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Effect of Cholesterol on Liposome Stability to Ultrasonic Disintegration and Sodium Cholate Solubilization

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Liposome disintegration by either ultrasonic vibration or sodium cholate solubilization was investigated as a function of cholesterol (CH) content in the egg phosphatidylcholine (PC) liposome membrane. Turbidity changes were used as an indication of the membrane stability to these stresses. First-order disintegration constants (k_u for ultrasonic stress and k_s for sodium cholate solubilization) were calculated to evaluate the membrane stability. A plot of k_u against membrane CH content gave a sigmoidal curve on which inflection points occurred at about 15 mol% and 33 mol% CH. In contrast, the plot of k_s against membrane CH content gave a biphasic curve with only one inflection point at about 17 mol% CH. These results can be explained in terms of the molecular packing model of phospholipids and CH proposed by Presti *et al.* (*Biochemistry*, **21**, 3831 (1982)). Disintegration by ultrasonic stress was little affected by the accumulation of CH-rich domains up to 15 mol% CH, but disintegration by sodium cholate was abruptly suppressed in the same CH content range. These results indicate that the mechanisms of disintegration of CH-rich domains and interfacial boundary phospholipid are entirely different between the two stresses.

In the liposome disintegration by sodium cholate, it was suggested that the penetration-saturation step of the surfactant molecule into the bilayer is rate-determining for pure PC liposomes, while the lamellar-micellar transition step is rate-determining for CH-rich liposomes.

Keywords—liposome stability; cholesterol; egg phosphatidylcholine liposome; ultrasonic vibration; sodium cholate; turbidity change; disintegration mechanism

The physical integrity of liposomes is affected by a variety of external stresses such as ultrasonic vibration, surfactants and freezing.¹⁻⁶⁾ Ultrasonic vibration, used for the size reduction of vesicles, transforms large multilamellar liposomes into small vesicles. An explanation of this rearrangement process has been given.⁷⁾ The destruction of liposomal structures by bile salt surfactants may be one of the important factors influencing drug carrier activity after oral administration.^{3,4)}

The ability of liposomes to resist degradation by externally applied stresses depends on lipid composition, in particular cholesterol (CH), and also vesicle size and surface charge.⁸⁾ We studied the disintegration of phosphatidylcholine (PC)/CH liposomes caused by ultrasonic vibration and sodium cholate solubilization. The time course of vesicle disintegration was followed by measuring the turbidity changes. Disintegration rate constants obtained from turbidity measurement were used to evaluate the stability of liposomes to these externally applied forces.

Experimental

Materials—PC was extracted from egg yolk and purified by column chromatography on silicic acid (Mallinckrodt, St. Louis).⁹⁾ Sodium cholate was purchased from Katayama Chem. Co. (Osaka), and recrystallized from acetic acid as cholic acid. CH and all other chemicals were of reagent grade.

Preparation of Liposomes—Liposomes were prepared essentially by the method of Bangham *et al.*¹⁰⁾ In short, the required amounts of PC and CH (0–57 mol%) were taken in a round-bottomed flask, and dried to give a thin film. Tris buffer (pH 7.4, I.S. 0.1 with NaCl) was added to make a final PC concentration of 1×10^{-3} M based on phosphorus,¹¹⁾ and the mixture was shaken gently then dispersed in a vortex mixer for 3 min.

Disintegration Rate of Liposomes by Ultrasonic Vibration—Turbidities of the multilamellar liposomes (MLV) prepared above varied significantly even for liposomes of identical lipid composition. Therefore liposome samples were briefly sonicated (probe-type sonifier, model UR 200P, 20 kHz, Tomy Seiko Co., Tokyo) for 2.5 min to obtain reproducible initial turbidities. This preliminary sonication was uniformly applied to all of the liposomes containing 0–57 mol% CH. The resulting suspensions were allowed to equilibrate at room temperature for 30 min, which was taken to be $t=0$ for the calculations of disintegration kinetics. Ultrasonic vibration was continuously applied for various periods to each sample, the volume of which was always maintained at 10 ml in a 50 ml glass centrifuge tube under a nitrogen stream at 40 °C. The samples were left standing for 30 min at room temperature, then turbidity measurements were carried out in a 1 cm quartz cell at 400 nm, using a Hitachi 124 spectrophotometer.

Disintegration Rate of Liposomes by Sodium Cholate—The disintegration kinetics of liposomes in sodium cholate solution was also followed by measuring the turbidity changes. Turbidity was monitored at 660 nm by a stopped-flow apparatus (model RA-401, Union Giken, Osaka) equipped with a UV-VIS detector.⁵⁾ Liposomes were first subjected to ultrasonic vibration to give various initial turbidities for the cholate disintegration studies. An equal volume of the liposome suspension containing 3×10^{-4} M phosphorus was quickly mixed with a 0.02 M sodium cholate solution (Tris buffer, pH 7.4, I.S. 0.1, 25 °C).

The concentrations of lipid and surfactant are diluted to half in the cell of the apparatus. Accordingly, the initial turbidity was presumed to be half of the original turbidity.

Turbidity reduction by ultrasonic vibration or surfactant solubilization was treated as a pseudo-first-order process, and first-order liposome disintegration rate constants, k_u and k_s , respectively, were obtained from the turbidity measurements; k_u was calculated from the initial slope of the plots shown in Fig. 1, and k_s was obtained from the linear plots of turbidity disappearance.

Results and Discussion

Disintegration Behavior under Ultrasonic Vibration

The sonication system used for the disintegration of liposomes gave reproducible turbidity changes ($\pm 5\%$ scattering). Unlike the disintegration of vesicles in surfactant solution, in which the bilayer was assumed to be disintegrated by successive stripping of shells from the vesicles, yielding mixed micelles, ultrasonic vibration merely tears the vesicles into smaller bilayer fragments and ultimately finer vesicles. These small vesicles contribute to the total turbidity of the suspension. Because of this, turbidity reduction at the early stages of disintegration was used to calculate k_u values. The rate constant (k_u) was assumed to represent membrane stability to ultrasonic stress: the smaller the rate constant, the more resistant the bilayer membrane.

Figure 1 shows the turbidity reductions of CH containing liposomes. At less than 17 mol% CH, linearity was generally poor for long sonication times. As the CH content increased to more than 33 mol%, the reduction showed an initial slow stage and a subsequent faster stage. Decreasing the sonication stress by using a bath-type sonicator, instead of a probe, prolonged the initial slow stage significantly.²⁾ The reason for this is not clear.

The k_u values were calculated from the initial slope, and also from the terminal linear portion at CH contents of more than 33 mol%. Figure 2 shows a plot of k_u vs. mol% CH in the liposomes. The sigmoidal shape of the k_u vs. mol% CH plot had three distinct regions. These regions are defined by the two inflection points at about 15 mol% CH and about 33 mol% CH. From 0 to 15 mol% CH, the membrane stability remained almost unchanged (stage I). From 15 mol% CH to 33 mol% CH, a rather sharp increase in membrane stability occurred (stage II). Increasing CH beyond 33 mol% caused only a moderate increase in membrane stability (stage III). At stage III there was little difference between the increments of the stability obtained from the initial and terminal slopes of the turbidity reductions (Fig. 1).

Presti *et al.*¹²⁾ proposed a model for the molecular packing of CH and phospholipids in membranes. According to their model, phases are formed as a function of the CH content in

Fig. 1. First-Order Plots of the Turbidity Change due to Liposome Disintegration by Ultrasonic Vibration

The PC concentration is constant at 1×10^{-3} M. Numbers indicate CH content (mol%). All liposome suspensions were subjected to a prior sonication of 2.5 min. Points are the mean values of 3–5 determinations and were reproducible better than $\pm 5\%$.

Fig. 2. The Effect of CH Content on the Disintegration Constant (k_u) of Liposomes by Ultrasonic Vibration

k_u was calculated from the initial slope (●—●). k_u was also obtained from the subsequent linear portion for liposomes containing 33.3, 40.0 and 50.0 mol% CH (○—○). PC concentration, 1×10^{-3} M. ↓ shows an inflection point at which the membrane stability anomalously changes.

the bilayer: under 20 mol% CH, CH-rich domains are formed and surrounded by a continuous phospholipid domain or phase. At about 20 mol% CH, the continuous PC domain disappears, but interfacial boundary phospholipid remains, separating the CH-rich domains. Further increasing the membrane CH causes the fraction of interfacial boundary phospholipid to gradually diminish and become zero at *ca.* 33.3 mol%. Uncomplexed phospholipid molecules finally disappear at 50 mol% CH.

Based on this model, the relationship between k_u and mol% CH can be interpreted as follows. The membrane stability, *i.e.* k_u , changed very little in stage I, where CH-rich domains are present but do not occupy a large fraction of the membrane. The inflection point observed at 15 mol% CH, which probably corresponds to the first critical ratio in Presti's model, reflects a critical ratio under which the contribution of the CH-rich domains produced in the continuous PC phase to the stability is still minor in the egg PC liposomes. In stage II, the stability increased dramatically with increasing CH content. This is a reasonable reflection of an accumulation of the CH-rich domains, in other words, a decrease of the interfacial boundary phospholipid. The second inflection point observed at 33 mol% CH coincides with the second critical point (33.3 mol% CH) in the model, at which the bilayer contains only CH-rich domains. Further incorporation (> 33 mol%) of the sterol into liposomes results in a mild increase in membrane stability (stage III) where the CH-rich domains are transformed to the 1:1 hydrogen-bonded complexes up to about 50 mol% CH. Membranes containing 50 mol% CH are stabilized about four-fold compared to CH free membranes. This also suggests strong interactions among resulting PC/CH 1:1 complexes. It is therefore reasonable to consider that tearing of bilayers by sonication occurs mainly in the free phospholipid domain.

The ultrasonic velocity has been considered to be a good measure of the mechanical properties of membranes.^{13,14)} In the liquid-crystalline state of dipalmitoylphosphatidylcholine (DPPC) liposomes, the limiting value of ultrasonic velocity shows an abrupt increase

in the region of 15 to 30 mol% CH. The k_u values also start changing abruptly near 15 mol% CH. When egg PC liposomes are used instead of DPPC liposomes, it seems that the stabilizing effect of CH against ultrasonic vibration is not sensitive to the fatty acid composition of phospholipids but rather to the disappearance of interfacial boundary phospholipid. It is noteworthy that a macroscopic mechanical property represented by the ultrasonic disintegration rate constant (k_u) is consistent with Presti's model.

Disintegration Behavior in Sodium Cholate

A mechanistic sequence for the disintegration of liposomes by surfactants has been proposed, in which the mode of action of sodium cholate was classified into type B.¹⁵⁾ We reported previously that the turbidity disappearance of pure PC liposomes clearly followed first-order kinetics despite the presence of polydispersed vesicles in terms of size.⁵⁾ An explanation was given for the linearity of the first-order plots by introducing the average size factor ($\bar{\phi}$). The reduction rate of the total turbidity (T_{tot}) is expressed by

$$-\frac{dT_{\text{tot}}}{dt} = \bar{k} \sum_{i=j}^m \frac{A_i - A_{i-1}}{A_i} A_i n_i \quad (m > j) \quad (1)$$

where $\bar{k}$ is the size-independent disintegration rate constant, A_i is the scattering coefficient of the i -th vesicle, n_i is the number of the i -th vesicle, and m and i denote the size classes of the largest and the j -th vesicles sensitive to the turbidity changes at a certain wavelength. The absorbance ($T = An$) of a dispersed system containing monodispersed particles is given by^{16,17)}

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

$$K = K_0 \left(\frac{a}{\lambda} \right)^p \quad (3)$$

where I_0 and I are the intensities of the incident and transmitted light, respectively. a is the particle radius, L is the length of scattering path, K and K_0 are the total scattering coefficient and the size-independent component of the scattering coefficient, respectively, and p is the exponent of the wave-length (λ). The term $(A_i - A_{i-1})/A_i$ in Eq. 1 can be therefore represented based on Eqs. 2, 3 and $T = An$ as

$$\phi_i = \frac{A_i - A_{i-1}}{A_i} = \frac{a_i^{p+2} - a_{i-1}^{p+2}}{a_i^{p+2}} \quad (4)$$

If ϕ_i , which is intrinsically size-dependent, is defined as an average size factor ($\bar{\phi}$), Eq. 1 can be represented as a first-order kinetic equation with regard to T_{tot} , *i.e.*

$$\begin{aligned} -\frac{dT_{\text{tot}}}{dt} &= \bar{k} \bar{\phi} \sum_{i=j}^m A_i n_i \quad (m > j) \\ &= \bar{k}_a T_{\text{tot}} \end{aligned} \quad (5)$$

Accordingly, the experimentally obtained first-order disintegration rate constant ($\bar{k}_a$) contains an average size factor $\bar{\phi}$, which may be useful to characterize the disintegration behaviors of dispersed systems with different size distributions.

The turbidity changes of liposomes containing 0–57 mol% CH followed first-order kinetics. Figure 3 shows the pseudo-first-order rate constant (k_s) obtained as a function of the initial turbidity and CH content at the constant sodium-cholate concentration of 0.01 M. The egg-PC/bile salt molar ratio was maintained at 0.1 throughout, *i.e.* excess of bile salt. The k_s value depended on the initial turbidities, which reflect different size distributions of vesicles, even when the composition of PC and CH was fixed, and the curves appeared to be generally biphasic. Values of k_s for PC liposomes (CH free) were most sensitive to the initial turbidity.

Fig. 3. Effects of Initial Turbidity and CH Content on the Disintegration Constant (k_s) in Sodium Cholate Solution

The concentrations of PC and sodium cholate were always maintained at 3×10^{-3} and 2.0×10^{-2} M, respectively. CH content (mol%): 0 (a), 9.2 (b), 14.0 (c), 29.6 (d) and 57.1 (e). The temperature was 25°C .

Fig. 4. The Effect of CH Content on the Disintegration Constant (k_s) at Different Initial Turbidities

Initial turbidity: 1.0 (●), 0.5 (○) and 0.25 (△). ↓ shows an inflection point at which the membrane stability anomalously changes.

Incorporating CH into the membrane decreased the biphasic nature of the curve (Fig. 3); at about 50 mol% CH, k_s was little affected by the initial turbidity.

In connection with the theory described earlier, the dependency of k_s on the initial turbidity reflects the difference of the size factor; the relationship $k_s = \bar{k}_a = \bar{k}\phi$ holds because $\bar{k}$ is assumed to be a size-independent disintegration constant for a single bilayer. However, as the CH-rich domains were accumulated, the dependency of k_s on the initial turbidity gradually diminished, suggesting that the size factor ϕ apparently becomes rather constant despite the fact that various initial turbidities were used. These results (Fig. 3) raise the question of why $k_s = \bar{k}\phi$ does not always hold for PC/CH membranes as the CH content increases, because we previously estimated ϕ_i to increase 2.5 times as the vesicle size decreased by half in radius in model calculations⁵⁾ and such changes of ϕ_i should be intrinsically applicable irrespective of CH content. Therefore, the physical meaning of the parameter $\bar{k}$ involved in k_s may differ between the pure PC liposomes and the CH-rich liposomes. This discrepancy could be accounted for by a stepwise disintegration mechanism of the bilayer. Step I is the penetration-saturation step of bile salt molecules followed by step II, the lamellar-micellar phase transition step.

As shown in Fig. 4, the relationship between CH content and k_s was biphasic when the initial turbidity was maintained at 1.0 O D. The same tendency was also seen with other initial turbidities (0.25 and 0.5). Accumulation of CH-rich domains in the membrane is responsible for the abruptly increasing stability up to about 17 mol%, and as interfacial boundary phospholipid appears, the membrane shows relatively minor increments of the stability. It was also noted that the k_s dependency showed an inflection point at about 17 mol% irrespective of the initial turbidity. It seems likely that with CH-rich liposomes (e.g. 1:1 molar ratio) the amount of the surfactant molecule that penetrates into the bilayer is limited and the transition rate is slowed.

Accordingly, the larger the bilayer in radius, the slower the integration rate. If the $\bar{k}$ reflects the lamellar-micellar phase transition rate for the CH-rich liposomes, it seems

probable that $k_s = \bar{k} \bar{\phi} \simeq \text{constant}$ in consequence of the mutual compensation of the changes in the two parameters, $\bar{k}$ and $\bar{\phi}$. With CH-free liposomes, the penetration-saturation step is rate-determining and $\bar{k}$ is a size-independent parameter; it was assumed previously that the dependency of k_s on the initial turbidity is due to changes of $\bar{\phi}$. Furthermore, the size of the mixed micelles decreases readily. Although the consistency of the theory and results should be further examined, we assume that the disintegration rate of the pure PC liposomes is mainly governed by the former step and with increasing CH content in the membrane the latter step becomes predominant.

In conclusion, there is a marked difference between the liposome stabilities to ultrasonic vibration (physical stress) and the disintegrating action of cholic acid (chemical stress) as a function of the CH content. The role of CH incorporated in egg PC liposomes is often said to be to make the bilayer membrane "more solid-like," but the amounts of CH used are not always rationalized in the literature. The observed variation of the membrane stability may provide useful criteria for compounding CH in liposomes and in assessing their response to external stresses.

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