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Emulsion and Vesicle Formation of Retinol and Retinyl Palmitate with Egg Yolk Phosphatidylcholine

NOBUYA ITHO,* HIROAKI KOMATSU,† TETSUROU HANDA,*,[1] AND KOICHIRO MIYAJIMA*

*Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-01 Japan; and †National Institute of Hygienic Sciences, Hoenzaka, Chuo-ku, Osaka, 540 Japan

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Spreading pressure measurements showed that retinyl palmitate (retinol palmitate, ReO-Pal) and egg yolk phosphatidylcholine (PC) were practically immiscible and separated into PC bilayers and ReO-Pal liquid. On the contrary, substantial amounts of retinol (ReOH) were incorporated into PC bilayers. The molar ratios of PC in the outer surface and the inside of sonicated particles of these lipid mixtures were determined on the basis of ^{1}H-NMR measurements in the presence of a paramagnetic cation Pr^{3+}. The higher ratio for the PC/ReO-Pal particles than 1.4 (the maximum value for vesicle structure) showed that part of the PC in the inner leaflet of vesicle bilayers was moved to the outer leaflet by addition of the neutral lipid, indicating formation of emulsion particles. Production of emulsion particles in the sonicated dispersion of PC/ReO-Pal mixtures was confirmed by electron microscopy (EM). Small angle X-ray scattering (SAXS) and EM measurements on (vortexed) hydrated lipid mixtures of PC/ReO-Pal demonstrated that excess PC, coexisting with emulsion particles, was organized into regular bilayer lamellas with a repeating distance of 64.8 nm. On the other hand, vesicles with irregularly stacked lamellar structures were observed in EM pictures on hydrated PC/ReOH mixtures. The irregular structures caused broad SAXS spectra with a repeating distance of about 70 nm and a sharp isotropic peak in ^{31}P-NMR spectra. Thus, esterification and hydrolysis of retinoids lead to remarkable changes in the structure organization with phospholipids.

Key Words: retinol; retinyl palmitate; phosphatidylcholine; emulsions; vesicles.

INTRODUCTION

Neutral lipids, retinoids (vitamin A and its derivatives), are fundamental mediators of proliferation of numerous cells (1, 2). When man is in good health, 90% of retinoids are stored in the liver and 10% are in an active state. Retinoids in the form of fatty acid esters of retinol fulfill either storage or transport functions. Retinol (ReOH) esterification is catalyzed by lecithin, retinol acyl transferase which transfers the sn-1 fatty acid of phosphatidylcholine (PC) to ReOH (3, 4). The main component of the sn-1 fatty acid of PC is palmitic acid; therefore, the main component of retinyl esters is retinyl palmitate (ReO-Pal), which is found in association with lipid droplets of liver cells and lipoproteins of animal plasma (5–7).

Retinol, being essential for reproduction, is transported by ReOH-binding proteins in plasma. The hydrophobic pocket in the protein minimizes cytotoxic effects of ReOH (8–10). ReOH induces structure and permeability changes of cell membranes and lipid bilayers (11–13). Details of interactions of ReOH and ReO-Pal with phospholipids have not been completely understood.

We have shown that neutral lipids with very low solubility in PC bilayers form emulsion particles besides the bilayer vesicles. On the other hand, incorporation of neutral lipids of appreciable solubility into the bilayers leads to structural perturbation and/or morphological change in the membranes (14–16). In the present work, we estimated interactions of PC with ReOH and ReO-Pal and studied emulsion production in PC/ReO-Pal mixtures and irregular structure formation in PC/ReOH mixed bilayers.

Two types of lipid dispersions were prepared: simply vortexed large multilamellas (hydrated lipid mixtures) and sonicated small particles (sonicated particles). Particle structure of mixed lipid was mainly investigated on the sonicated samples by ^{1}H-NMR, dynamic light scattering, and electron microscopic (EM) measurements. Sonicated small particles of single bilayers or oligo-bilayers gave a sharp peak in 31P-NMR spectra, owing to the rapid tumbling, and very weak peaks in small angle X-ray scattering (SAXS) spectra. We therefore studied the membrane structure with hydrated lipid mixtures (large particles).

EXPERIMENTAL

Materials

Egg yolk PC was kindly provided by Asahi Kasei Co., Ltd. (Tokyo). The purity (over 99%) was detected by thin layer chromatography (TLC). The acyl chain composition was 0.2% 14:0, 34.9% 16:0, 0.3% 16:1, 11.9% 18:0, 30.4%

[1] To whom correspondence should be addressed.

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18:1, 15.0% 18:2, 0.2% 20:2, 0.3% 20:3, 3.2% 20.4, 3.3% 22:6, and 0.3% others. ReOH purchased from Eastman Kodak was purified by alumina column chromatography. The solvent was gradually changed from petroleum ether to chloroform and the eluted fractions were assayed by TLC (solvent: chloroform/methanol = 95/5). ReO-Pal obtained from Wako Pure Chemical Industry, Ltd. (Osaka) was purified by alumina column chromatography with a mixed solvent of petroleum ether/acetone = 94/6. Each fraction was checked by TLC with the same mixed solvent. Deuterium oxide (purity >99.75%) was obtained from Wako Pure Chemical Industry, Ltd. Paramagnetic cation, Pr^{3+}, was purchased as praseodymium nitrate hexahydrate (99.5%). Water was doubly distilled with a quartz still.

Spreading Pressure Measurement

Spreading pressures of PC/ReOH and PC/ReO-Pal mixtures at air/water interface (i.e., surface pressure of a lipid monolayer in equilibrium with lipid in bilayer, liquid, or solid states) were obtained from the steady values of surface pressure after addition of a lipid or lipid mixture on water at 25°C. The details of the monolayer techniques have been described elsewhere (17).

Vortexed and Sonicated Lipid Dispersions

PC was dissolved with ReOH or ReO-Pal in chloroform. After evaporation of the solvent, the mixture was dried *in vacuo* for 10 h and then dispersed in water by a vortex mixer (hydrated lipid mixtures). An aliquot of the hydrated sample was sonicated for 30 min under a stream of nitrogen gas at 70°C (sonicated particles). The probe-type sonicator used was UC-200 from Tomy Seiko Company, Ltd. No free fatty acid resulting from the hydrolysis of PC during sonication was detected by TLC (solvent: chloroform).

^{1}H-NMR Spectra for Sonicated Particles in the Presence of Pr^{3+}

Proton nuclear magnetic resonance spectra were recorded on a Bruker AC300 spectrometer equipped with pulsed FT facilities at 25°C. The chemical shift of proton resonance was determined by reference to the residual HDO proton (4.6 ppm). Lipid mixtures at total concentration of 10 mM were sonicated in D_2O and the NMR spectra were measured immediately after addition of Pr^{3+} (20–30 μM). The measurement was completed within 30 min.

Dynamic Light Scattering (DLS)

Hydrodynamic diameters of sonicated lipid particles were measured on a Photal laser particle analyzer LPA-3100 connected to a photon correlator LPA-3000 at 25°C. The data were analyzed by histogram method, and the weight-averaged particle size were evaluated.

Electron Microscopy

A drop of sonicated or hydrated lipid dispersion was applied on a 400-mesh collodion coated copper grid and was negatively stained with ammonium molybdate. The grid thus prepared was observed on Hitachi H-500 electron microscope operating at 75 kV.

^{31}P-NMR Spectra for Hydrated Lipid Mixtures

^{31}P-NMR spectra for hydrated lipid mixtures (50 mM) were measured at 25°C on a Bruker AC300 FT-NMR spectrometer operating at resonance frequency of 121 MHz (pulse width: 45°). A composite ^{1}H decoupling pulse was employed. D_2O in a capillary tube was used for external lock. The chemical shift of ^{31}P resonance was recorded with reference to that of lysophosphatidylcholine micelles in the capillary tube.

Small Angle X-Ray Scattering

A hydrated lipid mixture (100 mM) was sealed between thin mica sheets in a custom-built metal holder and was set in the sample chamber of the 6-m point focusing small angle X-ray scattering camera system at the high intensity X-ray laboratory of Kyoto University. The X-ray source was a rotating-anode Cu$K\alpha$ radiation generator (γ = 1.54178 Å) with a power of 2 kW. The detector was a multiwire delay-line two-dimensional position-sensitive proportional counter. The scattered X-ray photons were detected for more than 20 min at 25°C. The scattering intensity–scattering vector profiles of circular average reflection data were recorded (18).

RESULTS

Spreading Pressure

Spreading pressure of a lipid mixture is surface pressure of a mixed monolayer in equilibrium with bulk lipid phases and is assumed to be the surface vapor pressure of the lipid mixture (17, 19). Figure 1 shows the spreading pressure of PC/ReO-Pal and PC/ReOH mixtures as a function of mole fraction. Spreading pressures of PC, ReOH, and ReO-Pal were 44.0, 24.0, and 5.7 mN/m, respectively. In PC/ReO-Pal mixtures, spreading pressure was independent of the mole fraction and was 44 ± 1 mN/m (Fig. 1A). On the basis of the surface phase rule (17), PC and ReO-Pal were presumed to be immiscible in the PC bilayer and ReO-Pal liquid phases.

On the other hand, spreading pressure for PC/ReOH mixtures changed with the mole fraction (Fig. 1B). The results indicated that PC and ReOH were miscible, consistent with the DSC results of Ortiz *et al.*, who found that the gel–liquid crystal phase transition temperature of DPPC decreased with the ReOH mole fraction (20).

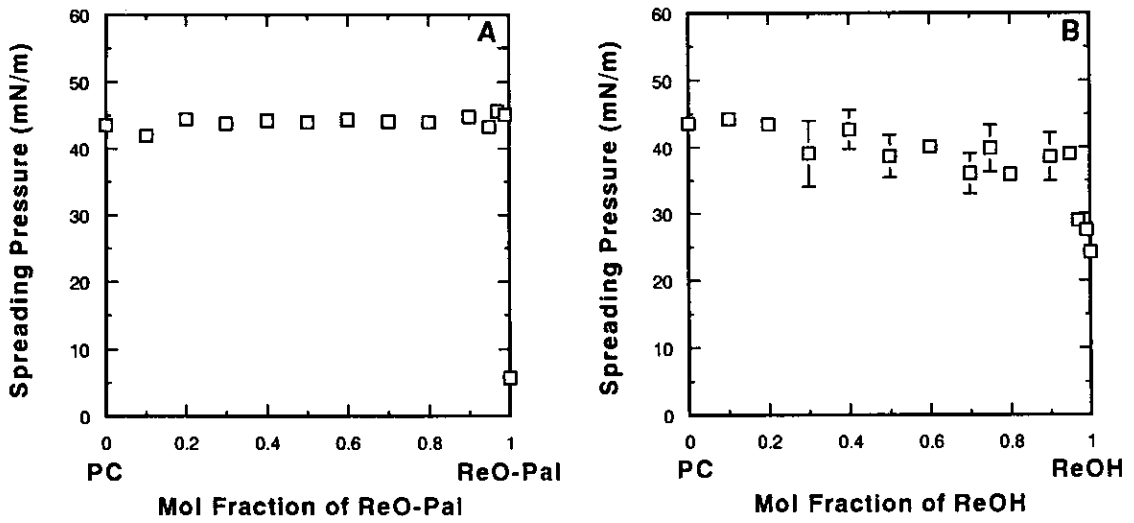

FIG. 1. Spreading pressure of (A) PC/ReO-Pal and (B) PC/ReOH mixtures at an air/water interface at 25°C.

^{1}H-NMR Spectra for Sonicated Particles in the Presence of Pr^{3+}

When a nonpenetrating paramagnetic cation, Pr^{3+}, is added to PC vesicles, the ^{1}H-NMR signal of the $-N^+(CH_3)_3$ shows two distinct peaks which arise from the groups in inner and outer leaflets of vesicular bilayers (21, 22). The signal of the inside is not affected by the cation, while the signal from the surface, being in contact with Pr^{3+}, shifts to downfield. Such downfield shifts were observed in the sonicated particles of both PC/ReOH and PC/ReO-Pal mixtures.

The ratio of PC molecules in the outer and inner leaflets of single bilayer vesicles is calculated as a function of the vesicle diameter, d (nm), as

$$\text{(out/in) ratio} = \frac{(d/2)^3 - [(d/2) - 2]^3}{[(d/2) - 2]^3 - [(d/2) - 4]^3}. \quad [1]$$

The equation is based on the assumption that vesicles consist of outer and inner leaflets (lipid monolayers) of 2 nm (23). With increasing d, the (out/in) ratio decreases from the maximum value 1.4 at $d = 28$ nm and eventually converges to unity. For large multibilayer vesicles ($d > 100$ nm), the ratio is predicted to be less than unity because only the outermost leaflets are in contact with the paramagmetic cation.

Table 1 shows the (out/in) ratios observed for sonicated particles and those calculated by Eq. [1]. The intensity ratio of the outer and inner signals was stationary for at least 4 h in all sonicated dispersions. For the PC vesicles, the value was 1.40, which agreed with the calculated value and was consistent with the reported value (24). Here d values were obtained by the DLS measurement. For PC/ReO-Pal mixtures, the ratio and the particle diameter increased with the mole fraction of ReO-Pal. ReO-Pal was insoluble in PC bilayers and was presumed not to affect the permeability to Pr^{3+}. These results showed that a part of PC in the inside was moved to the surface of the sonicated particles, indicating formation of emulsion particles composed of PC and ReO-Pal. In PC/ReOH mixtures, sonicated particles with higher ReOH molar fractions (>0.5) did not stably disperse, but aggregated and precipitated. The (out/in) ratio at PC/ReOH of 1/1 was close to the calculated value for single bilayer vesicles with d of 48 nm.

Electron Microscopic Pictures for Sonicated Particles

Figure 2 shows negatively stained EM pictures of sonicated particles for PC/ReO-Pal (3/7) (Fig. 2A) and PC/ReOH (1/1) (Fig. 2B) mixtures. Emulsion particles (average diameter, about 100 nm) were observed in the PC/ReO-Pal dispersion. On the other hand, small unilamellar vesicles with average diameter of about 50 nm were seen in the PC/ReOH dispersion. These results clearly showed that ReOH

TABLE 1

Ratio of ^{1}H-NMR Peak Areas for Choline Methyl Groups of PC from Outer Surface and inside of Sonicated Particles

Lipid (molar ratio)	Diameter (nm) (weight average) [by DLS]	(out/in) Ratio: Observed [by ^{1}H-NMR]	(out/in) Ratio: Calculated for vesicles [by Eq. [1]]
PC	28 ± 11	1.40	1.40
PC/ReO-Pal (5/5)	78 ± 24	1.35	1.11
PC/ReO-Pal (4/6)	112 ± 38	1.50	<1.08
PC/ReO-Pal (3/7)	120 ± 55	2.39	<1.07
PC/ReOH (5/5)	48 ± 15	1.14	1.20

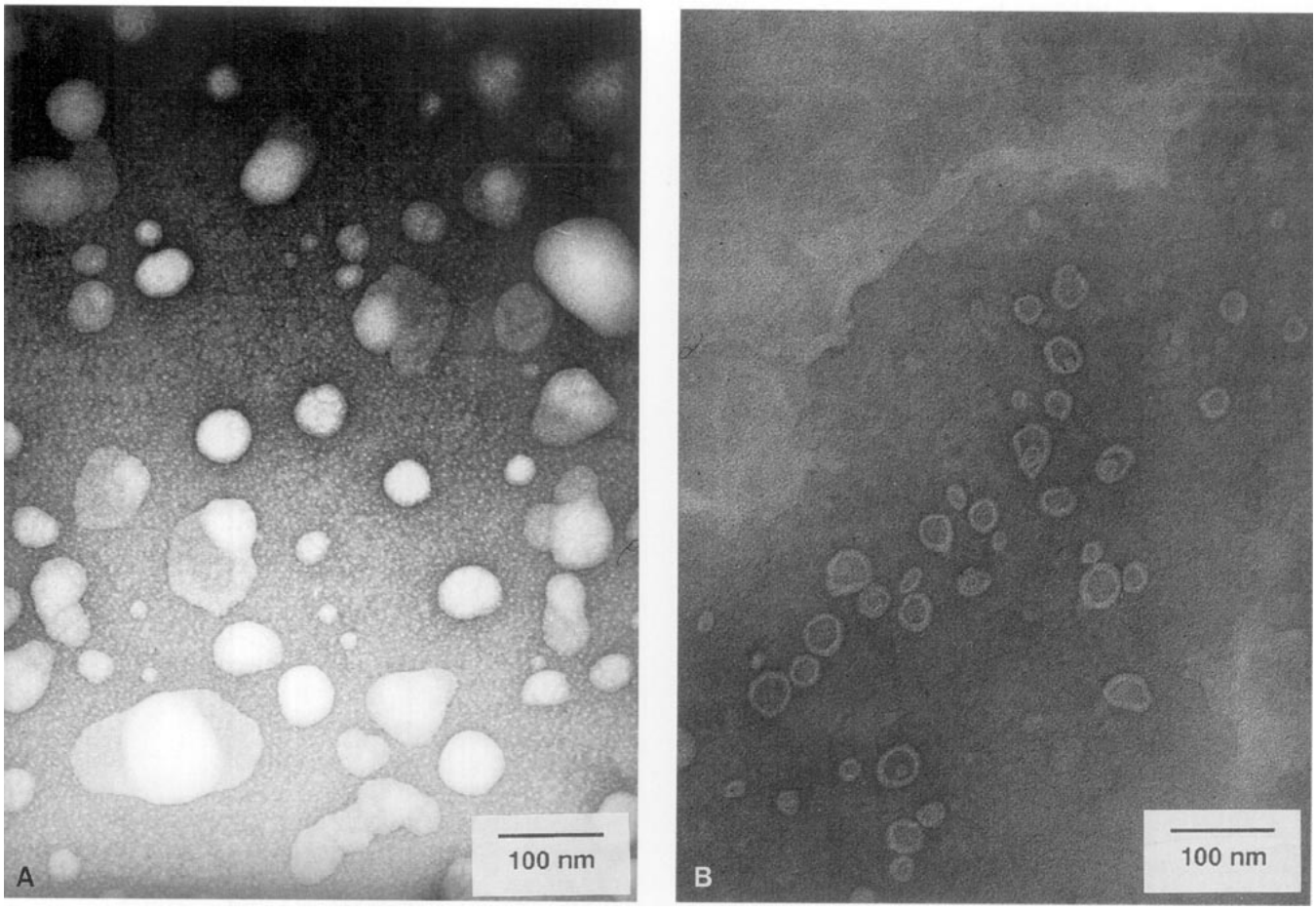

FIG. 2. Electron microscopic pictures of sonicated particles for (A) PC/ReO-Pal (molar ratio = 3/7) and (B) PC/ReOH (molar ratio = 1/1) mixtures.

and ReO-Pal had different effects on the structure organization of PC.

SAXS of Hydrated Lipid Mixtures

Scattering intensities for hydrated lipid mixtures are represented as a function of scattering vector (q) in Fig. 3. The q value at the peak positions correlate with the repeated distance (spacing) of lattice plane, d, as $d = 2\pi/q$. Phospholipids organized into multilamellar (multibilayer) structures give rise to reflections with repeated distances which relate as 1:1/2:1/3:1/4. Reversed hexagonal and reversed cubic configurations will give different reflection profiles. Hydrated PC bilayers gave peaks at d = 64.8 and 32.1 Å, which were assigned to the first and second reflections of multilamellar structure (Fig. 3A, photon detection time = 20 min). In PC/ReO-Pal mixtures, similar structures were observed (Fig. 3B, photon detection time = 20 min). Liquid ReO-Pal did not give any peak in SAXS spectra.

In PC/ReOH mixtures, lamellar structures similar to those of PC were observed when the ReOH molar fraction was less than 0.4. Solid ReOH did not give any peaks in SAXS spectra. At the molar fraction of 0.5, weak and broad scattering peaks were observed at d = 71.4 and 35.8 Å (Fig. 3C, photon detection time = 240 min). These reflections were presumed to arise from perturbed multilamellar structures of the PC/ReOH mixtures. No other distinct peaks were detected in the measuring period of 240 min.

^{31}P-NMR Spectra for Hydrated Lipid Mixtures

Phosphatidylcholine when organized into multilamellar structures gives rise to an asymmetric ^{31}P-NMR line shape with a high-field peak and a low-field shoulder (25, 26). The hydrated multilamellas of PC showed an asymmetric line shape of 50 ppm, characteristic of an axially symmetric chemical shift tensor. When ReOH or ReO-Pal was added, a sharp symmetric line appeared at 0 ppm (Fig. 4), indicating that a part of the signal originating from PC in regular multilamellas was replaced by a signal characteristic of PC molecules undergoing a rapid isotropic motion that led to a nearly complete averaging of the chemical shift anisotropy. ReO-Pal was more effective for such a spectral change than ReOH: 5 mol% of ReO-Pal gave a clear symmetric sharp

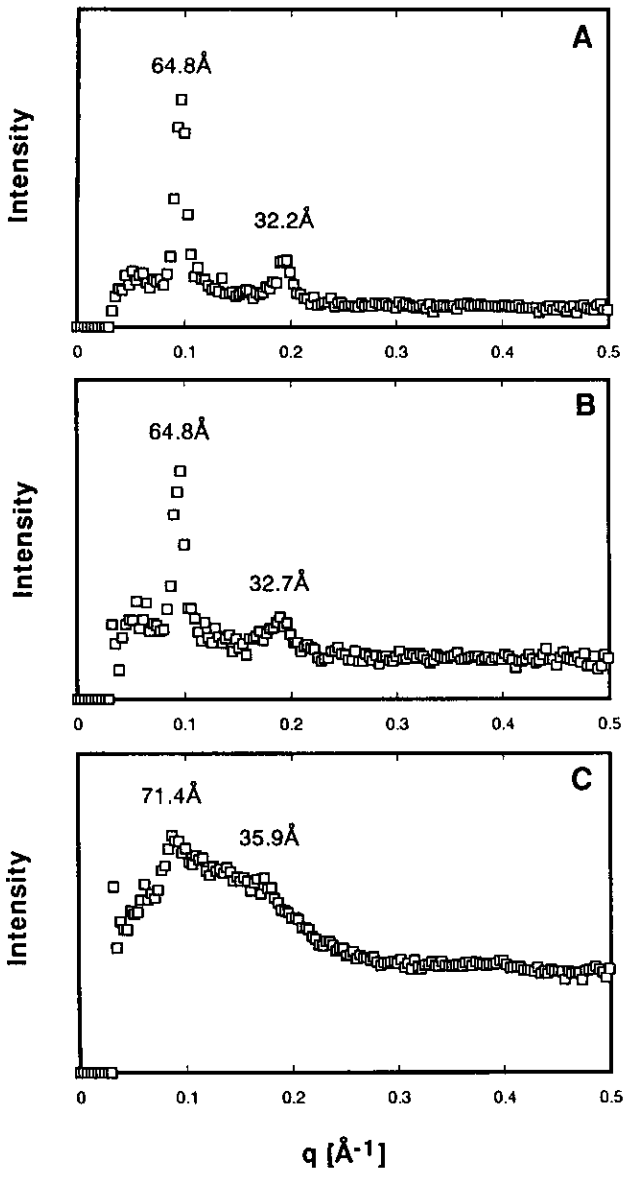

FIG. 3. SAXS spectra for hydrated mixtures of (A) PC, (B) PC/ReO-Pal (mole ratio = 1/1), and (C) PC/ReOH (mole ratio = 1/1). Time for X-ray photon detection; (A) 20 min, (B) 20 min, and (C) 240 min.

line (Fig. 4, left side) and such a symmetric line was not detected until more than 20 mol% of ReOH was added to PC multilamellas (Fig. 4, right side).

Electron Microscopic Pictures for Hydrated Lipid Mixtures

EM pictures in Fig. 5 show that emulsion particles coexist with multilamellar vesicles in hydrated mixtures of PC/ReO-Pal (1/1, Fig. 5A) and that large vesicles with irregularly stacked multilamellas were formed in hydrated mixtures of PC/ReOH (1/1, Fig. 5B).

DISCUSSION

Interactions of PC with ReOH and ReO-Pal

Neutral lipids with limited solubility in PC bilayers, such as triglycerides (15), cholesterylesters (14), and ubiquinone-10 (27), give minimum disturbance on the bilayer structure. Excess neutral lipids separated from the bilayers form droplets in aqueous medium. The droplets were stabilized by PC monolayers spread from the bilayers to the surface (formation of emulsion particles). Thus, in stable emulsions, emulsion particles composed of neutral lipid cores and PC monolayers coexist with bilayers (vesicles) consisting mainly of PC. The coexistence brings about formation of eutectic monolayers of PC and neutral lipids at the emulsion surface (14, 15). A constant composition of emulsion surface irrespective of particle size is maintained by this mechanism.

On the other hand, addition of neutral lipids with large solubility often causes structural change of PC bilayers. Incorporation of such neutral lipids into the bilayers shifts the hydrophilic–lipophilic balance toward lipophilic nature and induces structural perturbations. Diglycedides (28), α-tocopherol (29), and menaquinone-4 (16) in PC bilayers lead to formation of reversed hexagonal and reversed cubic structures.

Spreading pressure for PC/ReO-Pal mixtures showed that these lipids were practically immiscible and separated into the PC bilayers and Reo-Pal droplets, indicating the possibility of emulsion formation. In contrast, spreading pressure of PC/ReOH mixtures showed that these lipids were mutually miscible. Gel/liquid crystal phase transition temperature of DPPC decreases by incorporation of ReOH (20). Substantial amounts of ReOH in phospholipid bilayers shift the hydrophilic–lipophilic balance and causes structural perturbations and/or morphological changes (12, 30).

Emulsion and Vesicle Formations

Results in Table 1 and Fig. 2 show that sonication brings about production of emulsions in PC/ReO-Pal mixtures and formation of unilamellar vesicles in PC/ReOH mixtures.

In the hydrated mixtures of PC/ReO-Pal, emulsion particles coexisted with multilamellar vesicles (Fig. 5A). The solubility of ReO-Pal in the vesicle membranes was very limited, and the multilamellas gave very similar SAXS profiles to those of pure PC multilamellas (Figs. 3A and 3B). The PC monolayers at the emulsion surface are assumed not to give any reflection peak in the SAXS spectra. These results showed that regular multilamellar structures of PC coexisted with emulsion particles. The sharp isotropic lines in ^{31}P-NMR spectra of the hydrated mixtures (Fig. 4A) were therefore attributed to the emulsion formation. Figure 5A showed formation of some small particles (diameter, about 50 nm) even in the hydrated mixtures of PC/ReO-Pal (with-

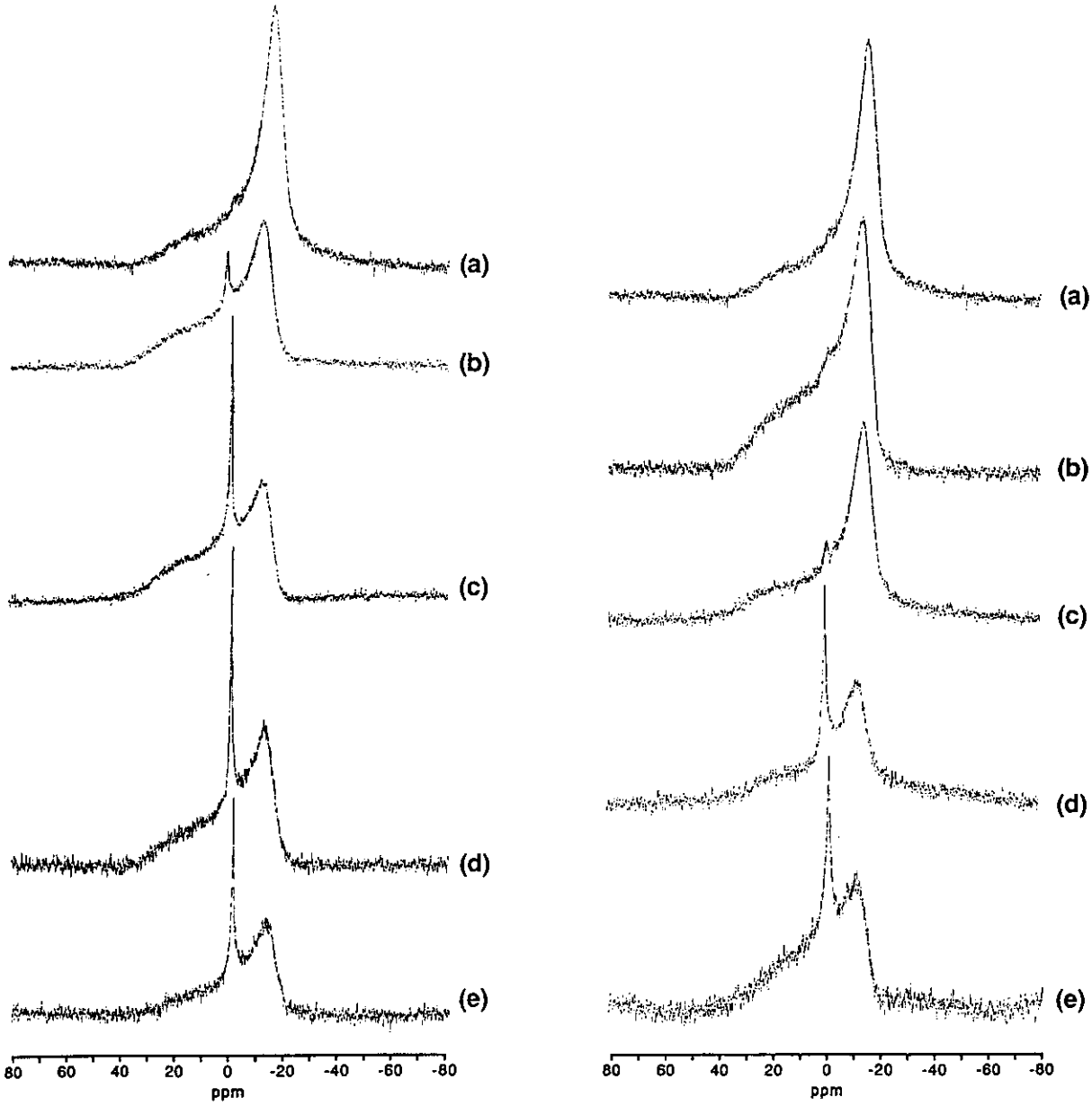

FIG. 4. ^{31}P-NMR spectra for hydrated lipid mixtures. (Left) PC/ReO-Pal mixtures: mole fractions of ReO-Pal are (a) 0, (b) 0.05, (c) 0.1, (d) 0.3, and (e) 0.5. (Right) PC/ReOH mixtures: mole fractions of ReOH are (a) 0, (b) 0.1, (c) 0.2, (d) 0.3, and (e) 0.5.

out sonication). Rapid tumbling of such small particles may be a plausible reason for the isotropic lines in NMR spectra (25). Hydrated PC lamellas did not give any sharp lines in the spectrum (Figs. 3A and 3B). Other possible reasons are that PC head groups in emulsion monolayers are in conformations different from those in a regular bilayer state (26) or that lateral motion of PC in curved monolayers of emulsion particles is very rapid and averages the chemical shift tensor. Further work on conformational and motional properties of lipid molecules in emulsion monolayers is necessary.

Hydrated mixtures of PC/ReOH produced large vesicles with irregularly stacked multilamellas (Fig. 5B). Such irregular multibilayers are presumed to result in the broad peaks at 71.4 and 35.9 Å in SAXS spectrum (Fig. 4C). Much weaker peaks, compared with those of the PC and PC/ReO-Pal samples, also indicated that regular multilamellar structure was remarkably disrupted in the PC/ReOH mixtures. SAXS and ^{31}P-NMR data showed that appreciable amounts (<20 mol%) of ReOH were solubilized without any structural or morphological change. Between 20 and 50 mol% of the neutral lipid, irregular (by SAXS) and isotropic (by ^{31}P-NMR) structures appeared. Further addition of ReOH led to separation of lipid mixtures from aqueous medium. Local concentration or clustering of the neutral lipid with small

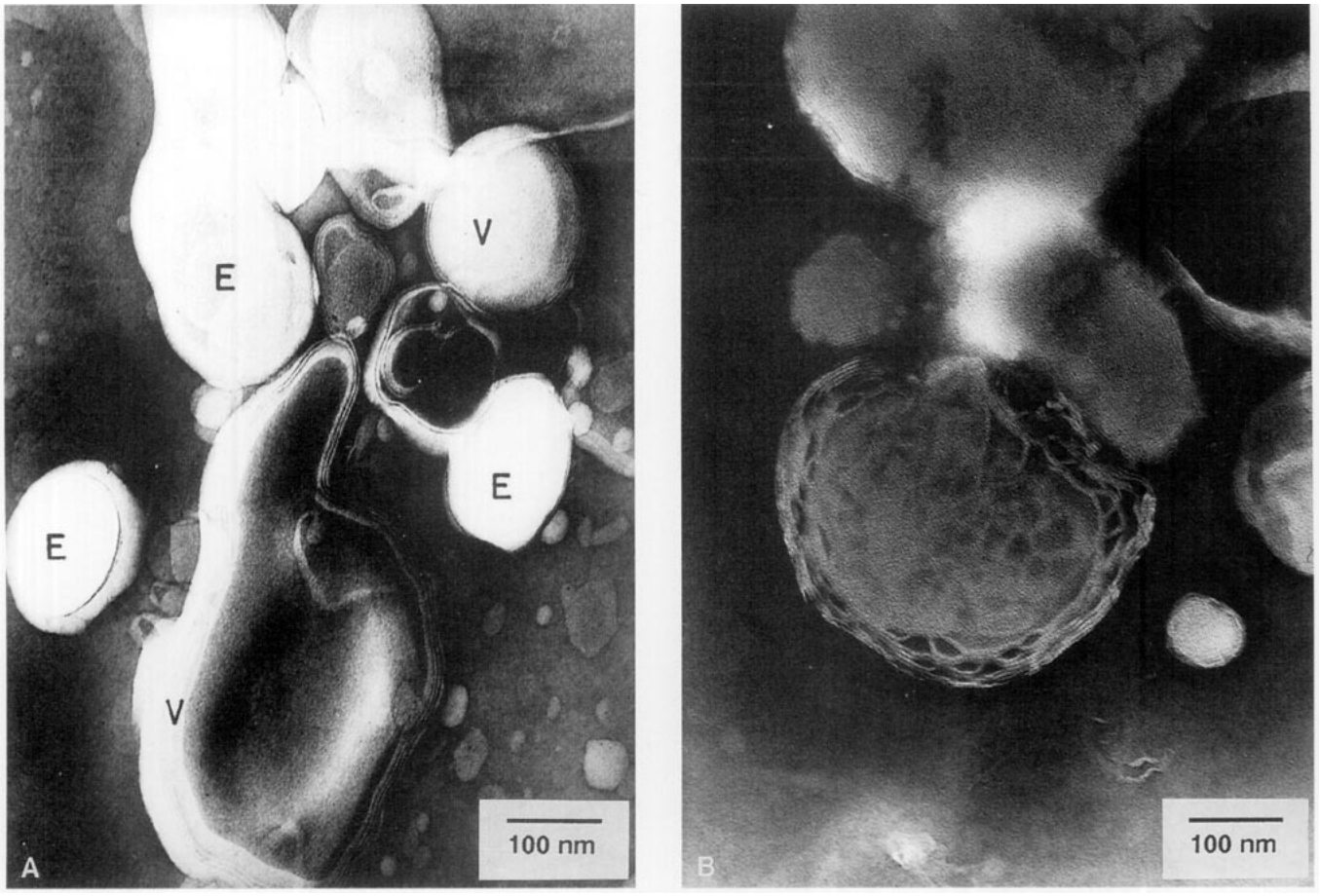

FIG. 5. Electron microscopic pictures for hydrated lipid mixtures. (A) PC/ReO-Pal (molar ratio = 1/1) and (B) PC/ReOH (molar ratio = 1/1). The mixture in (A) was observed at a different contrast and the particles V and E were judged to be vesicular and emulsion particles, respectively.

hydrophilic group are assumed to induce structural perturbations and formation of reversed micelles in bilayers (31), giving irregular structures in SAXS and EM results. Such structures may be precursors for reversed cubic and hexagonal arrangements of lipid molecules (31). Lipid assemblies with the reversed arrangement tend to aggregate, fuse (12), and separate from an aqueous medium because of lipophilic nature of the outer surface (16).

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REFERENCES

1. Jetten, A. M., *in* "Growth and Maturation Factors" (J. Guroff, Ed.), Vol. 3, pp. 251–293. Wiley, New York, 1985.
2. Roberts, A. B., and Sporn, M. B., *in* "The Retinoids" (M. B. Sporn, A. B. Roberts, and D. S. Goodman, Eds.), Vol. 2, pp. 210–286. Academic Press, Orlando, 1984.
3. Saari, J. C., and Bredberg, D. L., *J. Biol. Chem.* **15,** 8636 (1989).
4. Shi, Y.-Q., Furuyoshi, S., Hubacek, I., and Rando, R. R., *Biochemistry* **32,** 3077 (1993).
5. Harrison, E. H., Blaner, W. S., Goodman, D., and Ross, A. C., *J. Lipid Res.* **28,** 973 (1987).
6. Ruotolo, G., Zhang, H., Bentsianov, V., and Le, N.-A., *J. Lipid Res.* **33,** 1541 (1992).
7. Beer, F., *J. Lipid Res.* **33,** 915 (1992).
8. Muccio, D., Waterhous, D. V., Fish, F., and Brouillette, C. G., *Biochemistry* **31,** 5560 (1992).
9. Bychkova, V. E., Berni, R., Rossi, G. L., Kutyshenko, V. P., and Ptitsyn, O. B., *Biochemistry* **31,** 7566 (1992).
10. Noy, N., Slosberg, E., and Scarlata, S., *Biochemistry* **31,** 11118 (1992).
11. Stillwell, W., Ricketts, M., Hudson, H., and Nahmias, S., *Biochim. Biophys. Acta* **688,** 653 (1982).
12. Battaglia, K. B., Fliesler, S., Li, J., Young, J. E., and Yeagle, P. L., *Biochim. Biophys. Acta* **1111,** 256 (1992).
13. Meeks, R. G., Zaharevitz, D., and Chen, R. F., *Arch. Biochem. Biophys.* **207,** 141 (1981).
14. Handa, T., and Nakagaki, M., *Adv. Colloid Interface Sci.* **38,** 45 (1992).
15. Handa, T., Saito, H., and Miyajima, K., *Biochemistry* **29,** 2884 (1990).
16. Handa, T., Asai, Y., Komatsu, H., and Miyajima, K., *J. Colloid Interface Sci.* **153,** 303 (1992).
17. Nakagaki, M., Tomita, K., and Handa, T., *Biochemistry* **24,** 4619 (1985).

18. Hayashi, H., Hamada, F., Suehiro, S., Masaki, N., Ogawa, T., and Miyaji, H., *J. Appl. Cryst.* **21,** 330 (1988).
19. Handa, T., Ichihashi, C., and Nakagaki, M., *Prog. Colloid Polym. Sci.* **71,** 26 (1985).
20. Ortiz, A., Aranda, F. J., and Fernandez, C. G., *Biochim. Biophys. Acta* **1106,** 282 (1992).
21. Fernandez, M. S., Celis, H., and Montal, M., *Biochim. Biophys. Acta* **323,** 600 (1973).
22. Lee, Y., and Chan, S. I., *Biochemistry* **16,** 1303 (1977).
23. Huang, C., and Mason, J. T., *Proc. Natl. Acad. Sci. U.S.A.* **75,** 308 (1978).
24. Huang, C., *Biochemistry* **8,** 344 (1969).
25. Burnell, E. E., Cullis, P. R., and DeKruijff, B., *Biochim. Biophys. Acta* **603,** 63 (1980).
26. Thayer, A. M., and Kohler, S. J., *Biochemistry* **20,** 6831 (1981).
27. Handa, T., Asai, Y., Miyajima, K., Kawashima, Y., Kayano, M., Ida, K., and Ikeuchi, T., *J. Colloid Interface Sci.* **143,** 205 (1991).
28. Das, S., and Rand, R. P., *Biochemistry* **25,** 2882 (2986).
29. Nakajima, K., Utsumi, H., Kazama, M., and Hamada, A., *Chem. Pharm. Bull.* **38,** 1 (1990).
30. Ortiz, A., Aranda, F. J., Villalain, J., and Fernandez, C. G., *Biochim. Biophys. Acta* **1112,** 226 (1992).
31. Cullis, P. R., and DeKruijff, B., *Biochim. Biophys. Acta* **559,** 399 (1979).