Eutectic mixed monolayers in equilibrium with phospholipid-bilayers and triolein-liquid phase

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ABSTRACT Triolein (TO) and phospholipids (egg yolk phosphatidylcholine, egg yolk phosphatidylethanolamine, and bovine brain phosphatidylserine) had low mutual solubilities and separated into the TO-liquid phase and phospholipid-bilayers. Spreading pressures of the TO-phospholipid mixture (i.e., surface pressures of the mixed monolayer in equilibrium with the phase-separating lipid mixture) at the air/saline interface were independent of the lipid composition. On the other hand, collapse pressures of the mixed monolayer of TO and phospholipid (i.e., surface pressures of the mixed monolayer in equilibrium with the TO-liquid phase) at the interface changed with the monolayer composition and were lower than the spreading pressure. The experimental data indicated the spreading and collapse pressures as offering a phase diagram for the presence of equilibrium between the mixed monolayer, the phospholipid-bilayers and the TO-liquid phase. The diagram showed that TO and the phospholipids were miscible in the mixed monolayer, forming an eutectic mixed monolayer. When the mixed monolayer initially had the eutectic composition, no collapse of the monolayer was detected until the surface pressure reached the value of the spreading pressure. No specific complex between TO and the phospholipid is required to explain the stability and collapse of the mixed monolayers. The bulk immiscibility of the lipids elucidated by the spreading pressure-measurements, immediately leads to the phase behaviors observed.

INTRODUCTION

Some neutral lipids, such as triglycerides (TG) and cholesteryl esters have very low solubility in phospholipid bilayers (1, 2). The excess amount of the neutral lipid separates from the bilayers and forms droplets in an aqueous medium. The droplets are covered with phospholipid monolayers supplied from the bilayers (spreading monolayers), and stabilized as emulsion particles; in other words, the emulsion particles (neutral lipid cores plus surface monolayers of phospholipid) are in equilibrium with the phospholipid bilayers (3, 4). Examples of these emulsions are arterial lipid deposits, intracellular lipid storage droplets and plasma lipoproteins. In plasma, TG-rich lipoproteins, chylomicrons and very low density lipoproteins, attach to capillary endothelium, where lipoprotein lipases promote hydrolysis of TG (lipolysis). The solubility of TG in the emulsion monolayers is an important factor in rate-determination of the digestion (5).

On the basis of phase-behavior investigation of the TG-phosphatidylcholine (PC) emulsions (mixtures of emulsion particles and bilayer vesicles), Miller and Small have shown that the weight fraction of TG in the surface monolayers (3.7%) is independent of the TG content in the emulsion (6, 7). Similar solubilities of TG in the surface monolayers have been observed in chylomicrons and very low density lipoproteins (8). We have shown that TG and PC are freely miscible in the monolayers at the TG/saline interface, but the limited solubility due to the coexistence of the emulsion particles and bilayer vesicles (i.e., the eutectic composition of TG at zero degrees of thermodynamic freedom) (4). On the other hand, Smaby and Brockman have suggested the formation of a preferential packing array or complex of TG and PC (4/96 in mole ratio) in the mixed monolayer at the air/saline interface (9, 10).

In this work, we measured both collapse pressures of the mixed monolayer and spreading pressures of the bulk mixture of triolein (TO) and phospholipid (egg yolk phosphatidylcholine, PC, egg yolk phosphatidylethanolamine, PE, or bovine brain phosphatidylserine, PS) at the air/saline interface. The miscibilities of lipids in both monolayer and bulk phase (bilayers) were estimated, and the monolayer-bilayer equilibrium and the monolayer stability were evaluated.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei Co. (Osaka). The purity (over 99.5%) was determined by thin-layer chromatography. The small amount of impurity (0.5%) was identified as sphingomyelin by HPLC. Egg yolk phosphatidylethanolamine (PE) and bovine brain phosphatidylserine (PS) were purchased from Sigma Chemical Co. (St. Louis, MO). The purities of both PE and PS were over 97% as checked by thin-layer chromatography. Triolein (TO) obtained from Taiyo Chemical Co. (Kyoto) was purified by silicate (Wakogel C-200; Wako Pure Chemicals, Osaka) column chromatography to remove fatty acids, diglycerides, and monoglycerides by using chloroform/methanol (99/1) as an eluent. The purity of TO thus obtained was over 99%. Water was doubly distilled with a quartz still.

Measurements

Triolein, phospholipids (PC, PE, and PS) and mixtures of TO and phospholipid were dissolved in benzene. The solution was supplied from an Agla micrometer syringe on saline (10 mM Tris-HCl/150 mM NaCl, pH 7.0) in a Teflon-coated duralumin trough. The monolayer spread was left for 5 min for evaporating the solvent. The area per molecule of insoluble lipid or the average area per molecule of mixed lipid in the monolayer, A (10^{-2} nm^2 or Å^2) was calculated as $A = S/(nN_A)$, where S was surface area of the saline between two movable barriers, n was the moles of insoluble lipids spread on the surface, and

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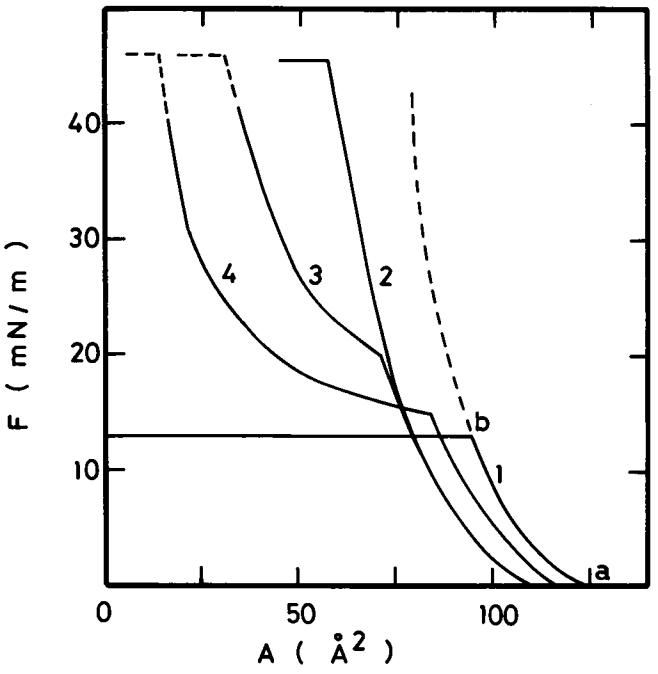

FIGURE 1 Surface pressure (F) – average area per molecule (A) curves of PC-TO mixed monolayer at the air/saline interface. Curves *1:* TO; *2:* PC; *3:* PC/TO = 1/1; *4:* PC/TO = 1/3 in mole ratio. The upper parts of the curves *3* and *4* (*broken lines*) were evaluated by referring to the experimental values of spreading pressure.

N_A was the Avogadro number. The surface pressure, F (mN/m), was measured by Whihelmy's plate method. The torsion balance used was Shimadzu T-NR. Temperature was kept constant at 25°C by circulating the thermostated water through glass tubing immersed in the trough. A ground plate of quartz was used after cleaning it in fuming nitric acid for more than one night.

Lipids or lipid mixtures were dissolved in benzene. After the solvent evaporation, lipids or lipid mixtures were dried in vacuo for 15 h. Spreading pressures of lipid or lipid mixture at the air/saline interface were obtained from the steady value of surface pressure after the addition of lipid or lipid mixture onto the interface. Phospholipids used in this study were hydrated within one minute at 25°C. The spreading of monolayer from the liquid triolein was completed within a few seconds. The details of the monolayer techniques have been described elsewhere (11, 12).

RESULTS

Collapse and spreading of lipid monolayer

Surface pressure (F)-area per molecule (A) curves (F-A curves) of TO and PC are shown in Fig. 1. The surface pressure of the TO monolayer became stationary (F = 13.0 mN/m) in the area per molecule-region below 95 Å^2, indicating a phase transition of the monolayer. The surface pressure at the monolayer transition point was identical with the spreading pressure of TO. The spreading pressure of phospholipid was close to the surface pressure at the inflectional point as observed in the F-A curve. The spreading pressure has been defined as the surface pressure of the monolayer in equilibrium with the bulk lipid-phase (liquid and bilayer phases for TO and phospholipid, respectively) (13, 14). The inflectional points of the TO and PC monolayers (lines *1* and *2* in Fig. 1), therefore, represent the monolayer-collapses to the bulk liquid of TO and the bilayers of PC, respectively (12, 13).

Phospholipids and TO have very low mutual solubilities: Solubilities of PC, PS, and PE in TO are 8×10^{-4}, 1.5×10^{-3} and $\sim 1 \times 10^{-2}$ mol/liter, respectively, at 25°C (3). TO is solubilized in the PC bilayers up to the weight fraction of 0.037 (6). The bulk mixture of TO and phospholipid (PC, PE, or PS), thus, consisted of the TO liquid and the phospholipid bilayers. Fig. 2 shows the spreading pressure of the TO-phospholipid mixture as a function of the phospholipid-mole fraction (*closed circles*). The spreading pressure of every phase-separating mixture was independent of the mole fraction because of zero degree of freedom for the mixture (13–15).

When two lipid components are immiscible in the binary bulk phase but miscible in the mixed monolayer, the monolayer shows a two-step collapse. The lower collapse pressure depends upon the monolayer composition, while the higher collapse pressure is independent of the composition and has the same value as the spreading pressure of the binary mixture (14, 15). At the lower collapse point, one component (TO in these cases) starts to separate from the mixed monolayer and forms a bulk phase (TO liquid). At the higher collapse point, another component (PC, PE, or PS) is segregated from the monolayer to form a different bulk phase (bilayers). Further compression of the mixed monolayer leads to the co-collapse of the two lipids, and the surface pressure and composition of the monolayer are constant (formation of an eutectic monolayer). The remained monolayer is in equilibrium with the two bulk phases (an eutectic monolayer in equilibrium with TO liquid and phospholipid bilayers). When the mixed monolayer initially has an eutectic composition, the lower and higher collapse points merge to give a single collapse point. The surface pressure at this point is equal to the spreading pressure (14, 15).

The collapse pressure of the mixed monolayer of TO and phospholipid (Fig. 1 and *open circles* in Fig. 2) changed with the monolayer composition, and was assumed to be the lower one. Higher collapse points were experimentally not obtained. At the surface pressure above 40 mN/m (the surface tension of the saline below 32 mN/m), the mixed monolayer often leaked through the edges of the trough and the movable barrier. The relation of the spreading and collapse pressures with the lipid composition (Fig. 2) indicated the eutectic compositions of PC-TO to be ~0.95, and of PE-TO and PS-TO to be close to unity in the phospholipid mole fraction. The phase diagram for the TO-PC mixture in Fig. 2 was similar to that obtained on the basis of the collapse pressure measurements (9). The experimental results of

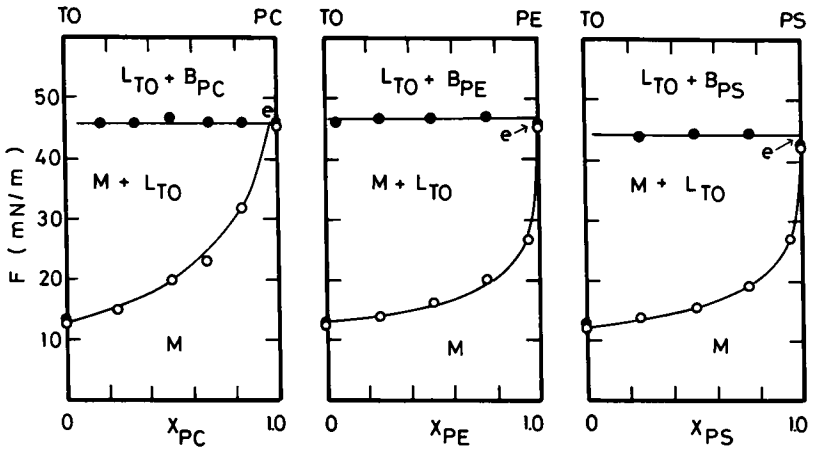

FIGURE 2 Surface pressure (F)-mole fraction (X) diagrams (phase diagrams) for TO-phospholipid mixtures. *Closed circles:* spreading pressure; *open circles:* collapse pressure. The solid lines in the diagrams were calculated by Eq. 2. L_{TO}: liquid phase of triolein; B_{PC}, B_{PE}, and B_{PS}: bilayers of PC, PE, and PS, respectively; *M:* mixed monolayer of TO and phospholipid. Points *e:* eutectic points.

spreading pressure showed the immiscibility of TO and PC in the bulk phases. The stability and collapse of mixed monolayer are influenced by the miscibility of lipids in the bulk phases (13, 14).

Average area per molecule of mixed monolayer

The average area per molecule of mixed monolayer at F = 10 mN/m is represented as a function of the mole fraction of lipid in Fig. 3. Both TO and phospholipids remained in the mixed monolayer below the collapse pressure of TO (13 mN/m). The average area of the PC-TO mixed monolayer showed a negative deviation from the ideal straight line, while small or negligible deviations were seen in the PE-TO and PS-TO mixed monolayers. These results suggested the presence of (net) attractive lateral interactions between PC and TO in the mixed monolayer, while those interactions between TO and PE or PS were small or negligible.

The average area per molecule (A) at 40 mN/m is represented in Fig. 4 as a function of the initial mole fraction of monolayer (X). At this surface pressure, the actual mole fraction of lipid in mixed monolayer were very close to the eutectic compositions (see footnotes in Table 1). The A-X relations at 40 mN/m were distinct from those at 10 mN/m in Fig. 3. The correlation give an area of 0 Å^2 at the TO mole fraction of unity. When the surface pressure of the mixed monolayer reached the value of the spreading pressure, the mixed monolayer was in equilibrium with both phospholipid bilayers and TO liquid and the composition of the monolayer was fixed at the eutectic value. The results in Fig. 4 suggest that the TO mole fractions in the eutectic monolayers were close to zero and almost all of TO was separated from the mixed monolayers just before the surface pressures attained to the eutectic values (i.e. spreading pressure: 45.8 mN/m for PC-TO, 47.0 mN/m for PE-TO, and 44.5 mN/m for PS-TO; see Fig. 2).

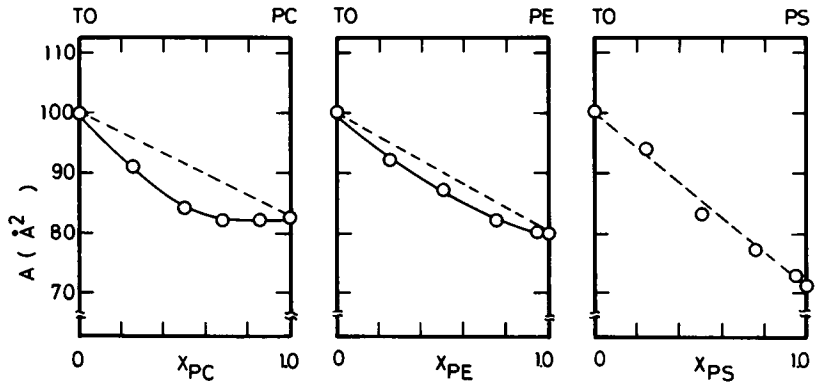

FIGURE 3 Average area per molecule of mixed monolayer (A) as a function of the mole fraction (X). Surface pressure is 10 mN/m. The broken lines were obtained by assuming the ideal mixing in the mixed monolayers.

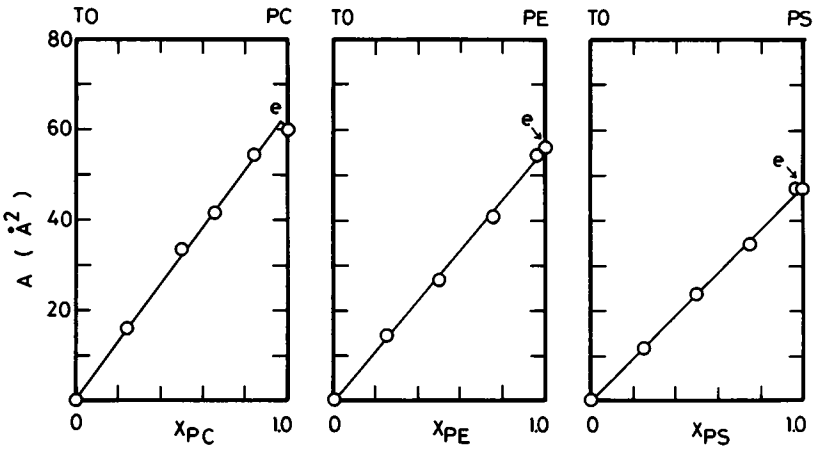

FIGURE 4 Average area per molecule of mixed monolayer (A) as a function of the mole fraction (X). Surface pressure is 40 mN/m, and the actual monolayer compositions are close to the eutectic ones. Points *e*: eutectic compositions. The slopes of the solid lines give the values of $(1/X^e)A^e$ (see Eqs. 4–5).

DISCUSSION

Equilibrium between mixed monolayer and TO liquid phase

When a mixed monolayer of TO and phospholipid was compressed to the (lower) collapse point, TO separated from the monolayer and a liquid phase was formed. The TO liquid phase contained very small fractions of phospholipid (less than 0.08 mol%, 3), and the chemical potential of TO, μ_{TO} was virtually identical with that of pure TO. When the mole fraction of TO in the mixed monolayer decreased, the equilibrium between the monolayer and the TO liquid was maintained by the increase in the surface pressure of the monolayer (11, 13–16):

$$d\mu_{TO} = A_{TO}dF + kT\,d\ln(f_{TO}x_{TO}) = 0 \qquad (1)$$

and

$$\int_{F_{TO}}^{F} A_{TO}\,dF = -kT\ln(f_{TO}x_{TO}). \qquad (2)$$

Here, F_{TO} is the collapse (or spreading) pressure of pure TO, and was 13.0 mN/m. A_{TO} is the area per molecule of TO at the corresponding F value, and was estimated on the extension of the line *ab* in Fig. 1. f_{TO} and x_{TO} are the activity coefficient and mole fraction of TO in the mixed monolayer, respectively. The mole fraction and moles of TO (n_{TO}) and PC (n_{PC}) in the mixed monolayer are correlated as $x_{TO} = [n_{TO}/(n_{TO} + n_{PC})]$. k is the Boltzmann constant. The collapse pressure calculated by Eq. 2 is shown as a function of the monolayer composition in Fig. 2 (*solid curves*). The activity coefficient of TO, f_{TO}, was assumed to be equal to unity (ideal mixing) for the PE-TO and PS-TO mixtures. In the PC-TO mixed monolayers, the Bragg-Williams equation was applied for the lateral interactions as

$$\ln f_{TO} = \omega(1 - x_{TO})^2 \qquad (3)$$

The ω value of −4.0 led to the best fit between the observed (13–30 mN/m) and calculated collapse pressures. The errors in the extrapolation of A_{TO} to the higher surface pressure (±5 Å²) brought about the uncertainty of ±0.2 in the ω value. The results showed that the interactions between PC and TO in the mixed monolayer were considerably attractive, and that PE and PS ideally mixed with TO as already indicated in Fig. 3. The eutectic compositions calculated by Eq. 2 with the spreading pressures are shown in Table 1. The theoretical values agreed with the experimental data (see Table 1 and Fig. 2).

Average area per molecule of eutectic monolayer

The average area per molecule of the eutectic monolayer (i.e., the mixed monolayer with surface pressure identical with the spreading pressure), A^e, is

TABLE 1 Mole fractions of phospholipid (X^e), average areas per molecule (A^e), and surface pressures (F^e) at the eutectic point, and lateral interaction parameters (ω) in the mixed monolayer

		Air/saline-interface		
	X^e *	A^e ‡	F^e	ω §
		A^2	*mN/m*	
PC-TO	0.960	61.0	45.8	−4.0
PE-TO	1.00	56.0	47.0	0
PS-TO	1.00	49.0	44.5	0

* Calculated by Eqs. 2 and 3. $X^e = 1 - X^e_{TO}$.
‡ Calculated by Eq. 5 at F = 40 mN/m. At this surface pressure, the mole fraction of phospholipid calculated in the mixed monolayers was very close to the eutectic composition (X^e).
§ The lateral interaction energy = ωkT. Uncertainty in ω is ±0.2 (see text).

$$A^e = X^e A^e_{PL} + (1 - X^e) A^e_{TO} \quad (4)$$

Here, X^e, A^e_{PL} and A^e_{TO} are the mole fraction of phospholipid (PL), the partial molecular areas of PL and TO at the eutectic point, respectively. At the higher collapse point (the eutectic surface pressure), part of TO in excess of the eutectic composition was segregated from the mixed monolayer, but PL remained in the monolayer. The observed average area per molecule, A, was, therefore,

$$A = (A^e/X^e)x^i \quad (X^i < X^e). \quad (5)$$

Here, x^i is the initial mole fraction of PL. When x^i approaches zero (TO monolayer) or X^e, A approaches zero or A^e, respectively. Eq. 5 was applied in Fig. 4 and the A^e values were calculated by the use of the theoretical values of X^e (Table 1).

Eutectic mixture or complex formation in monolayer

Collapse of a mixed monolayer depends not only upon the lipid-lipid interactions and miscibility in the monolayer but also upon those in the bulk lipid phases separated out (11–15). Diglycerides and α-tocopherol have large solubilities in the PC bilayers and the mixtures with PC form nonbilayer-phases besides the bilayers (17, 18). Triglycerides and phospholipids have limited mutual solubilities in the bulk phases (TG liquid and phospholipid bilayers). When the mixed monolayer of TO and PC was in equilibrium with the TO and PC bulk phases, the composition and surface pressure were fixed at the eutectic values.

On the basis of the collapse pressure measurements, Smaby and Brockman claim the formation of a preferential packing or complex in the mixed monolayer of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and TO at the air/saline interface. The TO/POPC ratio in the complex is 4/96 (9). They use Fowkes-Gaines' equation for surface osmotic pressure (9, 19, 20), and attempt to correlate the number of associated water molecules of TO and the collapse pressure. However, lipids with different number of the associated water molecules often give similar collapse pressures. The discrepancy has been adjusted by employing another parameter, the activity coefficient of water at the interface (9).

At the collapse point of the TO-PC mixed monolayer, the chemical potentials of TO and water are maintained constant in the mixed monolayers because of the equilibria with the respective bulk phases. The surface activity of water, however, changes with the value of the collapse pressure (i.e., surface osmotic pressure). It is difficult to obtain the activity coefficient of water in the monolayer without special assumptions for the molecular area, association, complex-formation and free state of water. On the other hand, the mole fraction (X_{TO}) and the molecular area (A_{TO}) of TO were experimentally determined. We, therefore, examined the equilibrium of TO represented by Eq. 1.

NMR studies on the PC-TO mixed bilayers have shown that the TO molecule has narrow resonances (indicative of rapid motions), and chemical shifts (indicative of hydrogen bonding with H_2O but not with PC) (1). While attractive interactions between PC and TO obtained in this work were strong ($\omega kT = -4kT$), no specific complex between the TO and PC molecules was required to explain the monolayer-bilayer equilibrium of the mixture. The bulk immiscibility of these lipids immediately led to the phase behaviors of the mixture observed.

The lateral interaction parameter and the eutectic composition at the air/saline interface were compared with those at the TO/saline (3) interface. It is known that the interactions between TO and PC at the TO/saline interface are repulsive (3), in contrast to the attractive ones at the air/saline interface. Therefore, the interactions and collapse behaviors of the TO-PC mixed monolayer at the air/saline interface can be considered as not directly correlated with those at the TO/saline interface. On the other hand, the spreading pressures at the air/saline interface offered information on the interactions and miscibility of TO and PC in the bulk phases. The bulk-behavior of the PC-TO mixture was closely correlated with the stability and phase-behavior of the mixed monolayer at both TO/saline and air/saline interfaces.

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REFERENCES

1. Hamilton, J. A. 1989. Interactions of triglycerides with phospholipids: incorporation into the bilayer structure and formation of emulsions. *Biochemistry.* 28:2514–2520.
2. Hamilton, J. A., D. T. Fujito, and C. F. Hammer. 1991. Solubilization and localization of weakly polar lipids in unsonicated egg yolk phosphatidylcholine: a ^{13}C MAS NMR study. *Biochemistry.* 30:2894–2902.
3. Handa, T., H. Saito, and K. Miyajima. 1990. Phospholipid monolayers at the triolein-saline interface: production of microemulsion particles and conversion of monolayers to bilayers. *Biochemistry.* 29:2884–2890.
4. Handa, T., Y. Asai, K. Miyajima, Y. Kawashima, M. Kayano, K. Ida, and T. Ikeuchi. 1991. Formation and structure of stably dispersed small particles composed of phosphatidylcholine and ubiquinone-10: coexistence of emulsion particles with bilayer vesicles. *J. Colloid Interface Sci.* 143:205–213.
5. Deckelbaum, R. J., J. A. Hamilton, A. Moser, G. B-Olvecrona, E. Butbul, Y. A. Carpentier, A. Gutman, and T. Olivecrona. 1990. Medium-chain versus long-chain triglycerol emulsion hydrolysis by lipoprotein lipase and hepatic lipase: implications for the mechanisms of lipase action. *Biochemistry.* 29:1136–1142.
6. Miller, K. W., and D. M. Small. 1982. The phase behavior of triolein, cholesterol, and lecithin emulsions. *J. Colloid Interface Sci.* 89:466–478.
7. Miller, K. W., and D. M. Small. 1983. Triolein-cholesteryloleate-

cholesterol-lecithin emulsions: structural models of triglyceride-rich lipoproteins. *Biochemistry.* 22:443–451.

8. Miller, K. W., and D. M. Small. 1983. Surface-to-core and interparticle equilibrium distributions of triglyceride-rich lipoprotein lipids. *J. Biol. Chem.* 258:13772–13784.
9. Smaby, J. M., and H. L. Brockman. 1985. Miscibility, chain packing, and hydration of 1-palmitoyl-2-oleoyl phosphatidylcholine and other lipids in surface phases. *Biophys. J.* 48:701–708.
10. Smaby, J. M., and H. L. Brockman. 1987. Regulation of cholesteryloleate and triolein miscibility in monolayers and bilayers. *J. Biol. Chem.* 262:8206–8212.
11. Nakagaki, M., K. Tomita, and T. Handa. 1985. Interactions of differently oriented lipids in monolayer: mixed monolayers of 16-(9-anthroyloxy)palmitic acid with phosphatidylcholine and cholesterol. *Biochemistry.* 24:4619–4624.
12. Handa, T., C. Ichihashi, and M. Nakagaki. 1985. Polymorphic phase transition and monomolecular spreading of synthetic phospholipids. *Prog. Colloid Polymer Sci.* 71:26–31.
13. Nakagaki, M., and N. Funasaki. 1974. The formation and collapse of mixed monolayer of triolein and tricaprylin. *Bull. Chem. Soc. Jpn.* 47:2482–2485.
14. Nakagaki, M., and T. Handa. 1976. The two-step collapse of mixed monolayers of cholesterol and its esters. *Bull. Chem. Soc. Jpn.* 49:880–885.
15. Handa, T., and M. Nakagaki. 1979. Miscibility of phospholipid and cholestrylacetate in mixed monolayer on aqueous surfaces. *Colloid Polymer Sci.* 257:374–381.
16. Defay, R., I. Prigogine, A. Bellemans, and D. H. Everett. 1966. Surface tension and adsorption. Longmans, Green & Co., London. 158–181.
17. Das, S., and R. P. Rand. 1986. Modification by diacylglycerol of the structure and interaction of various phospholipid bilayer membranes. *Biochemistry.* 25:2882–2889.
18. Nakajima, K., H. Utsumi, M. Kazama, and A. Hamada. 1990. α-tocopherol-induced hexagonal H_2 phase formation in egg yolk phosphatidylcholine membranes. *Chem. Pharm. Bull.* 38:1–4.
19. Gaines, G. L., Jr. 1978. The thermodynamic equation of state for insoluble monolayers. 1. Uncharged films. *J. Chem. Phys.* 69:924–930.
20. Fowkes, F. M. 1962. Ideal two-dimensional solutions 11. A new isotherms for soluble and gaseous monolayers. *J. Phys. Chem.* 66:385–389.