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Asymmetric disposition of detergents within vesicle bilayer and its effect on ion permeation through the membrane

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Phospholipid vesicles were prepared by detergent removal using hydrophobic porous beads, Amberlite XAD-2, or dialysis from detergent-phospholipid mixed micelles. The liposomes formed were found to be mostly unilammellar vesicles. The vesicle diameter was estimated, by both quasi-elastic light-scattering and gel-exclusion chromatography on Sephacryl S-1000, to be 80 nm for the vesicles formed by removal of octaethylene glycol monododecyl ether by the bead method. The effect of detergents within a bilayer on ion permeation was demonstrated. When the content of octaethylene glycol monododecyl ether reached a molar ratio of 0.2, the intrinsic ion selectivity of the phospholipid membrane between anion and cation was diminished. The ion permeability measured for vesicles with detergent incorporated into initially detergent-free vesicles was about 10-times greater than that for vesicles with detergent remaining following the process of detergent removal. This observation was explained by the different disposition of the detergent in the bilayer, that is, when vesicles were formed by the removal of detergent from mixed micelles, the residual detergent became distributed in both the outer and inner leaflets, and when the detergent was incorporated into initially detergent-free vesicles, the detergent became distributed only in the outer leaflet within the experimental time limits. This idea was supported by the NMR studies. It was also found that, as a detergent, octaethylene glycol monododecyl ether has a stronger effect on ion permeation than octyl glucoside.

Introduction

Since relatively homogeneous unilamellar vesicles can be prepared by the removal of detergent from phospholipid-detergent mixed micelles, the detergent removal method has been widely used for liposome preparation [1–15]. Dialysis has been commonly used to remove the detergent, but for those with a low c.m.c., such as Triton X-100, it takes more than 10 days to prepare phospholipid vesicles by removal of the detergent by dialysis [2]. Therefore, we have developed a new method using hydrophobic porous beads [10] which enables vesicles to be prepared within 1 h by removal of even long-chain surfactants, i.e., those with a low c.m.c., from mixed micelles.

Some detergents can remain in the membrane wall after vesicle formation by the detergent removal method. In such cases the remaining detergent sometimes seriously disturbs the barrier effect of the lipid membrane. In this paper, we discuss how the amount of residual detergent as well as the mode of disposition of detergent within the membrane can affect ion permeation through the membrane.

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Experimental

Materials

L-α-Phosphatidylcholine from egg yolk was purchased from Green Cross Co. (no less than 99% purity). Octyl β-D-glucoside and octaethylene glycol monododecyl ether ($C_{12}E_8$) used as detergents were purchased from Wako Chemicals and Tokyo Kasei Co. Ltd., respectively. $Pr(NO_3)_3$ was used as an NMR shift reagent. Tes (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) for the preparation of buffer solution was purchased from Sigma Co. [^{14}C]Octyl glucoside and [^{3}H]dipalmitoylphosphatidylcholine were from (New England Nuclear and [^{14}C]$C_{12}E_8$ was from CEA (France). All other reagents were of reagent grade from Wako Chemicals.

Methods

Vesicle size was determined by both quasi-elastic light scattering using a Coulter Submicron Particle Analyzer model N4 and gel-exclusion chromatography on Sephacryl S-1000 according to the method of Reynolds et al. [16]. The vesicle size was also estimated from the ion-trapped volume. ^{1}H-NMR spectra were recorded on a Varian XL-200 NMR spectrometer at 200 MHz. Ionic permeability was determined by electrometric measurement using an ionalyzer (Orion Research, model 701A) with solid membrane electrodes for Cl^- and Na^+ or a liquid membrane electrode for K^+, and with a Ross reference electrode.

Detergent and phospholipid concentrations were determined by monitoring their radioactivities using an Aloka LSC-903 liquid scintillation system. The micro method of Bartlett [17] was also sometimes adopted to determine phospholipid concentration. The buffer solution used was usually composed of 250 mM NaCl/1 mM EDTA/20 mM Tes (pH 7.0).

Solutions of mixed lipid-detergent micelles were obtained by incubating a detergent solution at the desired concentration with a lipid film deposited on the walls of a test tube. It is known that micellar solutions with detergent/lipid ratios of 10 : 1 or more produce virtually 100% single-walled vesicles, whereas lower ratios are insufficient for complete dispersal of the lipid and lead to the appearance of multilamellar liposomes in the final product [9]. Micellar solutions used in the present study generally contained 15 mM phosphatidylcholine and 150 mM $C_{12}E_8$. For the experiments using octyl glucoside, the initial detergent content was 175 mM to allow for the approximate concentration of 25 mM monomeric octyl glucoside that would be present because of this detergent's high c.m.c.

Detergent removal was carried out using either the dialysis method or the bead method under low light and nitrogen in order to avoid possible oxidation of phospholipid. In principle, the latter method uses the adsorption ability of detergents on hydrophobic porous beads [18,19] such as amberlite XAD-2. Usually, 1–3 g of the beads are packed into a short column (Econocolumn from Bio-Rad of dimensions 0.7 × 10 cm and containing polyethylene support films); 1 ml of mixed micelle solution is then passed rapidly through the column using a syringe. Since the small sample volume does not come into contact with all of the adsorbent when this procedure is used, multiple passes were employed to remove the detergent to the desired level. It should be noted that the short time of exposure to the bead surface in multiple passes is insufficient to achieve equilibrium [19]. Using our method, liposomes can be prepared within only 10 min or so. This is far more rapid than the dialysis method, which requires 24 h or more. Furthermore, this method enables the removal of detergents with a low c.m.c., or low monomer solubility, which can not be removed by the dialysis method. Detergent incorporation was carried out by adding an appropriate amount of detergent from the outside to initially detergent-free vesicles, which were prepared by dialysis from octyl glucoside-phospholipid mixed micelles and contained essentially no residual detergent. All experiments were carried out at $25 \pm 1°$C.

Results and Discussion

Fig. 1 shows the relationship between vesicle diameter or its dispersion parameter (standard deviation) and the number of passes made in the $C_{12}E_8$ removal process. After one pass, the apparent size of the mixed micelle became maximal with a very wide size range. With repeated passage, the size and size dispersion became smaller

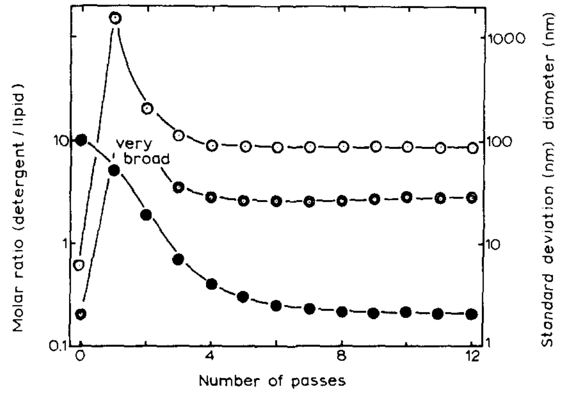

Fig. 1. Relationship between particle size (○), its dispersion index (standard deviation) (◉), detergent/lipid in molar ratio (●) and number of passes.

and reached a specific value. The diameter of the vesicles at this specific value was 85 nm when using $C_{12}E_8$ as a surfactant and 150 nm when using octyl glucoside.

The total ion-trapped volume was determined from the difference between C_∞ and C_0, the external concentration at zero time. Zero time was defined as the mid-point of the time required to exchange the external medium (replacing Cl^- by NO_3^- or Na^+ or K^+ by choline), which was usually about 7 min before the first potentiometric measurement. C_∞ was obtained by destruction of the vesicles by addition of a detergent. Trapped volume per mole of phospholipid was used as a measure of the apparent vesicle diameter. The values of the diameter measured by several methods are summarized in Table I. Since the values measured by a quasi-elastic light-scattering or gel chromatography almost agreed with those calculated from the ion-trapped volume of the vesicles, these vesicles were considered to be practically unilamellar vesicles. Electron micrographs also supported this consideration [20].

Ion permeabilities through the vesicle membrane were measured within a detergent concentration range for which unilamellar vesicle has been known to be formed [10,20]. Fig. 2 shows typical plots for the first-order efflux of Cl^- ion from vesicles containing specific amounts of detergent. When octyl glucoside/lipid = 0.39, the efflux rate constant (k) = $5.8 \cdot 10^{-5}$ s^{-1}. When octyl glucoside/lipid = 0.89, $k = 1.08 \cdot 10^{-3}$ s^{-1}.

The permeability coefficient (p) was obtained from the relation

$$p/k = \frac{\text{internal volume/vesicle}}{\text{membrane area/vesicle}} \tag{1}$$

In Fig. 3, permeability of Cl^- ion through the membrane of vesicles prepared by the use of $C_{12}E_8$ or octyl glucoside is plotted against the remaining detergent following the detergent-removal process. When the residual octyl glucoside was present at a molar ratio of less than 0.4, the ion permeability was rather small. With a molar ratio of detergent of 0.4 or more, the ion permeability increased abruptly. Residual $C_{12}E_8$ has a stronger effect on permeation than octyl glucoside.

In Fig. 4, the permeability of several types of ion in a logarithmic scale are plotted against the content of $C_{12}E_8$ in both the detergent removal process and detergent incorporation process carried out by incorporating the detergent in initially detergent-free vesicles. When the content of $C_{12}E_8$ reached a molar ratio of 0.2 the discrimination between small cation (Na^+) and small anion (Cl^-)

TABLE I

AVERAGE DIAMETER OBTAINED BY SEVERAL METHODS AND RESIDUAL DETERGENT

QELS: quasi-elastic light-scattering.

Method	Detergent	Detergent / Lipid	Diameter (nm) from: trapped vol.	S-1000 column	QELS
XAD-2 [a]	$C_{12}E_8$	0.25	70	78	85
XAD-2 [a]	octyl glucoside	0.20	140	100	150
Dialysis [b]	octyl glucoside	≈ 0.01	200	210	200

[a] Detergent was removed by using XAD-2.
[b] Detergent was removed by dialysis.

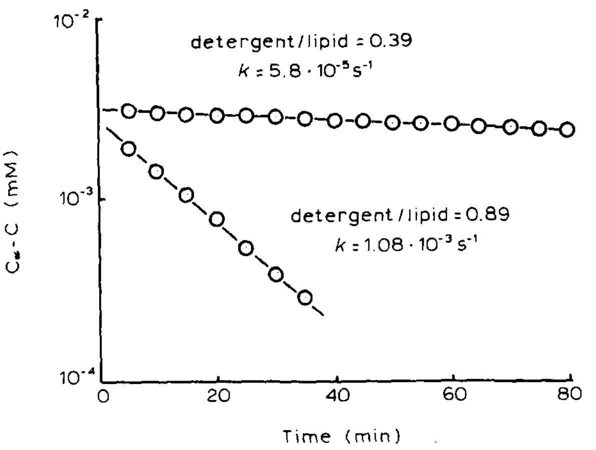

Fig. 2. Typical plots for the first-order efflux of Cl^- ion from vesicles containing detergents.

became reduced. It was also remarkable that the ion permeability of ions for vesicles with incorporated detergent was about 10-times greater than the permeability for vesicles with detergent remaining from the removal process.

This discrepancy may be ascribed to the asymmetric distribution of the incorporated detergent within the vesicle bilayers. Ideal mixing within the bilayer would make a linear function between the mole fraction of detergent in the bilayer (X_D) and the monomer concentration (C_{mono}) [21].

$$X_D = C_{mono}/\text{cmc} \qquad (2)$$

where C_{mono} is equal to the total free detergent concentration as long as one is below the cmc. In the previous paper [10], a linear relation was observed within the limits of experimental error, but the slope was only half of the theoretical slope of X_0 against 1/cmc, as predicted by Eqn. 2. We have presumed that only half the bilayer volume (the outer leaflet) was accessible to the detergent within the time taken for the experiment. This idea was supported by the subsequent NMR study. Fig. 5 shows the ^{1}H-NMR spectra near the choline methyl proton. In the case of the process of detergent incorporation into initially detergent-free

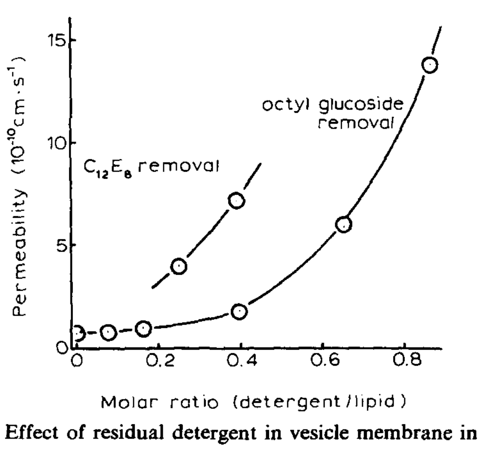

Fig. 3. Effect of residual detergent in vesicle membrane in the removal process on the permeability coefficient of Cl^-.

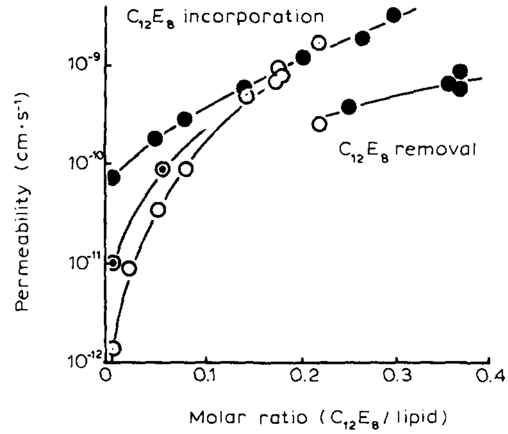

Fig. 4. Effect of $C_{12}E_8$ incorporated into initially detergent free vesicles and residual $C_{12}E_8$ in vesicle membrane in detergent removal process on permeability coefficient. ○, Na^+; ⊙, K^+; ●, Cl^-.

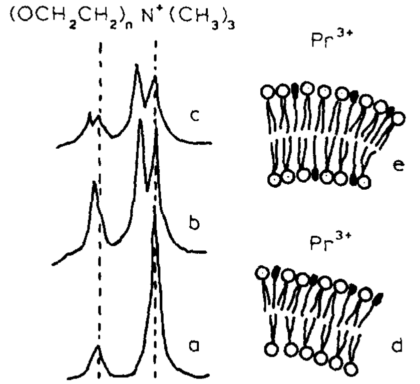

Fig. 5. ^{1}H-NMR spectra of $(OCH_2CH_2)_n$ of a detergent and choline group of a phospholipid. (a) Without Pr^{3+}; (b) Pr^{3+} added to vesicle in $C_{12}E_8$ incorporation process; (c) Pr^{3+} added to vesicle in $C_{12}E_8$ removal process. Schematic representation of disposition of $C_{12}E_8$ in the detergent incorporation process (d) and removal process (e).

vesicles, the signal belonging to the proton of ethylene oxide in $C_{12}E_8$ was completely shifted by a shift reagent $Pr(NO_3)$ which was added after preparation of vesicles. On the other hand, in the case of the process of detergent removal from mixed micelles, the signal for half the amount of detergent was shifted by adding the shift reagent, while the other half remained in its original place, in which the signal of the ethylene oxide proton of the detergent in the shift reagent-free condition was located.

These observations show that residual detergent is distributed in both the outer and inner leaflet, when vesicles are formed by the removal of detergent from mixed micelles, while, on the other hand, when a detergent is incorporated into initially detergent-free vesicles, the detergent becomes distributed only within the outer leaflet of the vesicles within the experimental time limit because of slow flip-flop of the detergent in the bilayer [22]. It was concluded that the different disposition of a detergent within a vesicle membrane could be responsible for the different characteristics of ion permeation through the vesicle membrane.

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References

1 Brunner, J., Škrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322–331
2 Allen, T.A., Romans, A.Y., Kercret, H. and Segrest, J.P. (1980) Biochim. Biophys. Acta 601, 328–342
3 Enoch, H.G. and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. 76, 145–149
4 Rhoden, V. and Godin, S.M. (1979) Biochemistry 18, 4173–4176
5 Schurtenberger, P., Mazer, N., Waldvogel, S. and Kanzig, W. (1984) Biochim. Biophys. Acta 775, 111–114
6 Zumbuehl, O. and Weder, H.G. (1981) Biochim. Biophys. Acta 640, 252–262
7 Milsmann, M.H.W., Schwendener, R.A. and Weder, H.G. (1978) Biochim. Biophys. Acta 512, 147–155
8 Nozaki, Y., Lasic, D.D., Tanford, C. and Reynolds, J.A. (1982) Science 217, 366–367
9 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840
10 Ueno, M., Tanford, C., and Reynolds, J.A. (1984) Biochemistry 23, 3070–3076
11 King, R.G. and Marchbanks, R.M. (1982) Biochim. Biophys. Acta 691, 183–187
12 Martin, D.W. and Ueno, M. (1985) Biophys. J. 47 (part II), 154
13 Schurtenberger, P., Mazer, N. and Kanzig, W. (1985) J. Phys. Chem. 89, 1042–1049
14 Almog, S., Kushnir, T., Nir, S. and Lichtenberg, D. (1986) Biochemistry 25, 2597–2605
15 Ueno, M. (1986) Med. Drug J. 22, 2045–2051
16 Reynolds, J.A., Nozaki, Y., and Tanford, C. (1973) Anal. Biochem. 130, 471–474
17 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
18 Holloway, P.W. (1973) Anal. Biochem. 53, 304–308
19 Cheetham, P.S.J. (1979) Anal. Biochem. 92, 447–452
20 Ueno, M., Tanaka, N., Adachi, I. and Horikoshi, I. (1986) The 39th Simposium on Colloid and Interfacial Chemistry, 1986, Tsukuba, Abstr. p. 388
21 Tanford, C. (1980) The Hydrophobic Effect, 2nd. Edn., p. 68, Wiley, New York
22 Billington, D., Coleman, R. and Lusak, Y.A. (1977) Biochim. Biophys. Acta 466, 526–530