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Effect of Doping Small Aromatic Molecules on the Physical Properties and Liposomal Structure of Lecithin

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The effect of doping aromatic compounds on the structure and physical properties of lecithin were studied using X-ray diffraction and broad-line ^{1}H-NMR methods and by the measurement of phase-transition behaviour using an optical method. To examine morphological change in the liposomal structures of doped egg-yolk lecithin systems, myelin figures were observed using a polarizing microscope. The lecithins used were L-α-dipalmitoylphosphatidylcholine (L-DPPC) and egg-yolk lecithin (egg-PC). Xylene and ephedrine were used as a hydrophobic aromatic dopant and a hydrophilic aromatic dopant, respectively. Xylene molecules were located between two ends of the hydrocarbon chains of lecithin molecules set toe-to-toe in the bilayer structure, *i.e.*, the central part of the bilayer. In contrast, ephedrine molecules with water molecules were located between two polar head groups of the adjacent bilayers, *i.e.*, the outer part of the bilayer. The doping of both aromatic compounds increased the stacking repeat distance of bilayer systems. The increase of the bilayer thickness due to the doping of aromatic compounds altered the thermotropic phase transition behaviour. The first phase transition point observed around 120° C for hydrated egg-PC shifted to a low temperature region, and the growing behaviour of myelin figures observed for these doped lecithin/water systems tended to grow in coiling and twisted structures.

Keywords: Doping effect of xylene or ephedrine; lecithin; liposome; NMR; X-ray diffraction

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INTRODUCTION

Clarification of the doping effects of a chemical compound, particularly of medicine, on the structure and physical properties of lipid bilayers is of basic importance for investigating various applications of this system as, for example, creating doped liposomes for therapy.

Phospholipids are amphiphilic, with a hydrophilic polar head part and hydrophobic hydrocarbon chain part. They are generally aggregated as stacked bilayer structures. It is well known that these molecules show both lyotropic and thermotropic phase transitions. The various phases observed for lipid stacked bilayer systems have been reported [1–6].

When doping long aliphatic compounds, such as long *n*-alkane, into the stacked bilayer system of lecithin, these compounds are reported to locate near the center of the lipid bilayer with their long axes parallel to those of the hydrocarbon chains of the lipid. Consequently, the surface area of the bilayer occupied by one lipid molecule is increased [7, 8].

In the present work, the effects of both cases of doping hydrophilic and hydrophobic small aromatic compounds, ephedrine and xylene, on the structure and properties of lipid condensed phases were investigated using X-ray diffraction, broad line ^{1}H-NMR and polarizing microscopic techniques.

For lecithin, xylene is a peculiar solvent, which causes the condensed phase of lecithin to reconstruct into a single crystal; single cyrstals of lecithin can be obtained after the xylene suspension is heated to about 50°C and then is kept at room temperature for several days, as noted with L-DPPC and DL-DPPC [4–6]. Ephedrine is a well-known drug for the treatment of asthma.

EXPERIMENTAL PROCEDURES

L-α-dipalmitoylphosphatidylcholine (L-DPPC) was obtained from Fulka Ltd., and the egg-yolk lecithin (egg-PC) from Sigma Co. It has been reported for egg-PC that approximately 70% of its β chains are oleic acid (unsaturated) with a double bond between the 9th and 10th carbon atoms and that 70% of its γ chains are palmitic acid (saturated) [9]. L-2-methylamino-1-phenylpropanol hydrochloride (ephedrine) was obtained from Dainippon Pharm. Co.

The sample of egg-PC with water content of 20 wt% was obtained by exposing egg-PC in a crystalline (anhydrous) state to ambient air with 70%

relative humidity during monitoring the increase of weight of it at room temperature. The xylene doped egg-PC sample was prepared by mixing egg-PC with water content of 20 wt% and excess xylene. The ephedrine doped egg-PC sample was prepared as follows. A lump of egg-PC with water content of 20 wt% was put in a vacuum dryer after weighing and water was evaporated during monitoring the sample weight to obtain an anhydrous sample. Then the anhydrous sample was mixed with about a half weight of 0.1 wt% water solution of ephedrine. From the mixture, water was evaporated in a vacuum dryer until it had got back the anhydrous state again. After that the doped sample was exposed to ambient air with 70% relative humidity until it had absorbed water up to the amount evaporated in the first step, which was monitored by weighing the sample.

The ^{1}H-NMR measurements were made by a JNM apparatus manufactured by JEOL Co., equipped with an autodyne detector of the Robinson type [10] and an electromagnet of essentially homogeneous and constant magnetic field (10^{-5}). The derivative curves of the absorption signal were recorded at 15 MHz. Therefore, the readily determined line width was that of the maximum slope of the absorption curve. The radio frequency magnetic field H_1 was kept at the lowest possible amplitude.

X-Ray diagrams were photographed with nickel-filtered Cu-Kα radiation (37.5 kV, 20 mA) using a flat-plate camera of $4 \sim 8$ cm passage.

The measurement of the transmittance of polarized light through optically anisotropic liquid crystals as a function of an external variable such as the temperature, is a simple way of observing thermotropic phase transition behaviour. Using a polarizing microscope, we measured the transmitted light intensity through the sample between crossed polarizers as a function of temperature (thermal depolarization analysis). The sample of liquid crystal deposited on a glass slide was inserted into a micro furnace (Mettler FP-52). The transmitted light was detected by a photo-electric cell and its intensity was recorded on a recording chart against temperature increasing at the rate of 3°C/min.

The morphology of myelin figures grown in the doped lipid and water system was observed under a polarizing microscope.

RESULTS AND DISCUSSION

(i) ^{1}H-NMR Studies

The observed right halves of the derivative envelopes of ^{1}H-NMR absorption signals from L-DPPC in various states are shown in Figure 1

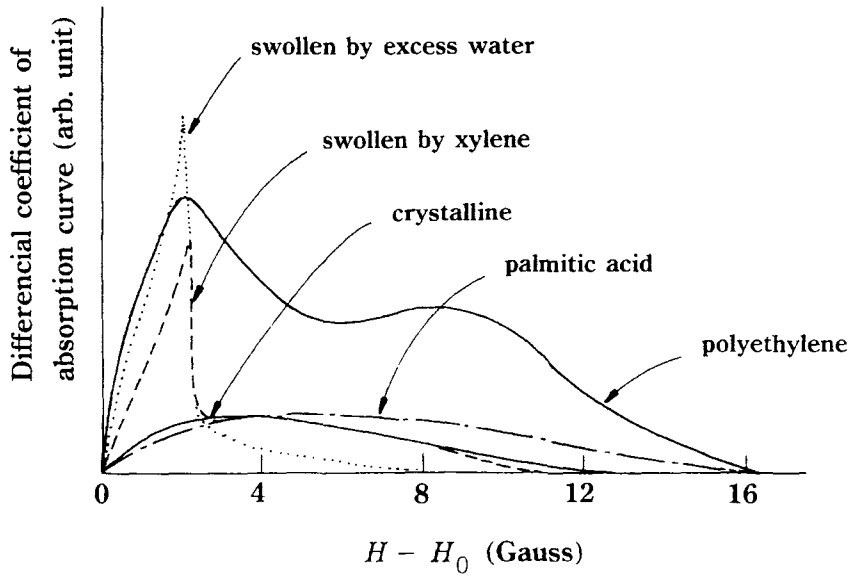

FIGURE 1 Derivative envelopes of ^{1}H-NMR absorption signal from L-DPPC in various states and related compounds recorded at 15 MHz.

together with those from crystalline palmitic acid and crystalline polyethylene as references. All measurements were performed at room temperature of approximately 25°C. The line width, *i.e.*, twice the distance from the peak of envelope to the vertical axis, shows the mobility of molecules in the specimen. When the line width of a narrow component is much narrower than the modulation width used, it is observed to be the same as the modulation width. All line widths of the narrow components in Figure 1 show the same value as that of the modulation used because a wide modulation is used for obtaining a clear envelope from a small amount of specimen. The derivative envelope of high density linear polyethylene consists of a broad component from the crystalline part and a narrow component from the amorphous part [11]. The line widths of the broad components of polyethylene, palmitic acid and L-DPPC in the crystalline state are approximately 16, 12, and 8 gauss, respectively. This indicates that the mobility of molecules in the polyethylene crystal is smaller than those of palmitic acid and L-DPPC. In the envelope of L-DPPC in the crystalline phase, a narrow component is not observed. When the specimen of L-DPPC was swollen by xylene at room temperature, a narrow component due to excess xylene molecules was additionally observed but the line shape of the broad component did not change. This indicates that doped xylene

molecules are in an ordered state in the crystal lattice of the L-DPPC and that the molecular arrangement in the crystalline state of L-DPPC is scarcely changed by the doping, except for an increase in bilayer thickness as observed by X-ray measurement, as shown later. On the contrary, when the specimen of L-DPPC was swollen by water at room temperature, the line shape of the obtained derivative envelope was drastically changed. The broad component was greatly reduced and the narrow component greatly increased. This indicates that the molecular arrangement in the water doped L-DPPC had drastically changed and that rotational motion around the hydrocarbon chain had occurred.

To clarify the effect of doping ephedrine to the condensed phase of lecithin, hydrated egg-yolk lecithin was used since the doping was carried out by use of a water solution of ephedrine. The hydrated egg-yolk lecithin showed drastic motional narrowing which could not be measured by the broad line NMR.

(ii) X-Ray Diffraction Studies

L-DPPC was used for the clarification of the doping effect of xylene and egg-PC was used for those of ephedrine and xylene in X-ray diffraction studies. In the X-ray diffractions from the crystalline phases of L-DPPC and egg-PC, a series of reflections from the bilayer thickness, 5.17 nm and 5.47 nm, respectively, up to higher order ones were observed. Strong reflections of 0.433 nm and 0.496 nm from the L-DPPC and 0.442 nm and 0.501 nm from the egg-PC, along with multiple other reflections were observed in the wide angle region (Figs. 2a, d). The diffraction patterns are closely similar to those of the reported X-ray diffraction studies of L-DPPC [12] and egg-yolk lecithin [1, 13]. It is indicated that the basic structures of these two lecithins are stacked bilayers in which the hydrocarbon chains are tilted as reported. When the crystalline specimen of L-DPPC was swollen by xylene with excess xylene molecules, the dimension of the reflections from the bilayer thickness increased from 5.17 nm to 6.07 nm. When we took the direction along the bilayer thickness as *c*-axis and the other two directions to be *a*-axis and *b*-axis in a bilayer plane, an increase of dimension along the *c*-axis was observed but the dimension and intensity distribution of *hk0* reflections did not change. That is, strong reflections in the wide angle region at $(0.433\ \text{nm})^{-1}$ and $(0.496\ \text{nm})^{-1}$ were still observed. The intensities of *hkl* reflections became weak and the *00l* reflections became slightly diffuse and weak as shown in Figure 2b. The doped xylene molecules appear to scarcely affect the side-by-side packing of the L-DPPC molecules and to be located in

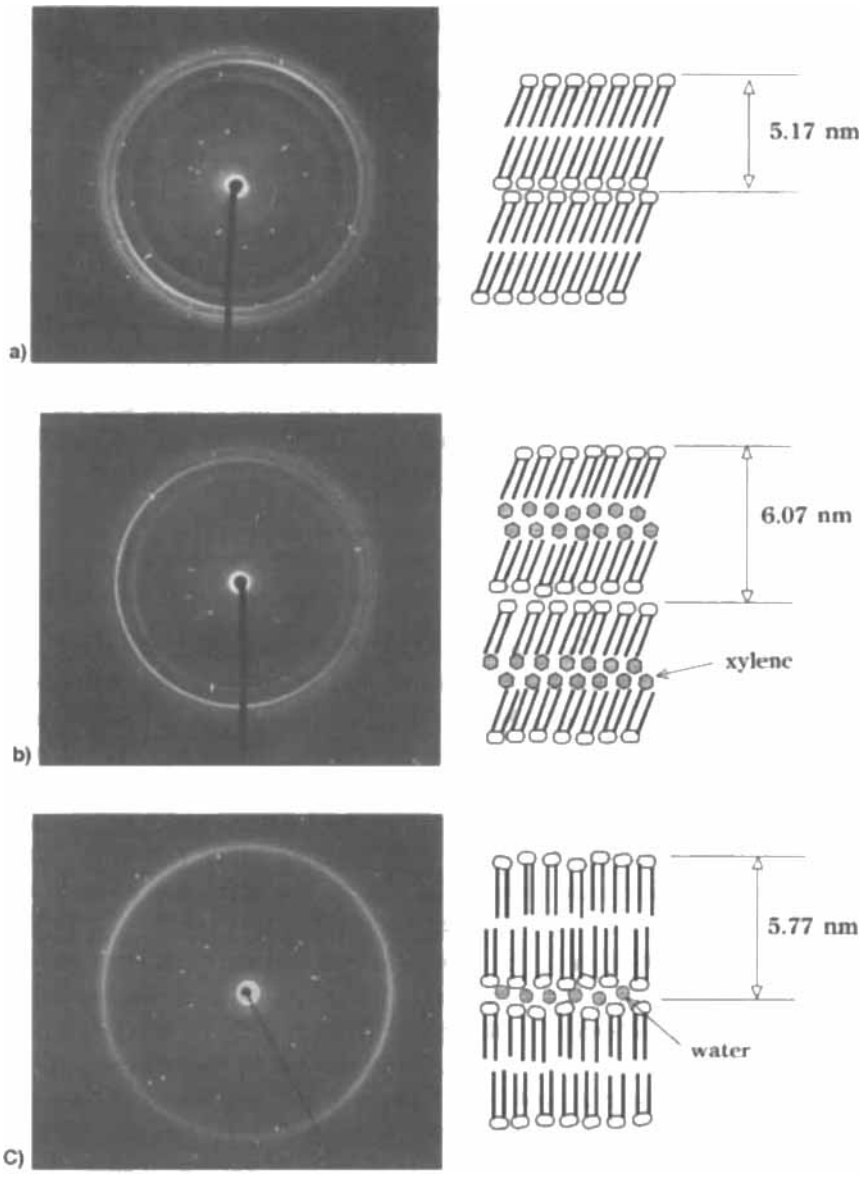

FIGURE 2 X-Ray diffraction patterns from L-DPPC (a ~ c) and from egg-PC (d ~ g) obtained by use of a flat camera with camera lengths of 7.31, 7.31, 8.11, 7.23, 5.89, 5.89, and 6.78 cm, for a, b, c, d, e, f, and g, respectively. Spots are reflexions from mica used to seal the specimen. A schematic representation of molecular arrangement proposed from the X-ray diffraction pattern appears beside each photo. a) L-DPPC in crystalline phase, b) L-DPPC in crystalline phase doped with xylene, c) L-DPPC in hydrated state, d) egg-PC in crystalline phase, e) egg-PC in hydrated state, f) egg-PC in hydrated state doped with ephedrine, g) egg-PC in hydrated state doped with xylene.

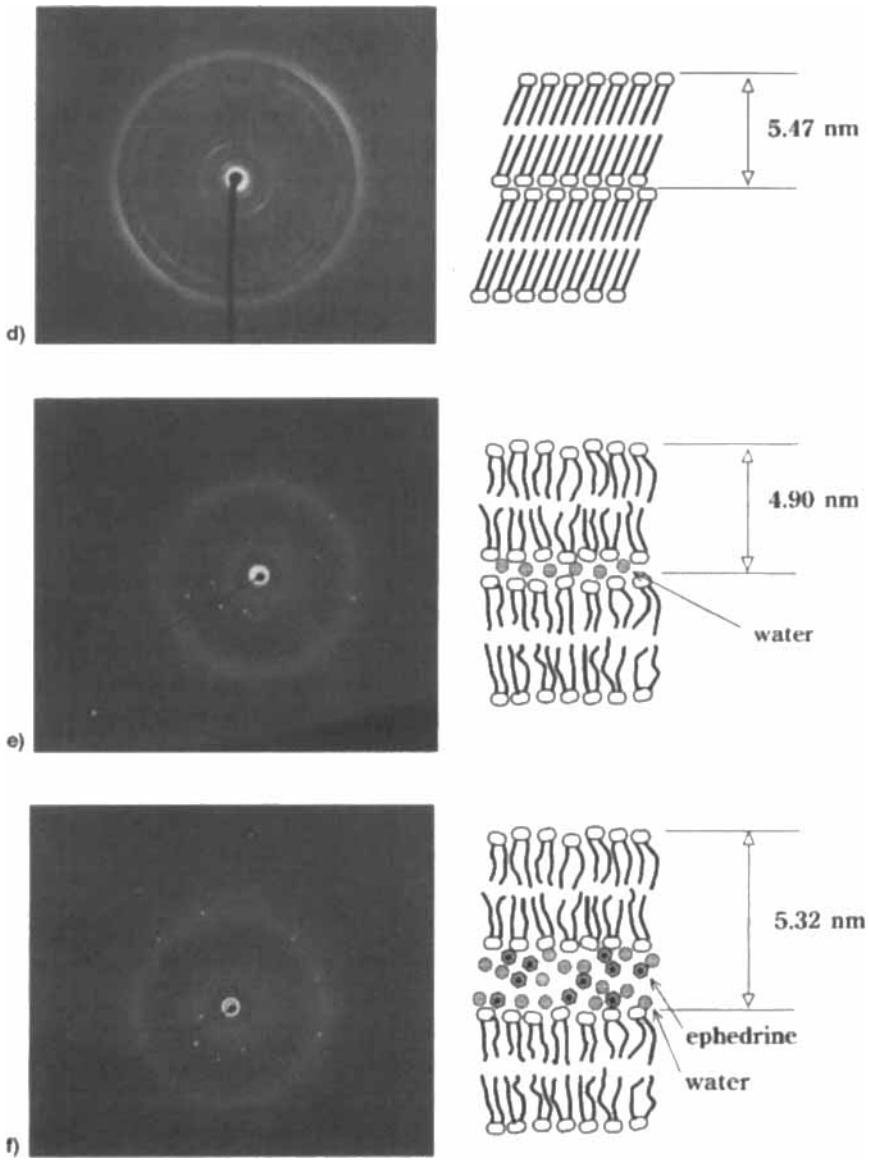

FIGURE 2 (Continued).

the central part of the bilayer (Fig. 2b). This result was in good agreement with the result from the ^{1}H-NMR measurement that the doped xylene maintained the molecular packing of the lecithin in the crystalline phase except for the increase of the bilayer thickness caused by the location of

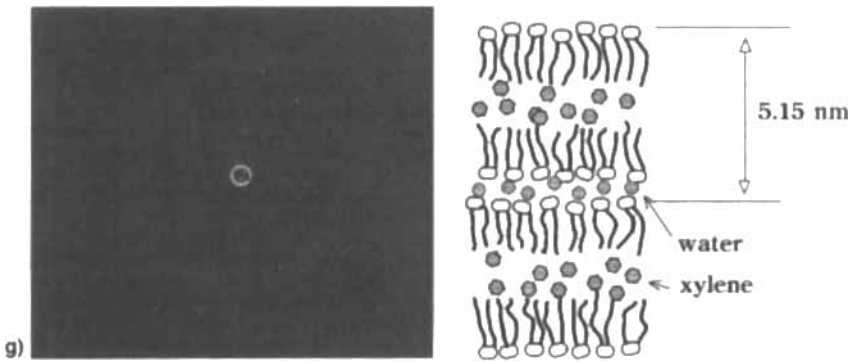

FIGURE 2 (Continued).

xylene molecules in the central part of the lipid bilayer. In contrast, when the crystalline specimen of L-DPPC was swollen by water with excess water molecules, the intensity distribution of all the reflection patterns was drastically changed, *i.e.*, most reflections disappeared except for those of lower order *00l* reflections from the bilayer thickness of 5.77 nm and that at $(0.415\,\text{nm})^{-1}$ which was the mean lattice distance of the side-by-side packing of hydrocarbon chains (Fig. 2c). This observed X-ray pattern shows that the arrangement of hydrophilic polar head groups become disordered by the doped water molecules, and the hydrocarbon chains which are tilted against the normal direction of the bilayer plane in the crystalline state are transformed to be perpendicular against the bilayer plane. The arrangement of these hydrocarbon chains consists of close side-by-side packing and each chain is parallel along the bilayer normal on the average. The drastic narrowing of the broad component of L-DPPC doped with water in the NMR measurement came from the rotational motion around the long axis of the hydrocarbon chain.

Figure 2d shows the X-ray diffraction pattern from crystalline egg-PC as described above. The long axes of hydrocarbon chains tilt 30° from the perpendicular direction to the bilayer plane [13].

When the crystalline egg-PC was swollen by approximately 20 wt% of water, the X-ray diffraction pattern from it was drastically changed and most of the sharp reflections has disappeared. It consisted of a strong reflection from the bilayer thickness of 4.90 nm together with its 2nd and 4th order reflections and a diffuse halo at about $(0.46\,\text{nm})^{-1}$ from the hydrocarbon chains in a disordered state (Fig. 2e). This indicates that the specimen is transformed from a crystalline phase to a liquid crystalline phase, Lα phase, by lyotropic phase transition.

Figure 2f shows the X-ray diffraction pattern from the hydrated egg-PC doped with ephedrine. By the doping, the bilayer thickness increased from 4.90 nm to 5.32 nm and a diffuse halo at $(0.46\,\text{nm})^{-1}$ was still observed. Therefore, it is supposed that ephedrine molecules are located with the water molecules in the space between the two bilayers consisting of polar head groups.

Figure 2g is the X-ray diffraction pattern from the hydrated egg-PC doped with xylene. By the doping, the bilayer thickness increased from 4.90 nm to 5.15 nm and a diffuse halo at $(0.46\ \text{nm})^{-1}$ was again observed. Xylene molecules are, therefore, supposed to be located in the space between the ends of hydrocarbon chains as is observed by doping to crystalline L-DPPC.

(iii) Thermotropic Phase Transition Behaviour by Optical Method

Using a polarizing microscope, we measured the transmitted light intensities of the samples prepared by the same methods as used in X-ray measurements, *i.e.*, hydrated egg-PC doped with xylene or ephedrine, against increasing temperature. Temperature increased at the rate of 3°C/min from room temperature to slightly above the melting temperature above 200°C.

The results are summarized in Figure 3. The difference in absolute intensities of the observed transmitted light primarily occurred from the amount of measured specimens, *i.e.*, thickness, and the anisotropy of the molecular arrangement, *i.e.*, optical anisotropy. With increasing temperature, the transmitted light intensities of the hydrated egg-PC drastically decreased at the first thermotropic phase transition point of about 120°C, increased at the second thermotropic phase transition of about 135°C and then gradually decreased again up to the melting point. As for these phase transitions observed over 100°C, the water content of the same egg-PC scarcely effects the phase transition behaviour. At the first phase transition, the molecular arrangement in the liquid crystalline state is transformed to an almost optically isotropic state. At the second phase transition, the seemingly isotropic state is transformed to develop aggregated spherulites as is reported for monohydrated L-DPPC powder specimens [6]. The phase transition behaviour is a typical one observed for lecithin.

For egg-PC doped with ephedrine, the first thermotropic phase transition point shifted towards a lower temperature of about 90°C compared to hydrated egg-PC, and a small peak, which appeared to be the second thermotropic phase transition, was observed near the first phase transition

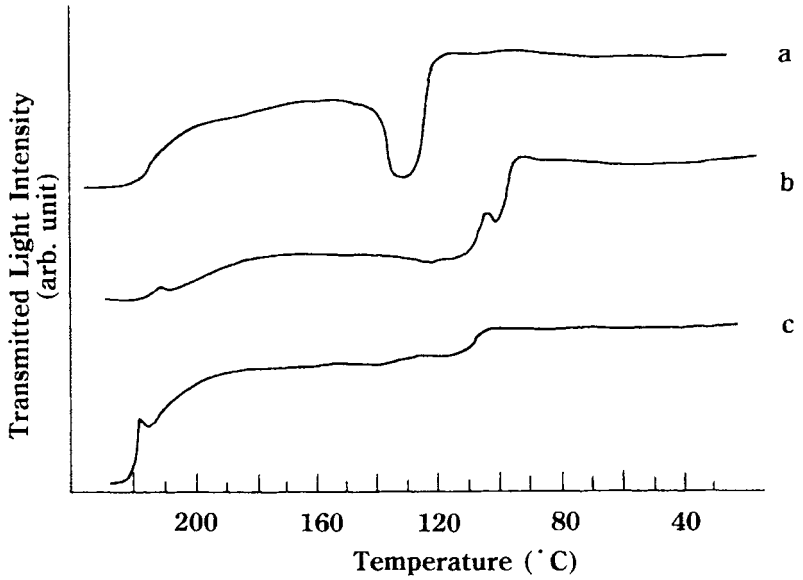

FIGURE 3 Temperature dependence of transmitted light intensities through samples between crossed polarizers. The light intensities were monitored by use of a polarizing microscope under the same measuring condition. The temperature was increased at the rate of 3°C/min. a) egg-PC in hydrated state, b) hydrated egg-PC doped with ephedrine, c) hydrated egg-PC doped with xylene.

point. The transmitted light intensity then gradually decayed beginning at about 170°C.

For egg-PC doped with xylene, only a first thermotropic phase transition at about 110°C was observed and melting began to occur gradually at about 180°C. After a small peak at about 220°C, due probably to a pretransitional effect, the specimen completely melted. As shown in Figure 3, the doping of each substance makes the first thermotropic phase transition point shift to a low temperature. It is suggested that the increase in bilayer thickness by doping aromatic compounds creates weaker intermolecular interactions in the specimen, causing its physical properties to change.

(iv) Morphology of Myelin Figures

The specimens used for observation of the growing myelin figures were prepared by the same methods as described above. They were hydrated egg-PC, hydrated egg-PC doped with ephedrine and hydrated egg-PC doped with xylene. Observations of the myelin figure growth were made using a polarizing microscope. Myelin figures were obtained as follows. At first, a

small lump of lecithin was placed on a slide glass. Then a cover glass was placed over the lump and pressed slightly until the lump thickness was reduced to approximately 50 μm. Water was used as the hydrophilic medium to grow myelin figures. A drop of water was placed on the edge of the cover glass of the preparation, so that the excess liquid water medium spread over into the gap of the slide and cover glasses. As soon as the surface of the lecithin lump came into contact with the medium, myelin figures of a simple rod-like form were observed to begin growing all together in the pure egg-PC/water system as shown in Figure 4a. The growth rate of the myelin figures was reported to be diffusion-limited [14]. In the cases of egg-PC doped with ephedrine and egg-PC doped with xylene, the textures of myelin figures were more complicated and the initial growth rates were approximately one order of magnitude slower than that of pure egg-PC in the initial growth stage. In the ephedrine doped egg-PC/water system, the prominent feature is the appearance of helical and coiling forms as shown in Figure 4b. The helical and coiling forms are also observed even for the myelin figures of the pure egg-PC/water system, but the most prevailing texture there is a simple rod-like structure. In the xylene doped egg-PC/water system, the myelin figures appear as an aggregation of helical and coiling forms as shown in Figure 4c.

The appearance of "pathological" myelin figures by doping can formally be explained as a result of changes in the elastic properties and surface energy of the bilayer system induced by doping. Coiled or twisted forms are elastically unfavorable due to an increased strain, but the increased contact area of rods is favorable to surface energy. An explanation of the morphological changes observed is still open to questions, together with the problem of whether the bilayer system is mechanically softened by doping.

CONCLUDING REMARKS

Small hydrophobic and hydrophilic aromatic dopants, xylene and ephedrine, respectively, increase the repeat distance of a lipid stacked bilayer system. Xylene molecules are located in the central part of the lipid bilayer and ephedrine molecules are located in the outer part of the bilayer along with water molecules. If the coiling and twisting in myelin figures results from these dopants, liposomes consisting of similar lipids and dopants as used here would show the same molecular arrangement, and easily create lipid bilayer liposome systems with curvature to enclose a drug.

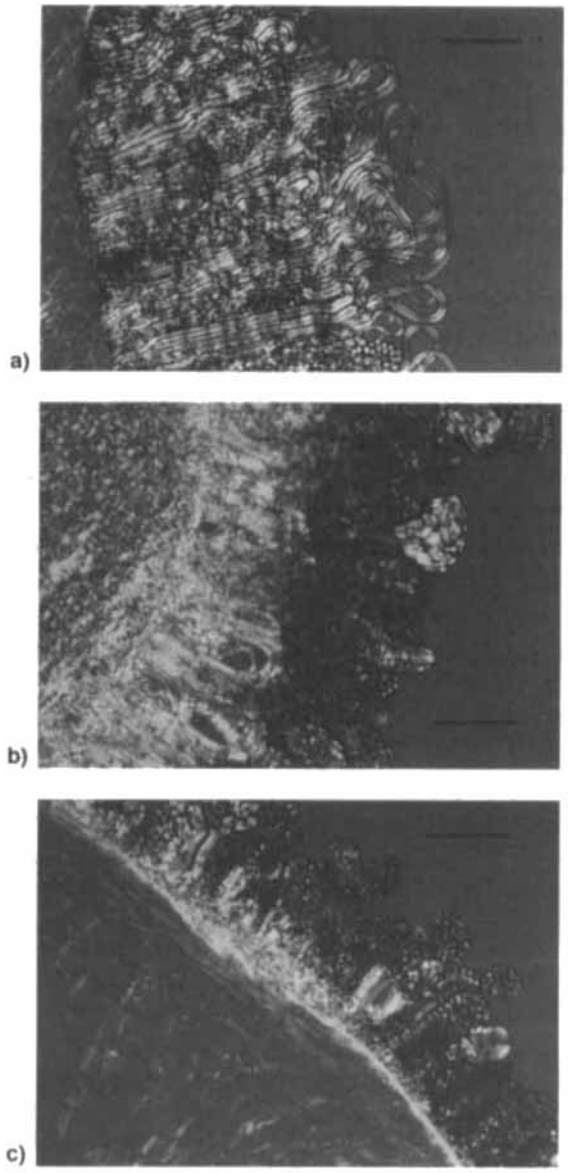

FIGURE 4 Photos of myelin figures in initial growth state. a) egg-PC/water system after about 15 min from starting to grow, b) ephedrine doped egg-PC/water system after about 45 min, c) xylene doped egg-PC/water system after about 45 min. Bars represent 200 μm. (See Color Plate X).

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