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KINETIC ASPECTS AND MECHANISM OF LIPOSOME DISINTEGRATION IN POLYOXYETHYLENE LAURYL ETHER AND SODIUM CHOLATE SOLUTIONS

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The disintegration behaviour of liposomes in polyoxyethylene lauryl ether (PLE) and sodium cholate solutions was studied by the turbidity disappearance method. In maximally solubilized systems of liposomes, the molar ratios (phosphatidylcholine/surfactant) were 0.43 and 1.8 for PLE and sodium cholate, respectively. The disintegration process of either unilamellar or multilamellar liposomes followed first-order kinetics. Based on a physical model in which liposomes heterogeneous in size were assumed to disintegrate from the outermost shell one by one, a mathematical expression of the turbidity disappearance rate was introduced and applied to explain the data thus obtained. Model calculations suggested that the number of disintegrated shells would not be so large, even if up to 50% reduction of the initial turbidity was observed. From the dependence of the pseudo-first-order rate constant (k_{obs}) on the surfactant concentration for unilamellar liposomes, it was assumed in general that k_{obs} consists of the contributions of the monomer and micellar fractions: for PLE, both fractions shared in the disintegration, but only the micellar fraction with sodium cholate. Furthermore, in the latter case, k_{obs} depended on the initial liposome concentration. These results are likely to be consistent with the proposed modes of surfactant action classified as type A and type B (Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79).

Introduction

The oral administration of therapeutic agents such as insulin, antitumor drugs and antihemophilia factors has been attempted in the form of liposomal preparations, since these agents would be unstable or little absorbed in the gastrointestinal tract [1–3]. However, for the physical integrity of liposomes, the environment of the gastrointestinal tract is likely to be very severe, since they certainly encounter drastic change of pH, digestive enzymes and bile salts which may destroy the bilayer structure of liposomes [4–6]. Thus, the extent to which liposomes retain their structural integrity under such unfavourable conditions would be a key factor in the design of a reasonable liposome preparation for entrapping any agents. Phospholipid-surfactant mixed micelle systems have also been investigated with a view to the extraction of membrane-fixed proteins and lipids [7,8].

Little attention, however, has been given in detail to the kinetic aspects of liposome disintegration in the frequent use of liposomes as model membranes and when they are envisaged as micro-carriers for biologically active substances. This may be due to their complex structure and

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Abbreviation: PLE, polyoxyethylene lauryl ether.

the difficulties of handling a heterogeneous system.

The present study is concerned with the disintegration kinetics of liposomes in the solution of surfactant, polyoxyethylene lauryl ether (PLE) and sodium cholate. The kinetics data are discussed on the basis of a physical model of the liposome disintegration processes. The difference between the modes of action of PLE and sodium cholate for liposome disintegration is also described.

Materials and Methods

Phosphatidylcholine was extracted from egg yolk and purified by column chromatography on silicic acid (Mallinckrodt, St. Louis, MO) [10]. Polyoxyethylene lauryl ether (PLE) was purified from commercially available Brij 35 (Katayama Chem. Co., Osaka) by the method described elsewhere [11] and subsequently recrystallized from acetone to remove remaining butanol. Sodium cholate was obtained from Tokyo Kaseikogyo Co., Tokyo, and recrystallized from diluted acetic acid as cholic acid.

Phosphatidylcholine was dispersed in Tris buffer (pH 7.4, $I = 0.1$) as described previously [12]. In short, multilamellar liposomes were first prepared by shaking on a vortex mixer for 20 min. The turbid suspension was then sonicated as follows: liposomes were divided into four grades by sonication for periods of 30, 60 or 120 min by a bath-type sonifier (Model B-12, Branson), or 60 min by a probe-type sonifier (Model UR 200P, 20 KHz, Tomy Seiko Co.). Titanium fragments from the probe were removed by centrifugation at $10\,000 \times g$ for 10 min. The suspensions were allowed to stand overnight for equilibration at room temperature. The turbidities of the resulting suspensions were as follows: 1.99 (30 min, bath-type) and 1.79 (60 min), 1.00 (120 min) and 0.32 (60 min, probe-type) (hereafter called unilamellar liposomes) at 660 nm at a phosphatidylcholine concentration of $3 \cdot 10^{-3}$ M throughout. The liposome concentration was then expressed in terms of the phosphorus concentration [13].

For solubilization studies, an appropriate aliquot of multilammelar liposome suspension subjected to bath-type sonication for 60 min was introduced in the surfactant solution prepared in Tris buffer, in the range $(0.5\text{–}2.0) \cdot 10^{-2}$ M (final concentration) for PLE and sodium cholate, and the mixed system was shaken in a thermostatically controlled water bath (25°C) for 2 days. The turbidity was then measured at 660 and 800 nm.

The disintegration kinetics of liposomes was followed by the turbidity change, which was monitored at 660 nm by a Hitachi 124 spectrophotometer and a stopped-flow instrument (RA-401, Union Giken, Osaka) equipped with a UV-VIS detector. An aliquot of the liposome suspension maintained at $3 \cdot 10^{-3}$ M phosphorus concentration was introduced in the PLE and sodium cholate solutions (Tris buffer, pH 7.4, $I = 0.1$). Temperature was 25°C. Slight increases in the initial turbidity caused by both PLE and sodium cholate were observed in the case of unilamellar liposomes. The point at which the turbidity returned to the original level was assumed to be $t = 0$ for kinetic measurements. Higher turbidities lasted for 200–400 ms.

Results

Solubilization of phosphatidylcholine by PLE and sodium cholate

Figs. 1 and 2 indicate the turbidity change of liposomal suspensions as a function of the phosphatidylcholine concentration in the PLE and sodium cholate solutions. Zero turbidity means that liposomal vesicles were completely solubilized, and the turbidity began increasing with increasing liposome concentration when the solubilizing capability of the surfactant was exceeded. The turbidity change was measured at different wavelengths, 660 and 800 nm, and little difference in the break points was found.

The respective concentrations of the phosphatidylcholine-surfactant system at that point were plotted, as shown in Fig. 3, in which the slope represents the molar ratio between phosphatidylcholine and surfactant in its maximally solubilized system. The molar ratios (phosphatidylcholine/surfactant) obtained were 0.43 and 1.8 for PLE and sodium cholate, respectively, which indicates that about 2 mol PLE and 0.5 mol sodium cholate were required to solubilize 1 mol phosphatidylcholine. The latter value is almost coincident with the result of Small et al. [14].

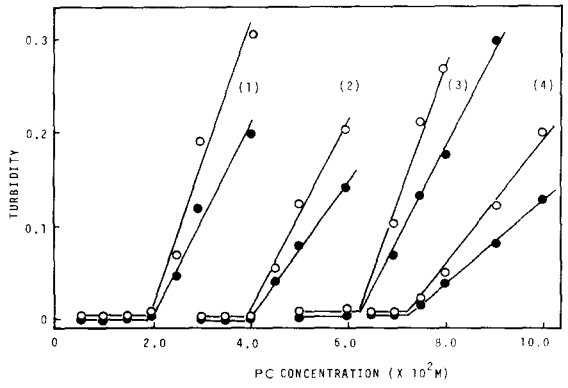


Fig. 1. Turbidity change of liposomes dispersed in solutions with varied PLE concentrations (pH 7.4 Tris buffer, 25°C). PLE concentrations: (1) $5 \cdot 10^{-3}$ M, (2) $1 \cdot 10^{-2}$ M, (3) $1.5 \cdot 10^{-2}$ M, and $2 \cdot 10^{-2}$ M. ○, absorbance at 660 nm; ●, absorbance at 800 nm. Liposomes used are multilamellar ones subjected to bath-type sonication for 60 min. PC, phosphatidylcholine.

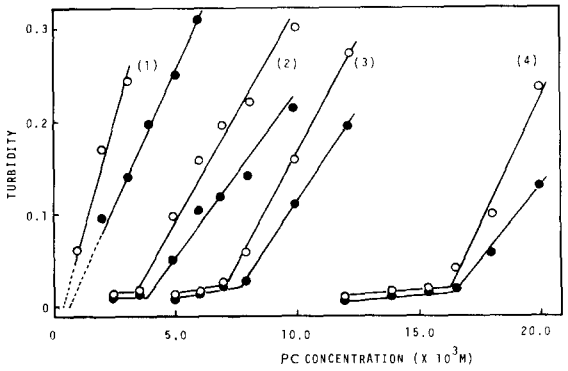


Fig. 2. Turbidity change of liposomes dispersed in solutions with varied sodium cholate concentrations (pH 7.4 Tris buffer, 25°C). Sodium cholate concentrations: (1) $6 \cdot 10^{-3}$ M, (2) $8 \cdot 10^{-3}$ M, (3) $1 \cdot 10^{-2}$ M, and $1.5 \cdot 10^{-2}$ M. ○, absorbance at 660 nm; ●, absorbance at 800 nm. Liposomes used are multilamellar ones subjected to bath-type sonication for 60 min. PC, phosphatidylcholine.

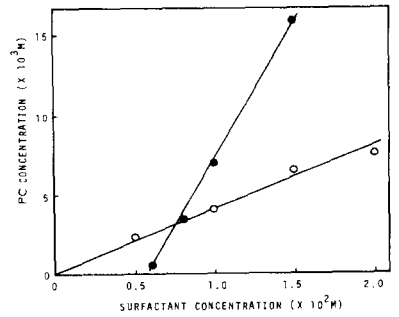


Fig. 3. Solubility change of liposomes at various PLE (○) and sodium cholate (●) concentrations.

Disintegration kinetics of liposomes in PLE solutions

The disintegration process of liposomes in the surfactant solution was followed by the decrease in turbidity for the different sizes of vesicle subjected to the different sonication periods. Fig. 4 shows that the disintegration process followed first-order kinetics with regard to the remaining turbidity. The larger the vesicle size, the slower the disintegration rate. The pseudo-first-order rate constant, k_{obs}, was obtained as a function of PLE concentration, as shown in Fig. 5.

The k_{obs} value generally increased with increasing surfactant concentration: the smaller the vesicle size, the more dependent on the surfactant concentration the k_{obs} value. However, as the liposome size decreased, the curve of k_{obs} vs. surfactant concentration did not pass through the origin when extrapolated. For unilamellar liposomes, the dependence of k_{obs} on surfactant concentration was unchanged among different initial liposome concentrations, unlike the sodium cholate case described next.

Disintegration kinetics of liposomes in sodium cholate solutions

Liposomes employed in this experiment were unilamellar. As in the PLE solution, the turbidity change in the sodium cholate solution followed first-order kinetics as well, enhanced with increasing surfactant concentration. The first-order rate constants are presented as a function of surfactant

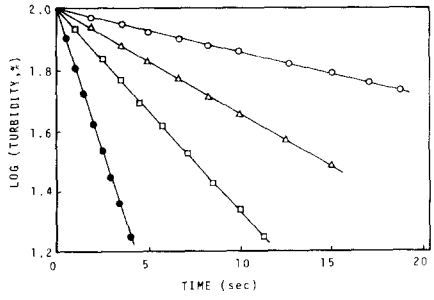


Fig. 4. First-order plots for the turbidity change of liposome disintegration in PLE solutions (25°C). Phosphatidylcholine (PC) concentration $3 \cdot 10^{-3}$ M; PLE concentration $2 \cdot 10^{-2}$ M. Unilamellar liposomes (●) and multilamellar liposomes sonicated for 30 min (○), 60 min (△) and 120 min (□). Turbidity change was measured at 660 nm.

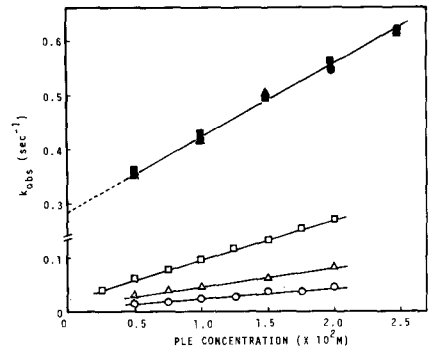


Fig. 5. Effect of PLE concentration on the apparent first-order disintegration rate constant, k_{obs}, for various liposomes (25°C). Open symbols: multilamellar liposomes (phosphatidylcholine concentration, $3 \cdot 10^{-3}$ M) sonicated for 30 min (○), 60 min (△) and 120 min (□). Closed symbols: unilamellar liposomes (probe-type sonication for 60 min; phosphatidylcholine concentration $1.5 \cdot 10^{-3}$ M (■), $3 \cdot 10^{-3}$ M (▲) and $4.5 \cdot 10^{-3}$ M (■)).

concentration in Fig. 6. The results, however, are entirely different from the disintegration behaviour caused by PLE: the k_{obs} dependence on the sodium cholate concentration was a function of the initial liposome concentration, converging on the abscissa at an estimated $(0.8–0.9) \cdot 10^{-2}$ M surfactant. This value approximately corresponds to the critical micelle concentration of sodium cholate [15,16].

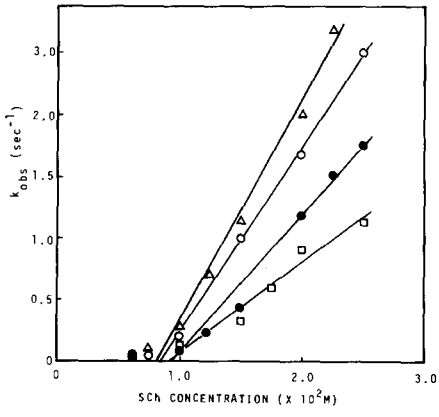


Fig. 6. Effect of sodium cholate (SCh) concentration on the apparent first-order disintegration rate constant, k_{obs}, for unilamellar liposomes (probe-type sonication for 60 min; phosphatidylcholine concentration $1.5 \cdot 10^{-3}$ M (△), $2.5 \cdot 10^{-3}$ M (○), $3 \cdot 10^{-3}$ M (●) and $5 \cdot 10^{-3}$ M (□)).

Discussion

Theoretical aspects of first-order disintegration kinetics

Phosphatidylcholines are solubilized by a variety of surfactants, forming mixed micelles [5,17–19]. Helenius and Simons [8] reviewed 'the solubilization of lipid bilayer', in which they suggested that the sequence of solubilizing processes was divided into three steps as a function of surfactant concentration: stage I begins with the incorporation of surfactant molecules into the bilayer, stage II gives the maximal incorporation of surfactant molecules, resulting in lamellar-micellar phase transition and subsequently in the formation of mixed micelles, and stage III produces further size reduction of mixed micelles due to the increasing ratio of surfactant/phospholipid. Although they mentioned that the above treatment concerned systems in equilibrium, it is very likely that the disintegration mechanism of liposomes also follows three stages in sequence, in which the collapse of the structural integrity of liposomes begins at Stage II.

When the disintegration kinetics of liposomes is followed by means of the turbidity change, the rate of the turbidity change perhaps reflects the process from stage II to stage III, depending on the concentration of surfactant added. Then, the question is raised as to why the disintegration process of the multilamellar liposomes clearly follows first-order kinetics as far as 50% reduction of the initial turbidity, despite their own size heterogeneity.

The physical model of liposome disintegration is based on the assumption that multilamellar liposomes are disintegrated by being stripped off one by one from the outermost shell to the next inner one, encountering the surfactant molecules. Assuming that liposomes from the same concentric bilayer are all identical in size and that the turbidity is proportional to the number of vesicles per unit volume, one may have

$$T_i = A_i n_i \qquad (1)$$

where T is the turbidity or the absorbance, A is the scattering coefficient and n is the number of liposomes. Subscript i denotes the number of the bilayer.

When an entire liposomal system bears a distribution of vesicles ranging from the m concentrics to unilamellar, the total turbidity, T_{tot}, is given by

$$T_{tot} = \sum_{i=1}^{m} A_i n_i \tag{2}$$

Differentiating Eqn. 2 yields

$$\frac{dT_{tot}}{dt} = \sum_{i=1}^{m} A_i \frac{dn_i}{dt} \tag{3}$$

As mentioned above, the disintegration of the outermost shell diminishes the number of liposomes in each size class and increases the number of liposomes reduced by one shell, and so the following equations may be written for the change of the vesicle number in each size class:

$$\begin{aligned}
\frac{dn_m}{dt} &= -k_m n_m \\
\frac{dn_{m-1}}{dt} &= k_m n_m - k_{m-1} n_{m-1} \\
&\vdots \\
\frac{dn_i}{dt} &= k_{i+1} n_{i+1} - k_i n_i \\
&\vdots \\
\frac{dn_1}{dt} &= k_2 n_2 - k_1 n_1
\end{aligned} \tag{4}$$

Substituting Eqns. 4 into Eqn. 3 gives

$$-\frac{dT_{tot}}{dt} = \sum_{i=1}^{m} k_i (A_i - A_{i-1}) n_i - k_1 A_1 n_1 \tag{5}$$

When the threshold of detection of the turbidity change at a certain wavelength is confined between the largest and the jth vesicle in size, Eqn. 5 may be reduced to

$$-\frac{dT_{tot}}{dt} = \sum_{i=j}^{m} k_i (A_i - A_{i-1}) n_i \quad (m > j) \tag{6}$$

The physical implication of Eqn. 6 may be understood in that the rate of the total turbidity change is the summation of the disintegration rate of the outermost shell alone in each size of liposome in which the turbidity term of $(A_i - A_{i-1})n_i$ represents the difference of turbidity between liposomes consisting of i shells and an inner core consisting of $i-1$ shells when the number of vesicles is n_i.

In order to be able to express Eqn. 6 in the form of apparent first-order kinetics concerning the total turbidity, T_{tot}, it is necessary to make some assumptions: when k_i can be assumed to be constant for the disintegration of the outermost shells of liposomes irrespective of their sizes, i.e., in respect of single layers, Eqn. 6 can be rewritten as

$$-\frac{dT_{tot}}{dt} = \bar{k}_i \sum_{i=j}^{m} \frac{(A_i - A_{i-1})}{A_i} A_i n_i \tag{7}$$

where $\bar{k}_i$ is the size-independent disintegration constant.

The absorbance, T, of a dispersed system containing monodispersed particles is given by [20,21]

$$T = \frac{\ln(I_0/I)}{L} = K\pi a^2 n \tag{8}$$

where I_0 and I are the intesity of the incident and transmitted light, respectively. a is the particle radius and L is the length of scattering path. K is the total scattering coefficient defined as

$$K = K_0 \left(\frac{a}{\lambda}\right)^p \tag{9}$$

where K_0 is the size-independent component of the scattering coefficient and p is the exponent of the wavelength, λ, dependent on vesicle size and refractive index.

From Eqns. 8 and 9 and $A_i = K\pi a_i^2$, the term of $(A_i - A_{i-1})/A_i$ in Eqn. 7 can be expressed as

$$\phi_i = \frac{A_i - A_{i-1}}{A_i} = \frac{a_i^{p+2} - a_{i-1}^{p+2}}{a_i^{p+2}} \tag{10}$$

in which the value of p is assumed to remain constant for vesicles within a limited range of radius. The value of p is usually around 1.5–1.7 for multilamellar liposomes and equal or less than 4 for unilamellar liposomes (Yotsuyanagi, T., Mizutani, J. and Ikeda, K., unpublished data; and Ref. 22).

If the size-dependent term, ϕ_i, were defined as an average size factor, $\bar{\phi}_i$, Eqn. 7 could be repre-

sented as first-order kinetics with regard to T_{tot}:

$$-\frac{dT_{tot}}{dt} = \bar{k}\bar{\phi}_i \sum_{i=j}^{m} A_i n_i$$

$$= \bar{k} T_{tot} \qquad (11)$$

where $\bar{k}$ is the apparent first-order disintegration constant, which is, therefore, experimentally determined for a particular liposomal system.

Perfect linearity of first-order plots for liposome disintegration observed in the PLE and sodium cholate solutions could be explained in such a manner that k_i and ϕ_i were assumed to be maintained constant within a particular range of size distribution.

However, theoretically ϕ_i should change with time as the size distribution shifts to smaller particles. It would be, therefore, worthwhile to make some sample calculations in order to estimate the magnitude of the size-dependent factor, ϕ_i, and its size dependence for possible situations using reasonable estimates for values of the parameters a and p. The ϕ_i values were calculated using Eqn. 10 where the initial radius of the vesicle and the exponent were assumed to be 0.5 μm and 1.5, respectively, and it is also assumed that the vesicle radius was successively reduced by 50 Å at every disintegration of the outermost shell [23].

As shown in Table I, a tendency was found for the ϕ_i value generally to increase with decreasing size of vesicles. However, it was not severely affected by the loss of outer shells during early stages of the disintegration, and approximately doubled with every disintegration of the vesicle to half its radius.

Naturally, the liposomal system contains vesicles of various sizes. Then, such a relative constancy of the ϕ_i value should also be maintained for each population with a different concentric shell, although the ϕ_i value itself differs. In this circumstance, the various vesicle sizes which reflect the total turbidity at $t = 0$ only decrease and the total number of vesicles in the system decreases with time because of solubilization. During such processes, the vesicles which are smaller than average would not make a significant contribution to the total turbidity, because the A_i value significantly decreases according to $A_i = K_0\pi(1/\lambda)^p a_i^{p+2}$. This would apparently lead the reduction of the size distribution range to be covered by turbidity measurements at a certain wavelength, which is likely to be one of the factors bringing about a good fit to the first-order plot.

TABLE I

SAMPLE CALCULATIONS OF THE MAGNITUDE OF THE SIZE-DEPENDENT FACTOR, ϕ_i

No. of shells stripped off	Change of vesicle radius (μm)	p	ϕ_i ($\times 10^2$)
1	0.500–0.495	1.5	3.45
2	0.495–0.490		3.49
3	0.490–0.485		3.52
4	0.485–0.480		3.56
5	0.480–0.475		3.60
6	0.475–0.470		3.64
⋮			
21	0.400–0.395	2.0	4.98
22	0.395–0.390		4.99
23	0.390–0.385		5.04
24	0.385–0.380		5.09
25	0.380–0.375		5.12
26	0.375–0.370		5.16
⋮			
51	0.250–0.245	2.5	8.69
⋮			
76	0.125–0.120	3.0	18.8
⋮			
88	0.065–0.060	3.5	35.6

On the disintegration process of various liposomal systems shown in Fig. 4, the number of shells stripped off during the whole period of well-established linearity is unknown. However, insofar as the ϕ_i values remain almost unchanged and are given by respective constants for the vesicles distributed from the m to jth size, it should be mentioned that ϕ_i is apparently presented as an average size factor, $\bar{\phi}_i$, that is weighted by the turbidity of each size when attempts are made to fit the obtained data to first-order kinetics. The results and the theoretical considerations, in contrast, suggest that the number of disintegrated shells would not be so large, but rather small, even if more than 50% of the initial turbidity were lost.

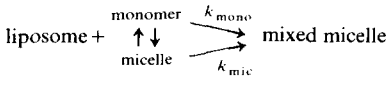
It should be pointed out again that first-order

disintegration kinetics of multilamellar liposomes was attributed to the relative constancy of the size-dependent factor defined by Eqn. 10 in comparison with the turbidity change with time, and the approach should be considered as the first approximation to the disintegration model of liposomes.

Modes of action of PLE and sodium cholate

As shown in Figs. 5 and 6, notable differences between the disintegrating action of PLE and that of sodium cholate against unilamellar liposomes were observed: (1) in the PLE solution, the relationship between k_{obs} and the surfactant concentration intercepted the ordinate by extrapolation, while sodium cholate initiated its action above the critical micelle concentration, and (2) the k_{obs} value depended on the initial liposome (phosphatidylcholine) concentration in the sodium cholate solution but not in the PLE solution. The results suggest that, kinetically speaking, the monomer of PLE also contributes to the disintegration, as well as micellar PLE, unlike the case with sodium cholate.

To generalize the contributions of the monomer and micellar fractions of surfactants, the disintegration reaction was illustrated as



Scheme I

where k_{mono} and k_{mic} are the bimolecular rate constants.

When the critical micelle concentration (CMC) is assumed to change little with the molar ratio of surfactant and lipid, k_{obs} may be expressed as

$$k_{obs} = k_{mono}[CMC] + k_{mic}[S_t - CMC]/N \quad (12)$$

where S_t is the total concentration of surfactant and N is the aggregation number.

Since $S_t \gg$ [CMC] in the present PLE solution, Eqn. 12 can be reduced to

$$k_{obs} = k_{mono}[CMC] + k_{mic}[S_t]/N \quad (13)$$

and one may also have

$$k_{obs} = k_{mic}[S_t - CMC]/N \quad (14)$$

for the sodium cholate case because of the negligible contribution of the monomer species.

Based on Eqns. 13 and 14, the k_{mono} and k_{mic} values were estimated for the disintegration of the unilamellar liposomes, as shown in Table II, where it is assumed that the values of critical micelle concentration for PLE and sodium cholate are $9.1 \cdot 10^{-5}$ M and $0.9 \cdot 10^{-2}$ M, and those of N are 40 and 5, respectively [19,24,25]. The reaction rate due to the monomer species of PLE was about 6-times greater than that of the micellar, and micellar sodium cholate was comparable to micellar PLE.

According to the classification of soluble amphiphiles [8], PLE and sodium cholate are categorized as type A and type B, respectively. Furthermore, it is suggested that type A surfactants may wedge their way into the bilayer structure [26], resulting in a pressure for increasing curvature and inducing the conversion from vesicles to small mixed micelles. Type B surfactants such as bile salts, on the other hand, may chop up the bilayer into small disc-like pieces where the bile salt molecules cover the hydrophobic edges that are exposed by fragmentation [18].

Such different modes of action of types A and B were first described for systems in equilibrium, but may be partly consistent with the present kinetic results: (1) the monomer species of PLE play a great role in vesicle disintegration, especially for unilamellar liposomes because of easier penetration to the arranged layer, and (2) only micellar sodium cholate can disintegrate the bilayer, and the rate of turbidity disppearance increases with phosphatidylcholine/surfactant ratio

TABLE II

THE DISINTEGRATION CONSTANTS, k_{mono} AND k_{mic}, OF UNILAMELLAR LIPOSOMES IN PLE AND SODIUM CHOLATE SOLUTIONS

Disintegration constants are expressed in $l \cdot mol^{-1} \cdot s^{-1}$.

Surfactant	Phosphatidylcholine concn. (mM)	k_{mono} ($\times 10^{-3}$)	k_{mic} ($\times 10^{-2}$)
PLE	1.5–4.5	3.1	5.6
Sodium cholate	1.5	–	10.0
	2.5	–	7.5
	3.0	–	6.5
	5.0	–	3.8

because the size of the bilayer fragmented off is considered to be dependent on the phosphatidylcholine/sodium cholate ratio, i.e., the larger the bilayer to be intercalated by sodium cholate molecules (micelles), the slower the rate of the turbidity disappearance.

There is controversy over the disintegrating action of surfactants against liposomes [5]. First, the effective concentration of surfactants is essentially the critical micelle concentration, which may vary with the molar ratio of surfactant and lipid [27]. Another possibility is that the effective concentration is the total surfactant concentration.

From the kinetic viewpoints based on the schematic model, the dependence of k_{obs} on the surfactant concentraton was expressed by Eqn. 13 in the PLE system and by Eqn. 14 in the sodium cholate system, in which the monomer and micellar contributions are involved. However, it may not be acceptable from the viewpoint of the reaction mechanism that micelles directly attack the highly arranged bilayers and disintegrate them into mixed micelles. Even in the sodium cholate case, it could be considered that, although making a much smaller contribution to the disintegration rate, the monomer of sodium cholate may wedge into the bilayer and trigger the disintegration of liposomes, as does the monomer of type A surfactants, because the liposomes were solubilized at the molar ratio of phosphatidylcholine/sodium cholate of 1–0.5 in equilibrium even when the concentration was far below its critical micelle concentration (see Fig. 2).

It is reasonable to assume that the partition of surfactant molecules into the bilayer occurs first and that subsequently various aggregates, the bilayer incorporated by surfactant and surfactant micelles incorporated by phosphatidylcholine at various molar ratios, are concurrently produced with very rapid transformation [28]. Therefore, the fraction of these aggregates which exist at various stages of mutual incorporation increases with increasing concentration of surfactant in the system. Thus, the contribution of the micellar fraction in the model should be regarded as the collective contribution of the various aggregates.

Accordingly, when the PLE and sodium cholate actions are expressed by Eqns. 13 and 14 from the kinetic viewpoint, the effective concentrations are the total concentration and the micellar concentration for PLE and sodium cholate, respectively.

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