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RESEARCH PAPER

Interaction of Soybean Oil with Phosphatidylcholine and Their Formation of Small Dispersed Particles

Yasuyuki Asai[1,]* and Sumio Watanabe[2]

[1]*Formulation Research Laboratory, Kawashima, Eisai Company, Limited, Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 501-61, Japan*
[2]*Tsukuba Research Laboratories, Eisai Company, Limited, Tokodai, Tsukuba, Ibaraki 300-26, Japan*

ABSTRACT

Stable aqueous dispersions of soybean oil (SO) were obtained by cosonication with dipalmitoylphosphatidylcholine (DPPC) in the SO mole fraction range 0.1–0.8. To clarify the dispersal mechanism, the dispersed particles were characterized, and the interaction between SO and DPPC was investigated using several physicochemical techniques. Dynamic light scattering (DLS) measurements showed that the diameter of the dispersed particles was 40–60 nm. The trapped aqueous volume inside the particles was determined fluorometrically using the aqueous space marker calcein. The trapped volume in the SO/DPPC particles decreased remarkably with the addition of SO into small unilamellar vesicles of DPPC. The decline in fraction of vesicular particles was also confirmed by fluorescence quenching of N*-dansylhexadecylamine in the DPPC membrane by the addition of the quencher* $CuSO_4$*. These results indicate that the excess SO separated from the DPPC bilayers is stabilized as emulsion particles by the DPPC surface monolayer. Monolayer-bilayer equilibrium of SO/DPPC mixtures was estimated by measurement of spreading and collapse pressures. The results showed that the coexistence of emulsion particles (surface monolayer of DPPC + core of SO) with vesicular particles (bilayer) was critically important for the formation of stably dispersed particles of the lipid mixture.*

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* To whom correspondence should be addressed.

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INTRODUCTION

Fat emulsions are widely used for parental nutrition in cases for which essential fatty acid deficiency is likely to be a problem. The choice of lecithins (phosphatides) from egg yolk or soybean as an emulsifier for parenteral emulsions has been advocated since these materials can be metabolized. However, little attention has been given to the fundamental properties of submicronized emulsions stabilized with lecithins (phosphatides) for parenteral formulations. Schuberth and Wretlined (1) studied various emulsifying agents for fat emulsions at a constant concentration of egg yolk phosphatides (1.2%), and Benita, Friedman, and Weinstock (2) reported on the effect of concentration of phosphatides and nonionic surfactant, such as polyoxyethylene-polyoxypropylene block copolymer (Pluronic F68). Further investigations for fat emulsion stabilized with lecithins are necessary to obtain such information as the mechanism of stabilization of formation of the emulsion and characterization of the emulsion particles.

In this study, to clarify the interaction between soybean oil (SO) and phosphatides, we prepared the dispersed particles of SO and dipalmitoylphosphatidylcholine (DPPC) as a model phosphatide by cosonication and characterized them to investigate the dispersal mechanism using several physicochemical techniques. The structure of SO/DPPC particles was investigated by dynamic light scattering (DLS), fluorescence quenching, and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of SO and DPPC were evaluated by differential scanning calorimetry (DSC) and surface monolayer techniques.

EXPERIMENTAL

Materials

The SO was purchased from Sigma Chemicals Company Limited (St Louis, MO). Calcein (3,3′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-fluorescein), L-α-dipalmitoylphosphatidylcholine (DPPC), and copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Wako-Chemical Company, Limited (Osaka, Japan). *N*-Dansylhexadecylamine (DSHA) was from Lambda Company, Limited (Graz, Austria).

Methods

Preparation of Dispersed Particles

The SO and DPPC were dissolved in chloroform. After evaporation of the solvent, water was added to give a final combined concentration of SO and DPPC of 5 mM. The mixtures were sonicated for 30 min under a stream of nitrogen gas at 50°C. A probe-type sonicator, model UD-200 (Tomy Seiko Co., Ltd., Tokyo, Japan) was used at a power setting of 100 W.

Determination of Particle Sizes

The DLS measurements of the sonicated dispersions of SO and DPPC were performed with a DLS-7000DL submicron analyzer (Ohtsuka Electronics Co., Ltd., Osaka, Japan) at 25°C. The data were analyzed by the histogram method (3), and the weight-averaged particle sizes were evaluated.

Determination of the Trapped Volume Inside the Dispersed Particles

A dried mixture of SO and DPPC was hydrated with a 70 mM calcein solution instead of water for the preparation of the dispersion. Untrapped calcein was removed by gel filtration (Sephadex G-50). The volume of the calcein solution trapped in the dispersed particles was determined fluorometrically (4) after solubilization of the lipid particles by the addition of 10% Triton X-100, and the aqueous volume trapped per mole of DPPC was evaluated. The DPPC in the dispersion was assayed by the method of Bartlett (5).

Fluorescence Quenching

Fluorescence quenching techniques were used to obtain information on structural changes (ratio of external to internal membrane) in the SO/DPPC dispersed particles. Fluorescence quenching techniques have been previously described by Matsuzaki et al. (6). In this study, $CuSO_4$ was used as a quencher for the DSHA fluorescence embedded in the lipid particles. The SO/DPPC dispersed particles containing 1 mol% of DSHA were titrated with small aliquots of 1 M $CuSO_4$. The fluorescence intensity I at 510 nm (with excitation at 330 nm) was measured as a function of the Cu^{2+} concentration $[Q]$. Assuming that only the fluorescence of the Cu^{2+}-accessible DSHA is quenched according to the Stern-Volmer equation (7), one can estimate the exposed fraction of DSHA P, so that

$$I_o \cdot [Q]/(I_o - I) = (1/P) \cdot [Q] + 1/KP$$

where, I_o is fluorescence intensity in the absence of the quencher, I is the intensity after quenched by Cu^{2+}, $[Q]$ is the concentration of Cu^{2+}, and K is the Stern-Volmer constant.

Solubility of SO in DPPC Membranes and of DPPC in SO

To investigate the solubility of SO in DPPC membranes and of DPPC in SO, DSC was performed using a Model DSC-100 (Seiko-Denshi Co., Ltd., Tokyo, Japan). SO/DPPC mixtures (total 1.5×10^{-6} mole) in 40 μl of water were placed in a DSC pan and sealed. An equal volume of the water was placed in the reference pan. Temperature scans were made from 10°C to 70°C, with constant heating rates of 2°C/min. All calorimetric data were obtained from samples during the heating phase.

Measurements of Collapse and Spreading Pressures

The SO, DPPC, and SO/DPPC mixtures were dissolved in benzene as the spreading solvent. The solution was added with an Agla micrometer syringe onto the double-distilled water. After complete evaporation of the solvent, the surface pressures of the monolayers were measured by Whilhemy's method using a surface tensiometer (Model CBVP-A3, Kyowa Kaimenkagaku Co., Ltd., Tokyo, Japan), and the surface pressure-area per lipid molecule curve was obtained. The collapse pressures of the monolayer (surface pressures at the transition point from monolayer to bilayer or solid states) were determined from the inflection points on the curves. The spreading pressures of SO/DPPC mixtures at an air/water interface (surface pressures at the transition point from bilayer or solid states to monolayer) were obtained from the steady value of surface pressure at 12–24 hr after the addition of the lipid or the lipid mixture on water. Both the collapse and spreading pressures were determined at 25°C. Details of the monolayer techniques have been described elsewhere (8,9).

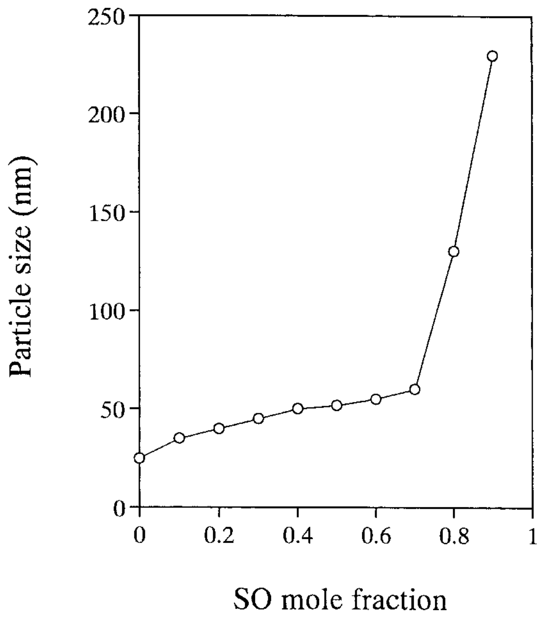

Figure 1. Weight-averaged diameter of dispersed particles represented as a function of mole fraction of SO (X_{SO}) in the mixture determined by dynamic light scattering (DLS).

RESULTS

Stably Dispersed Particles of SO and DPPC Mixtures

Figure 1 shows the diameter of the dispersed particles as a function of SO mole fraction (X_{SO}). Figure 2 shows

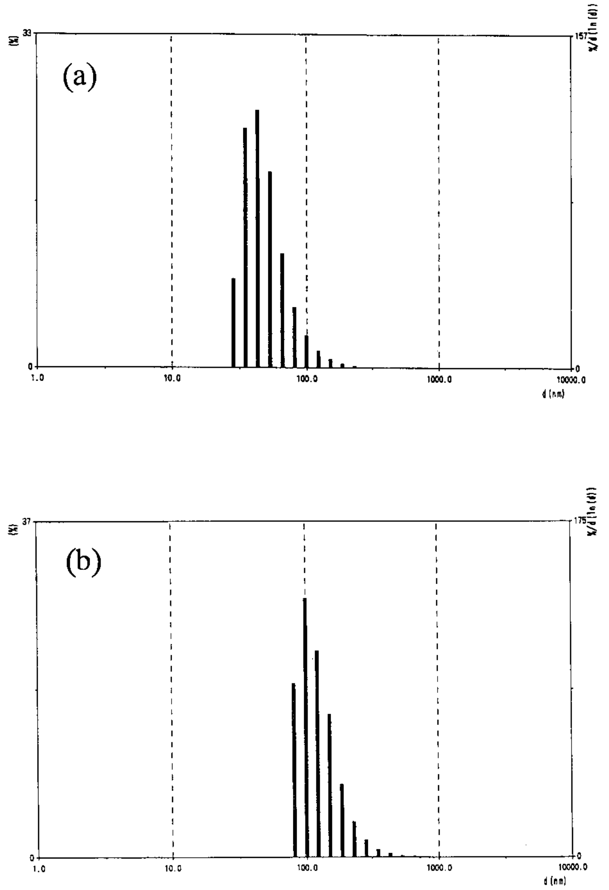

Figure 2. Histograms of weight-averaged particle size evaluated from dynamic light scattering (DLS) data for the dispersion SO/DPPC mixture. Mole fraction of SO (X_{SO}) in the mixture: (a) $X_{SO} = 0.5$, particle size 51.7 ± 24.4 nm (mean ± SD); (b) $X_{SO} = 0.8$, particle size 130.5 ± 67.8 nm (mean ± SD).

the DLS histograms of the particles of $X_{SO} = 0.5$ and 0.8, and the weight-averaged diameters were 51.7 nm and 130.5 nm, respectively. Separation of the dispersion to oil/water phases was not observed in the dispersions of SO and DPPC mixture in the range of $X_{SO} = 0$–0.8 within 72 hr after preparation. At $X_{SO} = 0.9$, the particle diameter was considerably larger at 230 nm, and separation was observed 72 hr after preparation. At $X_{SO} = 0.95$, the particle diameter was 270 nm, and the separation was detected within 24 hr after preparation.

Aqueous Space Inside the Dispersed Particles

Figure 3 shows the trapped volume of the particles per mole of DPPC at various X_{SO}. The trapped volumes of small unilamellar vesicles (diameter 20–50 nm), large unilamellar vesicles (diameter 200–1000 nm), and multilamellar vesicles (diameter 400–3000 nm) have been estimated to be 0.2 to 0.5, 7 to 10, and 3 to 4 liter · mole^{-1}, respectively (10). At $X_{SO} = 0$, small unilamellar DPPC vesicles (diameter 25 nm) had a trapped volume of 0.44 liter · mole^{-1}, which agrees with the reported value. The trapped volume of the dispersed particles of SO/DPPC mixture was highest at $X_{SO} = 0.3$ and then decreased sharply above $X_{SO} = 0.4$. The trapped volume was also calculated on the basis of total moles of SO and DPPC and is represented in the same figure. The dramatic drop in the trapped volume indicates that some structural change occurs in the dispersed particles as a result of the addition of SO.

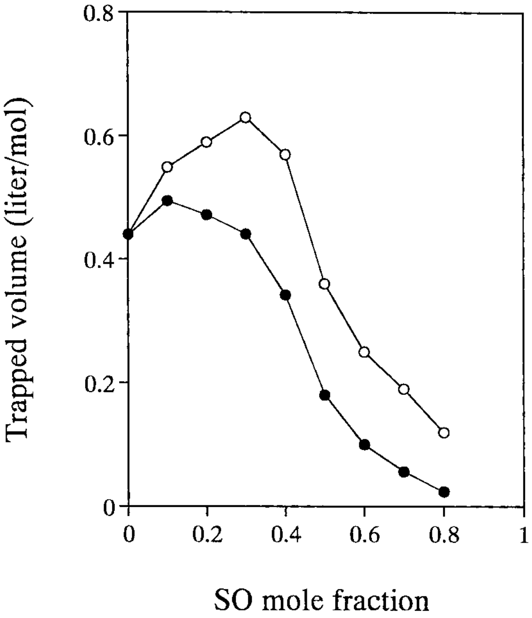

Figure 3. Trapped aqueous volume inside the dispersed particles represented as a function of mole fraction of SO (X_{SO}) in the mixture. Volume of inner space per mole of DPPC, ○; volume of inner space per total mole of the lipid (SO + DPPC), ●.

Fluorescence Quenching

The fluorescence characteristics of DSHA are known to be sensitive to the microenvironment around the probe, and the dansyl fluorophore is located in the vicinity of the glycerol backbone of the lipid bilayers (11). When the nonpenetrating fluorescence quencher $CuSO_4$ is added to SO/DPPC dispersed particles, it only quenches the fluorescence of the DSHA in the outer aqueous phase. In the modified Stern-Volmer plot, the $I_o \cdot [Q]/(I - I_o)$ versus $[Q]$ plots (the I values had been corrected for dilution) were linear. Figure 4 shows the ratio of the external to total (external plus internal) membrane P for SO/DPPC dispersed particles as a function of X_{SO}. DPPC liposomes, which served as a control, had a P ratio of 0.58, which

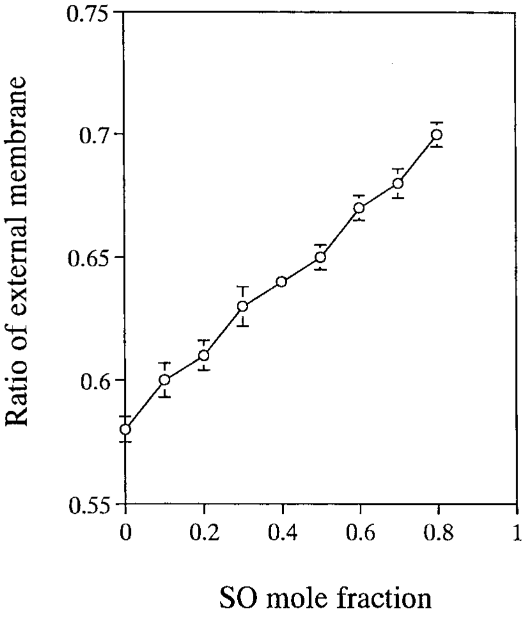

Figure 4. Ratio of the external to total (external plus internal) membrane in the lipid mixture determined by fluorescence quenching represented as a function of the mole fraction of SO (X_{SO}) in the mixture. Each point represents the mean ± SEM.

is in agreement with the molar ratio of PC molecules at the outer and inner surfaces of small unilamellar vesicles (12,13). The *P* value for the SO/DPPC dispersed particles increased with increases in the X_{SO}. These results suggest that some structural changes in the dispersed particles take place by the addition of SO.

Solubility of SO in DPPC Membranes and of DPPC in SO

Figure 5 represents the solubility of SO in DPPC membranes determined by DSC. The addition of SO decreased the phase transition temperature, and at X_{SO} values higher than 0.05, the phase transition temperature was constant at 37°C. This indicates that the solubility of SO in the DPPC membrane was equivalent to a mole fraction of 0.05. The phase transition enthalpy decreased with the increase in X_{SO}, and the phase transition was abolished at $X_{SO} = 0.95$. This indicates that, at $X_{SO} = 0.95$, DPPC was completely incorporated in SO, and that the solubility of SO in DPPC membrane was equivalent to a mole fraction of 0.05.

Collapse and Spreading Pressures of SO and DPPC Mixtures

Monolayer-bilayer equilibria of SO/DPPC mixtures were estimated on the basis of the measurements of collapse and spreading pressures. The collapse pressure is considered as the transition surface pressure from monolayer at the water surface to bilayer, while spreading pressure is considered as the transition surface pressure from bilayer to monolayer (14) and has the same value as the collapse pressure. The collapse and spreading pressures of SO were consistent with each other (13 mN/m). The collapse and spreading pressures of PC also were consistent with each other (47.0 mN/m), and the values agree with the reported collapse pressure of about 45.0 mN/m (14). The collapse and spreading pressures of a lipid mixture generally have different values and are dependent on miscibility of the lipids in the monolayer and bulk phase (15).

The collapse and spreading pressures of the SO/DPPC mixture were obtained as a function of X_{SO} and therefore gave a phase diagram for the monolayer (M)–DPPC bilayer (B)–SO solids (S) equilibrium (Fig. 6). The collapse pressure varied with X_{SO} in the mixed monolayer, while the spreading pressure was constant at 45.0 mN/m in the X_{SO} range of 0.05–0.95. On the basis of the surface phase rule (16), it was found that SO and DPPC were freely miscible in a mixed monolayer at an air/water interface (M), but only partially miscible in the bulk phases, that was, DPPC bilayers (B) and SO solids (S). The solubility of SO solid (S) in DPPC was evaluated from the inflectional point of spreading pressure *f* as the SO mole fraction of approximately 0.05. The solubility

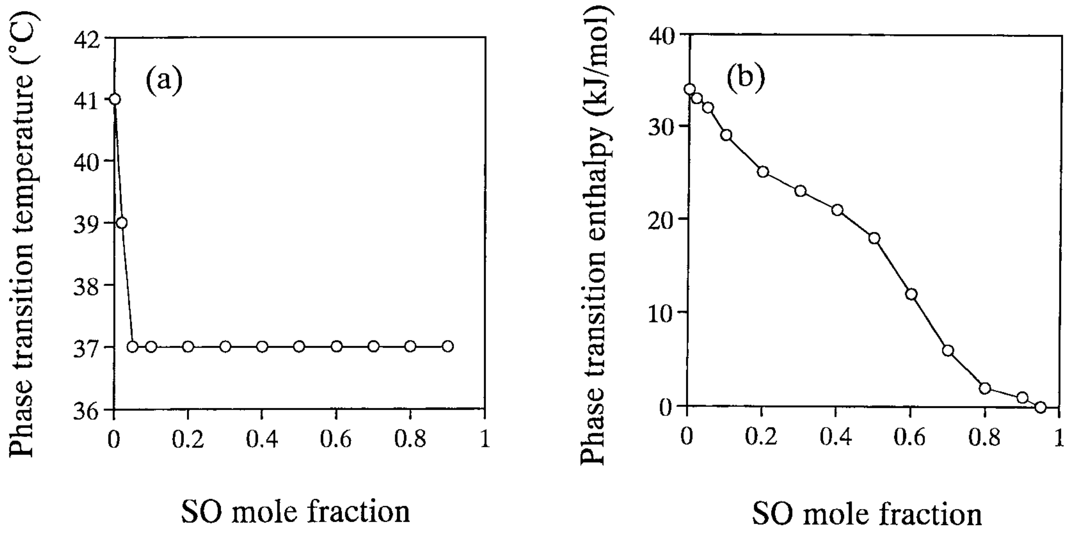

Figure 5. (a) Phase transition temperature represented as a function of mole fraction of SO (X_{SO}) in the mixture determined by differential scanning calorimetry (DSC); (b) phase transition enthalpy represented as a function of mole fraction of SO (X_{SO}) in the mixture determined by differential scanning calorimetry (DSC).

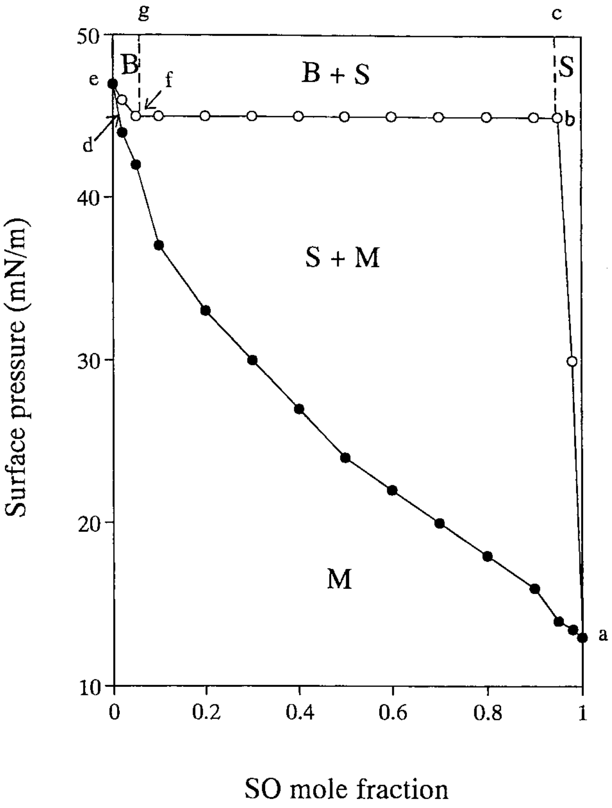

Figure 6. Monolayer-bilayer equilibria of SO/DPPC mixture in the presence of water. Spreading pressure, ○; collapse pressure, ●. The spreading pressure of the mixture is represented by the line *abfe*. The collapse pressure of the mixture is represented by the line *adg*. The stable dispersion containing an excessive amount of SO is represented by the line *bfd* (surface pressure = 45.0 mN/m), where the emulsion particles (combination of monolayer *d* and SO core *b*) coexist with the bilayer *f*. Without the bilayer, the monolayer has a lower surface pressure and a decreased effect on the stabilization of the emulsion.

of DPPC in the SO solid (S) was evaluated from the inflection point for the spreading pressure *b* as the DPPC mole fraction of approximately 0.05. These findings agree with the limited solubility of SO in DPPC bilayer membranes and of DPPC in SO determined by DSC. On the phase diagram in Fig. 6, a mixed monolayer exists in the region designated by M. Coexisting in the regions designated by S + M and B + S are SO solid and mixed monolayer and DPPC bilayers and SO solids, respectively. On the horizontal line *bf* at surface pressure of 45.0 mN/m, the system consists of DPPC bilayers *f*, which contains a limited amount (5%) of SO, and the SO solid phase *b*, which contains about 5% DPPC. The mixed monolayer *d*, which contains approximately 100% DPPC and has a surface pressure of 45.0 mN/m, is in equilibrium both with the bilayers *f* and the solid phase *b*. When the monolayer is formed on the surface of the SO-rich solid phase *b*, the hydrophobic solid can be stably dispersed in water and coexists with the bilayers *f* (liposomal vesicles).

DISCUSSION

DPPC and a Neutral Lipid, Soybean Oil

Soybean oil can be classified as a neutral lipid, and it forms monolayers with and without phospholipid. Neutral lipids have limited solubility in phospholipid bilayer membranes (17,18) and form separate phases in aqueous media, which are stabilized by the closely packed phospholipid monolayer surrounding the phases (15,19). This kind of equilibrium has been observed in the dispersions composed of phosphatidylcholine and ubiquinone-10 (14) or triglyceride (17,20). The monolayer-bilayer equilibria thus play important roles in the structural formations of phospholipid–neutral lipid mixtures in aqueous dispersions. Excess neutral lipid that separates from the PC bilayer membranes can be stably dispersed as small particles.

Structural Changes in the Dispersed Particles

It is presented that the alterations in structure of the dispersed particles from the vesicular structure occur on the basis of the trapped volume and fluorescence quenching measurements. An increase in X_{SO} of the dispersed particles leads to a reduction in the fraction of DPPC that participates in the formation of the liposomal bilayers, and it is suggested that the DPPC monolayers take part in the formation and stabilization of dispersed particles in water. Handa et al. (14) reported that the fraction of DPPC that forms bilayer vesicles ξ_1 may be calculated from the trapped volume v as follows:

$$\xi = (v/v_0) \qquad (1)$$

Here, v_0 is the trapped volume of small unilamellar vesicles ($v_0 = 0.44$ liter $\cdot$ mole^{-1}; see Table 1). The ξ_1 values calculated are presented in Table 1. The increased values of v in the range of $X_{SO} = 0.1$–0.4 are probably due to the increased size of the dispersed particles as a result of the increased X_{SO}.

The fraction ξ_1 is also calculated on the basis of the fluorescence quenching measurements (Fig. 4). The ξ_1 value is correlated with the ratio of external to total (ex-

Table 1

Fraction of DPPC Participating in the Formation of Vesicle Bilayers (ξ_1)

SO Mole Fraction (X_{SO})	Trapped Volume v (liter · mole^{-1} of PC)	ξ_1[a]	Ratio of Outer to Total Membrane p Determined by Fluorescence Quenching	ξ_1[b]
0	0.44	1.0	0.58	1.0
0.1	0.55	—	0.60	0.94
0.2	0.59	—	0.61	0.90
0.3	0.63	—	0.63	0.85
0.4	0.57	—	0.64	0.78
0.5	0.36	0.82	0.65	0.71
0.6	0.25	0.57	0.67	0.61
0.7	0.19	0.43	0.68	0.48
0.8	0.12	0.27	0.70	0.23

[a] Calculated by Eq. 1.
[b] Calculated by Eq. 2.

ternal plus internal) membrane p in SO/DPPC dispersed particles (14).

$$\xi_1 = [1/(1 - p_0)] \cdot [(1 - p) - s \cdot X_{SO}/(1 - X_{SO})] \qquad (2)$$

Here, p_0 is the ratio for the liposomal vesicles of DPPC and is 0.58; s is the solubility of DPPC in the separate solid phase of SO, equivalent to a mole fraction of 0.05 as determined by DSC (Fig. 5) and spreading pressures (Fig. 6). In Eq. 2, $(1 - p)$ is the fraction of DPPC that is inaccessible to the Cu^{2+} added to the outer aqueous phase of the dispersion, and $s \cdot X_{SO}/(1 - X_{SO})$ is the fraction of DPPC solubilized in the separate SO phase.

As seen in Table 1, Eq. 2 gives ξ_1 values that are close to the values evaluated by the trapped volume method. A large percentage of DPPC molecules are found in structural formations other than bilayer vesicles, and the SO separated from bilayers is stabilized by the DPPC monolayer as emulsion particles in aqueous media.

Stability of Dispersion and Lipid Composition

When the DPPC content is less than the solubility in SO (DPPC mole fraction less than about 0.05; see Figs. 5 and 6), the DPPC monolayer does not completely cover the hydrophobic SO particle surfaces. When $X_{SO} = 0.9$ or 0.95, the separation into oil/water phases was observed after preparation, and the dispersions were not stable. However, when the mole fraction of DPPC was higher (i.e., $X_{SO} = 0$–0.8), the PC monolayer covers the SO particles completely and stabilizes the dispersion. When DPPC is excessive, the monolayer is in equilibrium with the DPPC bilayers (liposomes), and the particle surface has the maximum value: the spreading pressure of the bilayers. Therefore, the coexistence of emulsion and liposomal particles is critically important for the stabilization of the particles in water.

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