting of the Biophysical Society of Japan, September, 1991, Sendai, Japan (Yamazaki et al., 1991) and 11th International Biophysics Congress, July, 1993, Budapest, Hungary (Yamazaki et al., 1993) .

MATERIALS AND METHODS

DPPC, 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphatidylcholine (pyrene-PC), and ethanol were purchased from Sigma Chemical Co., Molecular Probes Inc., and Wako Chemical Co., respectively. LUVs in 1,4-piperazinediethanesulfonic acid (Pipes) buffer (10 mM Pipes (pH 7.5), 140 mM NaCl, 0.002% NaN_3) were prepared by the method of reversed-phase evaporation (Szoka and Papahajopoulos, 1978) and were filtered through a 200-nm Nuclepore polycarbonate filter (Nuclepore Corp.). Ethanol solution in Pipes buffer was added to the preformed LUV suspension.

The excimer method was described in detail in the previous paper (Yamazaki et al., 1992c). For fluorescence measurements, a Hitachi F3000 spectrofluorimeter was used. Excitation wavelength of pyrene-PC was 347 nm. Emission wavelengths were 376 nm for monomer fluorescence and 481 nm for excimer fluorescence. The ratio of excimer to monomer fluorescence intensity (E/M) was calculated. Concentrations of DPPC in the samples for this measurement were 150 μM, which were determined by the phosphate analysis (Bartlett, 1959).

Specimens for HiRECM were prepared by the rapid cryofixation technique as follows. A drop of the suspension of LUV was deposited on a glow-discharged microgrid at 25°C. Excess suspension was blotted with a filter paper to form a thin film suspension. Immediately after that, the grid was plunged into liquid ethane cooled by liquid nitrogen so that the suspension was quickly frozen and fixed in a vitrified ice. The specimen was then transferred, kept at a liquid nitrogen temperature, onto a cryostage for inspection. Mechanical details of the HiRECM (JEM-4000SFX), equipped with a superfluid helium stage as well as a cryotransfer device, were described in the paper of Fujiyoshi et al. (1991). Electron micrographs were taken at accelerating voltage (E_a) of 300 kV and at a stage temperature of 4.2 K with 8- or 11-s exposure. The minimum dose system was used to minimize the irradiation damage upon specimens, and the total electron dose upon a specimen would be 80 electrons/Å^2 which does not seriously damage vitrified biological specimen at 4K (Fujiyoshi, 1986). A defocus value (Δf) was set at ca. 200 nm. The spherical (C_s) and the chromatic (C_c) aberration coefficient of the objective lens are 2.6 and 3.7 mm, respectively. The phase contrast transfer functions (for example, Amos et al., 1983), considering the modulated effects by the chromatic aberration (Hanssen et al., 1971), were calculated for several defocus values ranging from 84.9 (the Scherzer's focus; Scherzer, 1949) to 500 nm, at the conditions of the observation (E_a = 300 kV, C_s = 2.6 mm, C_c = 3.7 mm, ΔE = 4 eV; the maximum energy fluctuation of the incident thermal electrons). The contrast transfer functions have the same sign throughout the objective spacings greater than 1 nm for the defocus of the above range. The direct magnifications of the images were 40,000 ×. Calibration of the magnification was made by using the image of crystalline-chlorinated copper phtalocyanine taken under the same conditions as those for the lipid vesicles, since three times the separation of (020) lattice (13.04 Å (Brown, 1986)), 39.12 Å, is near the membrane thickness of the lipid bilayer. The membrane thickness was determined by measuring the distance between the centers of two black concentric lines of LUV in the enlarged electron-micrographs (magnifications; 400,000 ×) by use of 10-fold precise magnifier.

RESULTS AND DISCUSSION

The fluorescence intensity ratio E/M of pyrene-PC in DPPC LUV was plotted as a function of ethanol concentration (Fig. 1). As shown in Fig. 1, E/M value decreased with an increase in ethanol concentration and a discontinuity in the rate of decrease was observed at around 4.5% (w/v) ethanol above which the rate became much larger. As indicated in the previous paper (Yamazaki et al., 1992c), E/M value of pyrene-PC in DPPC vesicles in $L_{\beta I}$ structure is much lower than that in $L_{\beta'}$ structure, because structural restriction of $L_{\beta I}$ structure largely decreases collisions of pyrene molecules in the membrane and the discontinuity of the decrease of the E/M value appears at the same concentration of the transition from $L_{\beta'}$ to $L_{\beta I}$ structure as determined by the x-ray diffraction method and scanning densitometry. Therefore, the result of Fig. 1 indicates that $L_{\beta I}$ structure in the DPPC LUV was induced above 4.5% (w/v) ethanol. It was almost equal to that of the transition from $L_{\beta'}$ to $L_{\beta I}$ phase of DPPC MLV at the same temperature (25°C) determined by the scanning density meter (Ohki et al., 1990). The E/M values at all concentrations of ethanol in Fig. 1 did not change from 30 s to 72 h after mixing of LUV suspension with ethanol aqueous solution at 25°C. This result indicated that the induction of $L_{\beta I}$ structure in DPPC LUV was completed within at most

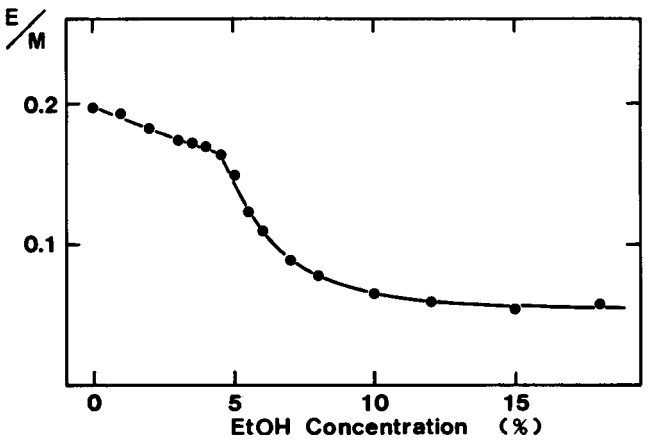

FIGURE 1 Ratio of excimer to monomer fluorescence intensities ([*E/M*] of pyrene-PC in DPPC LUV in the various concentrations of ethanol. Fluorescence intensities were measured at 10 min after mixing with ethanol aqueous solution at 25°C, and *E/M* values were calculated as explained in the text. Pyrene-PC concentration was 2.8 mol% in the LUV.

30 s after the addition of ethanol solution and that this $L_{\beta I}$ structure was stable for at least 72 h. Preliminary results by using a stopped-flow fluorescence spectroscopy method indicated that a half time of induction of $L_{\beta I}$ structure in DPPC LUV at 10% (w/v) ethanol was about 500 ms (Yamazaki et al., unpublished results).

In order to confirm the induction of $L_{\beta I}$ structure in DPPC LUV, we observed structures of DPPC LUVs in the presence of ethanol by HiRECM. Fig. 2 *a* shows images of DPPC LUVs in the absence of ethanol. These LUVs are considered to be $L_{\beta'}$ structures, since they were maintained at 25°C before the rapid freezing. These images are characterized by two black concentric lines separated by 3.8 ± 0.3 nm, which are interpreted as the polar head groups of the lipid and its contrast is mainly due to the phosphorus atom in the head group (Lepault et al., 1985; Kume et al., 1990). The observed value is a little smaller than that obtained from the electron density profile of the membrane of DPPC MLV by x-ray diffraction (4.2 nm) (McIntosch, 1980). In contrast, in the images of most DPPC LUVs in the presence of 15% (w/v) ethanol (Fig. 2 *b*), their membrane thicknesses were 2.7 ± 0.4 nm uniformly, which were much smaller than that of $L_{\beta'}$ structure. This short membrane thickness corresponds to that of the membrane in $L_{\beta I}$ structure, because it is almost same as that obtained from the electron density profile of DPPC MLV in $L_{\beta I}$ structure (3.0 nm) (Simons and McIntosch, 1984; Yamazaki et al., 1992b). These observations in HiRECM support the conclusion derived from the excimer method that $L_{\beta I}$ structure was induced in DPPC LUV at high concentrations of ethanol. Aggregations of LUVs were also observed in both cases, as expected on the basis of dynamic light scattering data (Wang and Thompson, 1982), which indicate that DPPC vesicles aggregate below main transition temperature (41°C) and disaggregate above this temperature. Images of aggregated LUVs were too complex to measure the membrane thickness of the LUV correctly. However, the difference in membrane thickness between 0 and 15% ethanol was qualitatively the same.

Some LUVs in the presence of 15% (w/v) ethanol had membrane domains which were thicker than those mentioned above (Fig. 2 *c*). They are considered to be membranes of $L_{\beta'}$ structure, because their thickness was about 3.8 nm. The reason for the appearance of the thick membrane in the thin membranes is not clear at present. Lepault et al. observed under a conventional electron cryomicroscope that DMPC LUV showed the L_{β} structure, even when it was maintained at a temperature higher than the main transition temperature before the rapid freezing (Lepault et al., 1985). They concluded that the freezing induced a rearrangement of the lipid hydrocarbon chain from L_{α} to L_{β}, although the mechanism of the rearrangement was not clear. In our case also, the freezing might be a factor inducing a rearrangement from $L_{\beta I}$ to $L_{\beta'}$ structure in a part of the membrane. The average membrane thickness of all LUVs, including these LUVs which have thick membrane domains, in the presence of 15% (w/v) ethanol was 3.0 ± 0.5 nm. It is evident that this membrane thickness was different from that in the absence of ethanol (3.8 ± 0.3 nm).

The exact estimation of the thickness of biological membrane have been so far determined by x-ray diffraction. Recently, Dorset et al. indicated the high-resolution analysis of the image of the phospholipid multilamellar array in the cryo-electron microscope by using the phase determination of electron diffraction intensities (Dorset et al., 1990). However, both methods can be applied to only the analysis of multilamellar structure of the phospholipid membranes and are not applicable for that of unilamellar vesicles which are major structure in the living cells. The results in this communication clearly showed that the membrane thickness of DPPC LUV in $L_{\beta'}$ structure determined by the HiRECM is largely different from that in $L_{\beta I}$ structure and that the values of both membrane thicknesses are very similar values to those obtained by the x-ray diffraction method, which indicates this method is useful for the detection of the phase transition between $L_{\beta'}$ and $L_{\beta I}$ structures qualitatively and also for semiquantitative determination of the membrane thickness of the phospholipid membranes. Tahara and Fujiyoshi recently simulated bilayer image of LUV in the HiRECM by using the MULTI SLICE program (Ishizuka and Uyeda, 1977) to clarify effects of the focus condition on the estimation of bilayer thickness. They showed that the simulated bilayer thickness was invariable under the defocus values from 66.3 nm (the Scherzer's focus of their condition) to 400 nm and that the effect of contrast transfer function was very small, which indicates the defocus value in the real observation of the LUV does not change the image magnification (submitted for publication). Their results provide an evidence that the estimation of the bilayer thickness by HiRECM is reliable.

In summary, results of two new methods, the excimer method and the HiRECM using liquid helium as a coolant, indicated directly the induction of $L_{\beta I}$ structure in DPPC LUV by ethanol, which was the first direct evidence of the

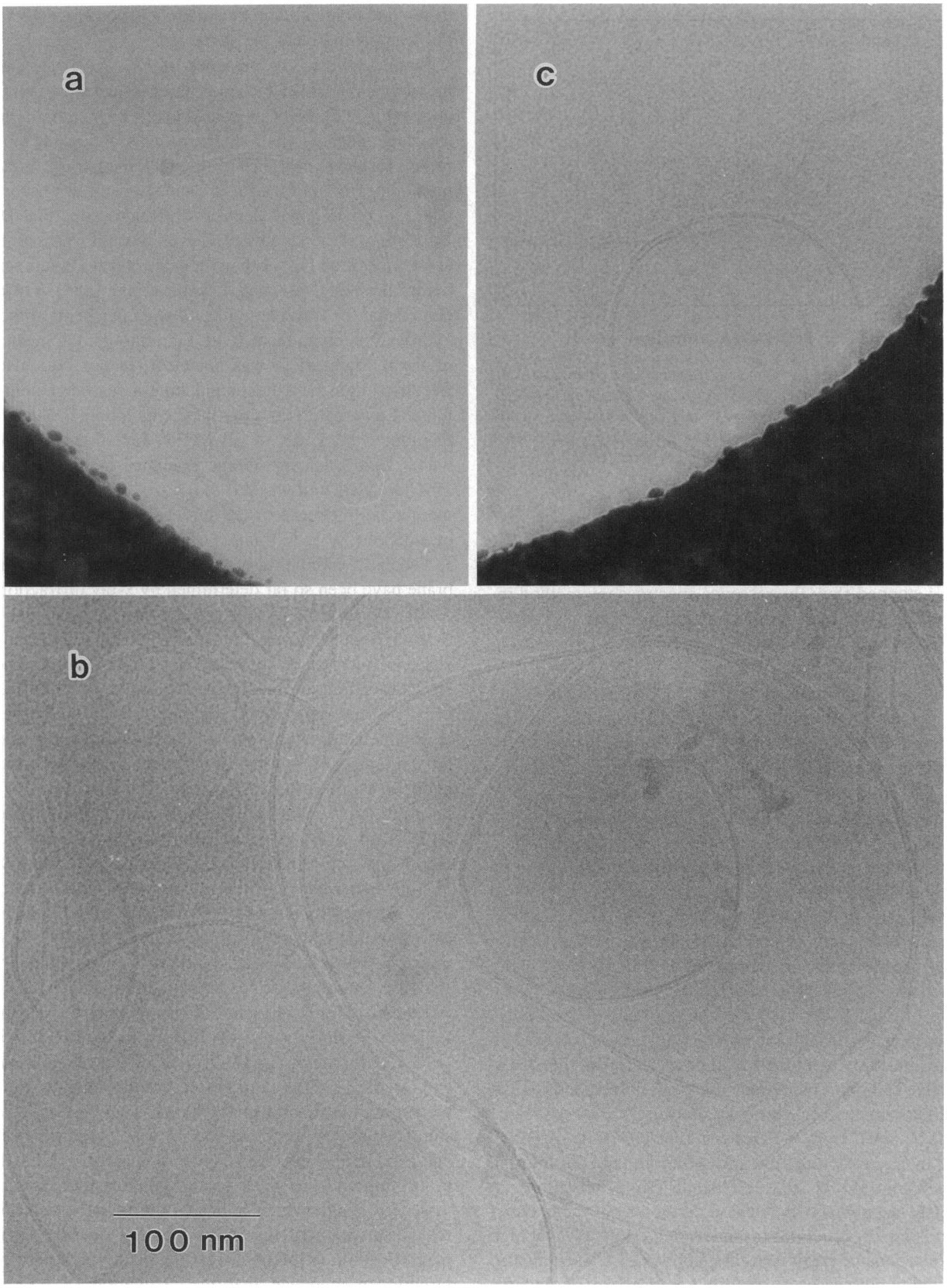

FIGURE 2 Electron micrographs of DPPC LUV in (*a*) 0% ethanol, (*b*) 15% (w/v) ethanol, and (*c*) 15% (w/v) ethanol. The bars represent 100 nm. These were taken under the following conditions: direct magnification, 40,000 ×; electron dose, ca. 80 electrons/Å^2; exposure time, 8 or 11 s; specimen stage temperature, 4.2 K; accelerating voltage, 300 kV.

existence of $L_{\beta I}$ structure in unilamellar vesicles of biological membranes. These methods enable us to investigate induction of $L_{\beta I}$ structure in other phospholipid membranes and biological membranes containing membrane proteins by various substances and to elucidate their mechanisms.

We thank Dr. Y. Fujiyoshi of Protein Engineering Research Institute for valuable advice, Prof. H. Yamagishi of Kyoto University for constant encouragement, and Prof. H. Hashizume of Shizuoka University for use of a Hitachi F3000 spectrofluorimeter. We thank N. Takahashi of Kyoto University for technical assistance.

This work was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (to M. Y.) and the Research Promotion Grants for Young scientists from Shizuoka University (to M. Y.).

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