Solubilizing Effects Caused by the Nonionic Surfactant Octyl Glucoside in Phosphatidylcholine Liposomes

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ABSTRACT: The mechanisms governing the interaction of the nonionic surfactant octyl glucoside (OG) on phosphatidylcholine (PC) liposomes were investigated. Permeability alterations were detected as a change in 5(6)-carboxyfluorescein (CF) released from the interior of vesicles, and bilayer solubilization was determined as a decrease in the static light scattered by liposome suspensions. A direct relationship was established in the initial interaction steps (10–50% CF release) between the growth of vesicles, the leakage of entrapped CF, and the effective molar ratio of surfactant to phospholipid in bilayers (Re). This dependence was also detected during the solubilization range of Re values between 1.3 and 3.0, where the decrease in the surfactant-PC aggregate size and in the light scattering of the system depended on the Re parameter and, hence on the composition of these aggregates. The free OG concentrations at subsolubilizing and solubilizing levels showed lower and similar, respectively, values than its critical micelle concentration (CMC). These findings indicated that the alterations in bilayer permeability were due to the action of surfactant monomers, whereas bilayer solubilization was determined by the formation of mixed micelles. This finding supports the generally accepted assumption that the concentration of free surfactant must reach the CMC for solubilization to occur.
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KEY WORDS: Carboxyfluorescein release, critical micelle concentration, mixed micelle formation, octyl glucoside, permeability alterations and bilayer solubilization, phosphatidylcholine liposomes, static light-scattering, surfactant/phospholipid molar ratios.

A number of studies have been devoted to improving our understanding of the principles that govern the interaction of surfactants with simplified membrane models, such as phospholipid bilayers (1–4). This interaction leads to the breakdown of lamellar structures and the formation of lipid–surfactant mixed micelles. A significant contribution has been made by Lichtenberg (5), who postulated that the critical effective surfactant/lipid ratio (Re) that produces saturation and solubilization depends on the surfactant critical micelle concentration (CMC) and on the bilayer/aqueous medium distribution coefficients (K), rather than on the nature of the surfactant. One of the most commonly used surfactants in membrane solubilization and reconstitution is octyl glucoside (OG) because of its reduced denaturing effect on proteins and its relatively high CMC value (6–11).

In earlier papers, we studied some parameters that are implicated in the interaction of different surfactants with liposomes at subsolubilizing and solubilizing concentrations (12–14). In this work, we seek to extend our investigations to correlate some physicochemical properties of surfactant-phosphatidylcholine (PC) aggregates formed during the interaction of OG with PC liposomes with the Re. This information may enhance our understanding of the complex phenomenon involved in the lamellar-to-micelle transitions during solubilization of PC liposome suspensions by this nonionic surfactant.

EXPERIMENTAL PROCEDURES

Materials and methods. PC was purified from egg lecithin (Merck, Darmstadt, Germany) according to the method of Singleton *et al.* (15), and was shown to be pure by thin-layer chromatography (TLC). The nonionic surfactant OG was purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was purchased from Rohm and Haas (Lyon, France). Piperazine-1,4 *bis*(2-ethanesulfonic acid) (PIPES) was obtained from Merck. PIPES buffer was prepared as 10 mM PIPES containing 110 mM Na_2SO_4 and adjusted to pH 7.20 with NaOH. Polycarbonate membranes and membrane holders were purchased from Nucleopore (Pleasanton, CA). The starting material 5(6)-carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY) and purified by a column-chromatographic method (16).

Unilamellar liposomes of a defined size (about 200 nm) were prepared by extrusion of large unilamellar vesicles obtained previously by reverse-phase evaporation (12). To study the bilayer permeability changes, vesicles containing CF were freed of unencapsulated fluorescent dye by passing through Sephadex G-50 medium resin (Pharmacia, Uppsala, Sweden) by column chromatography (13). The concentration range of phospholipid in liposomes was 1.0–10.0 mM, which was determined by TLC coupled with an automated flame-ioniza-

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tion detection system (Iatroscan MK-5; Iatron Lab. Inc., Tokyo, Japan) (17).

The size distribution and the polydispersity index (PI) of liposomes and surfactant-PC aggregates were determined with a photon-correlator spectrometer (Malvern Autosizer 4700c PS/MV; Malvern Co., Malvern, England). The measurements were made by distribution of particle number. Sample concentrations were adjusted to the appropriate range with PIPES buffer, and the measurements were taken at a lecture angle of 90° at 25°C.

The surface tensions of buffered solutions that contained increasing amounts of OG were measured by the ring method (18) with a Krüss processor tensiometer K 12 (Krüss GMBH, Hamburg, Germany). The CMC of the OG (18.0 mM) was determined from the abrupt change in the plot of surface tension vs. surfactant concentration.

To evaluate the alterations caused by OG on lipid bilayers, the effective Re in an aggregate (liposome or micelle) is calculated from the following equation (Ref. 5):

$$\mathrm{Re} = (S_T - S_W)/(PL - PL_{\mathrm{mon}}) \quad [1]$$

where PL is the phospholipid concentration (mM), S_T is the total OG concentration (mM), and S_W is the OG concentration in the aqueous medium (mM). The monomeric PL concentration (PL_{mon}) is negligible due to the low solubility of PL in water.

The determination of Re and S_W was carried out on the basis of the linear dependence between the surfactant concentrations required to achieve these parameters and the PL in liposomes, which is described by the equation:

$$S_T = S_W + \mathrm{Re}\,[PL] \quad [2]$$

where Re and S_W are the slope and the ordinate at the origin (zero PL) of each curve, respectively.

Permeability alterations and solubilization of liposomes. The permeability alterations, caused by OG, were determined by monitoring the increase in fluorescence intensity of the liposome suspensions due to the CF released from the interior of vesicles to the bulk aqueous phase (16). Fluorescence measurements were made with a Shimadzu (Kyoto, Japan) RF-540 spectrofluorophotometer. On excitation at 495 nm, a fluorescence maximum emission of CF was obtained at 515.4 nm. The presence of OG did not cause direct quenching of the aforementioned spectrofluorophotometric CF signal. OG solutions were added to equal volumes of liposome suspensions (lipid concentration ranging from 2.0 to 20.0 mM), and the resulting mixtures were left to equilibrate for 40 min. This interval was chosen as the minimum period of time needed to achieve a constant level of CF release. The experimental determination of this time interval will be shown in the Results and Discussion section. The fluorescence intensity measurements were taken at 25°C. The percentage of CF released was calculated from the following equation (Ref. 13):

$$\%\mathrm{CF\ release} = (I_T - I_0)/(I_\infty - I_0) \cdot 100 \quad [3]$$

where I_0 is the initial fluorescence intensity of CF-loaded liposome suspension in the absence of OG, I_T is the fluorescence intensity measured 40 min after adding OG to a liposome suspension, and I_∞ corresponds to the fluorescence intensity remaining after the complete destruction of liposomes by the addition of Triton X-100 aqueous solution (Rohm and Haas) (16).

With regard to liposome solubilization, it has been previously demonstrated that static light-scattering constituted a convenient technique for the quantitative study of the bilayer solubilization by surfactants (3,19,20). Accordingly, the solubilizing perturbation produced by OG in PC liposomes was monitored by this technique. The overall solubilization can be characterized by two parameters termed $\mathrm{Re}_{\mathrm{SAT}}$ and $\mathrm{Re}_{\mathrm{SOL}}$ which correspond to the Re ratios at which light-scattering starts to decrease with respect to the original value and shows no further decrease (21). These parameters correspond to the OG/lipid molar ratios at which the surfactant saturates liposomes and leads to a complete solubilization of these structures.

OG solutions were added to equal volumes of liposome suspensions, and the resulting systems were left to equilibrate for 24 h. This time was chosen as the optimum period needed to achieve a complete equilibrium surfactant/liposome for the lipid concentration range used (3,20). Light-scattering measurements were made in the spectrofluorophotometer at 25°C with both monochromators adjusted to 500 nm. The assays were carried out in triplicate, and the results given are average values.

RESULTS AND DISCUSSION

Mean vesicle size and stability of liposome suspensions. The vesicle size distribution of liposomes after preparation (PL ranging from 1.0 to 10.0 mM) varied little (around 200 nm). The PI, defined as a measure of the width of the particle size distribution obtained from the "cumulant analysis," remained below 0.1, which indicates that all liposome suspensions showed a homogeneous size distribution. The size of vesicles, after addition of equal volumes of PIPES buffer and equilibration for 24 h, showed values similar to those obtained after preparation, with a slight increase in PI (between 0.12 and 0.14). Hence, the liposome preparations appeared to be reasonably stable in the absence of surfactant under the experimental conditions used in solubilization studies.

Interaction of OG with liposomes. It is known that complete equilibrium may take several hours in surfactant/lipid systems (3,5). However, in subsolubilizing interactions, a substantial part of the surfactant effect takes place within approximately 30 min after its addition to the liposomes (22).

To determine the time needed to obtain a constant level of CF release from liposomes in the range of the PC investigated (1.0 and 10.0 mM), a kinetic study of the interaction of OG with liposomes was carried out. Liposome suspensions were

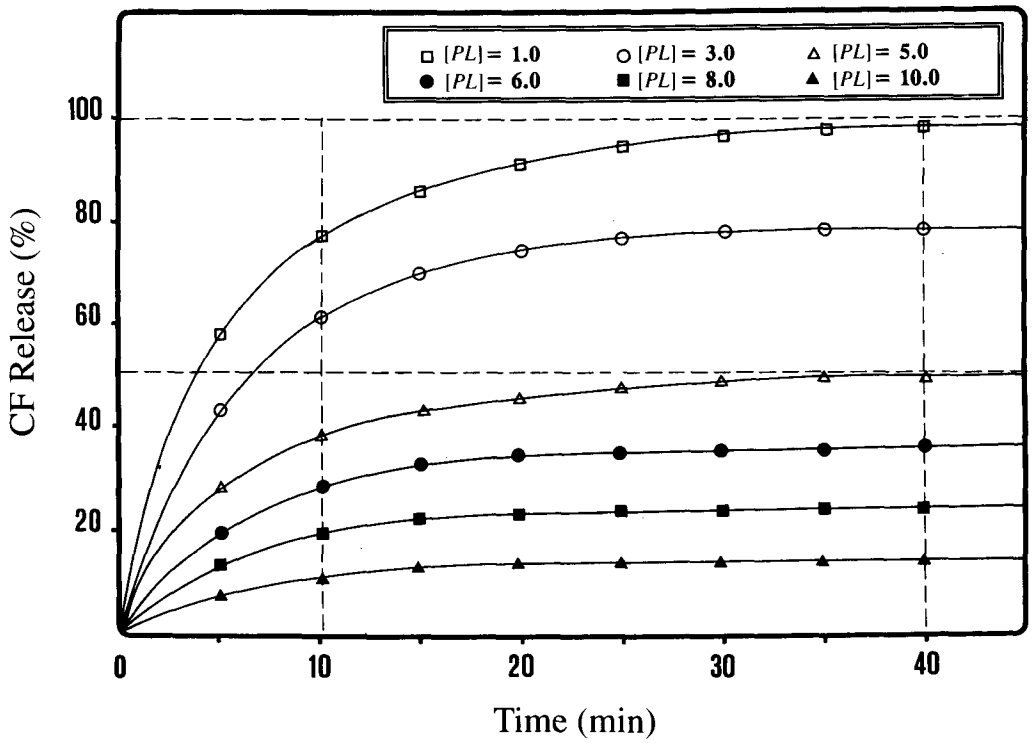

FIG. 1. Time curve of the release of 5(6)-carboxyfluorescein (CF) trapped in phosphatidylcholine liposomes caused by the addition of octyl glucoside (14.0 mM). *PL*, phospholipid concentration.

mixed with a constant subsolubilizing OG concentration (14.0 mM), and subsequent changes in permeability were studied as a function of time (Fig. 1). About 40 min was needed to achieve a constant level of CF release in all cases, despite the fact that approximately 80% of CF release took place during the first 10 min. Hence, changes in permeability were studied 40 min after addition of OG to the liposomes. The CF release of liposome suspensions in the absence of surfactant in this period of time was negligible due to the low spontaneous permeability of these bilayer structures.

To determine the Re and S_W parameters at subsolubilizing levels, a systematic study of permeability changes, caused by the addition of OG to liposomes, was carried out for different lipid concentrations. Changes in CF release were determined 40 min after surfactant addition. The results obtained are plotted in Figure 2. The surfactant concentrations that resulted in different percentages of CF release were graphically obtained and plotted for each of the PL whose lines corresponded to Equation 2, from which Re and S_W were determined. The regression coefficients (r^2) always showed higher values than 0.991, indicating that an acceptable linear relationship was established. The Re_{SAT} and Re_{SOL} values obtained (1.30 and 3.60, respectively) are in agreement with those previously reported (7,9).

In accordance with the procedure described by Urbaneja and colleagues (3,20), the solubilizing interaction of OG with liposomes was studied through changes in the static light scattered by these systems 24 h after addition of surfactant. Figure 3 shows the solubilization curves of liposome suspensions (*PL* ranging from 1.0 to 10.0 mM) arising from the addition of increasing amounts of OG. An initial increase in the scattered intensity of the system was always observed due to the surfactant incorporation into bilayers. Additional amounts of surfactant resulted in a fall in intensity until a low constant value was reached for bilayer solubilization. The arrows, indicating S_{SAT} and S_{SOL} (curve for *PL* 10.0 mM), corresponded to the surfactant concentration for saturation and solubilization of liposomes, respectively. The OG concentrations for different light-scattering percentages also were graphically obtained and plotted for each of the *PL*. An acceptable linear relationship was established in all cases (r^2 values higher than 0.990). The corresponding Re and S_W parameters were determined from these straight lines (Eq. 2).

Relationship between Re and S_W. Figure 4 shows the variation of S_W with Re throughout the interaction of surfactant/liposome (vesicles or mixed micelles). A progressive increase in S_W was observed as Re rises up to 100% CF release (Re = 1.18). Extrapolation of the curve (shaded area) led approximately to the initial S_W value for solubilization (100% light scattering, for Re_{SAT} = 1.30), which corresponded approximately to the surfactant CMC (S_W = 17.80 mM and OG CMC of 18.0 mM). This finding confirms that permeability alterations were determined by the action of surfactant monomers and supports the generally accepted assumption that the concentration of free surfactant must reach the CMC for solubilization to occur (5,23). The increase in Re resulted in a slight increase in S_W up to Re_{SOL}, which corresponds to the complete solubilization of liposomes through the formation of mixed micelles.

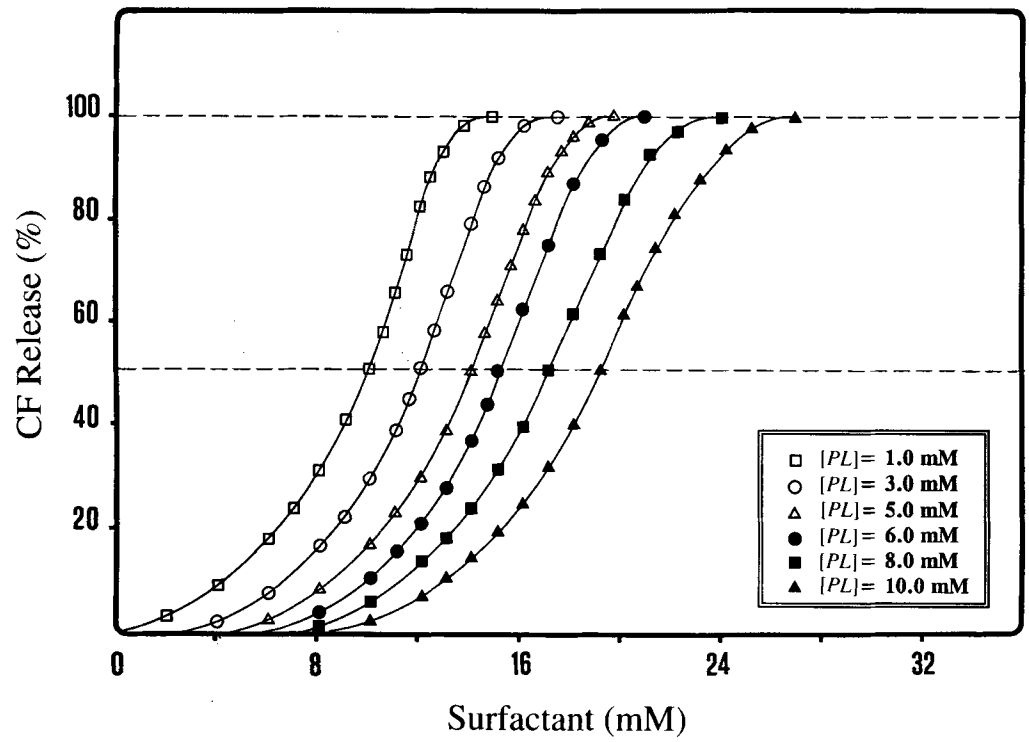

FIG. 2. Percentage changes in CF release of unilamellar liposomes (lipid concentration ranging from 1.0 to 10.0 mM), induced by the presence of increasing concentrations of octyl glucoside. See Figure 1 for abbreviations.

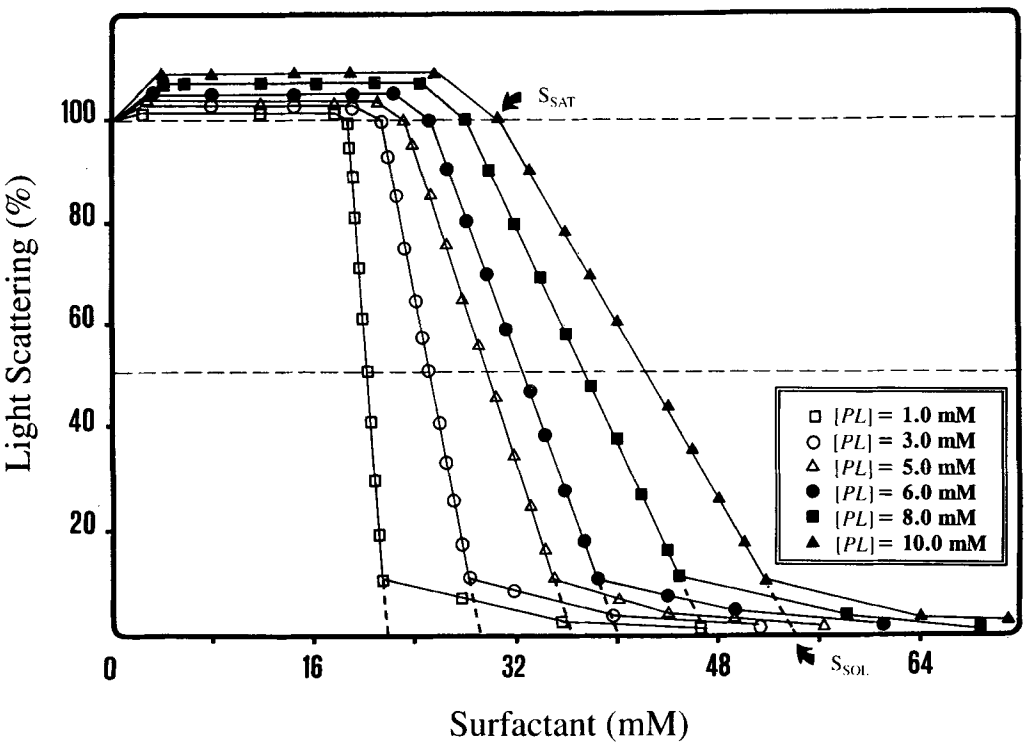

FIG. 3. Percentage change in static light-scattering of unilamellar liposomes (lipid concentration ranging between 1.0 and 10.0 mM), induced by the presence of increasing concentrations of octyl glucoside. The arrows indicating S_{SAT} and S_{SOL} (curve for phosphatidylcholine concentration 10.0 mM) corresponded, respectively, to the surfactant concentration for saturation and solubilization of liposomes.

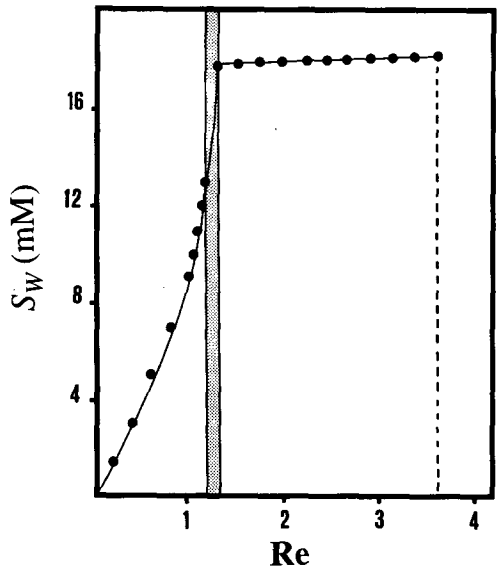

FIG. 4. Variation in the free surfactant concentration vs. the effective surfactant-to-phospholipid molar ratio (Re) during the overall interaction between octyl glucoside and phosphatidylcholine liposomes. S_W octyl glucoside concentration in the aqueous medium.

Dependence of the surfactant-PC aggregate size, CF release, and static light scattering on Re. A systematic investigation, based on dynamic light-scattering measurements of surfactant-PC aggregates was carried out throughout the process to elucidate the dependencies of the size of these aggregates (vesicles or micelles) and the changes in the percentages of both CF release and static light-scattering of the system on Re. The values obtained for 10.0 mM PC are given in Table 1. A progressive growth of vesicles was detected as the percentage of CF release increased, and the maximum increase was reached in the interval of CF release between 50–100%. The growth of vesicles occurred in a few seconds, with little further change over a period of hours. As for static light-scattering variations, the 100% value, corresponding to Re_{SAT} produced a slight fall in the vesicle size, even though with a monomodal distribution. When the light scattered by the system decreased, a sharp distribution curve appeared approximately at 57 nm, which indicates a new particle size distribution (PC-surfactant mixed micelles). The curve for these small particles rose up to 10% of scattered light, exhibiting (again at this point) a monomodal distribution, which corresponds to surfactant-PC mixed micelles (particles of 57 nm). These findings are in agreement with those reported by Almog *et al.* (7) for OG/PC liposome interactions. They demonstrated that, in the Re range between 1.4 and 3.2 (approximately 90–20% static light-scattering), vesicles and micelles coexisted (7).

Figure 5 shows variation in both percentage of CF release and vesicle size of liposomes vs. Re at subsolubilizing levels. The increase in Re led initially to a linear increase in both the percentage of CF release and the size of vesicles. However,

TABLE 1
Mean Size Distributions (nm) and Polydispersity Indexes of Surfactant-PC Aggregates (vesicles or mixed micelles) Resulting in the Overall Interaction of OG with PC Liposomes[a]

	Curve distribution (particle number)						
		1st peak		2nd peak		Average	Polydispersity
	Type[b]	(nm)	(%)	(nm)	(%)	(nm)	index
CF release (%)							
0	M	—	—	200	23.4	200	0.100
10	M	—	—	244	23.3	244	0.119
20	M	—	—	287	23.3	287	0.129
30	M	—	—	335	23.2	335	0.137
40	M	—	—	377	23.2	377	0.141
50	M	—	—	400	23.3	400	0.150
60	M	—	—	402	23.3	402	0.160
70	M	—	—	405	23.2	405	0.166
80	M	—	—	405	23.3	405	0.171
90	M	—	—	403	23.4	403	0.176
100	M	—	—	400	23.3	400	0.180
Light-scattering (%)							
100	M	—	—	365	23.2	365	0.203
90	B	57	2.0	357	21.3	331	0.239
80	B	57	4.3	345	19.3	293	0.252
70	B	57	5.5	303	18.0	245	0.243
60	B	57	6.4	258	16.9	203	0.231
50	B	57	7.5	220	15.9	168	0.226
40	B	57	8.7	177	14.6	132	0.215
30	B	57	12.4	122	10.9	87	0.210
20	B	57	18.3	109	5.1	68	0.205
10	M	57	23.1	—	—	57	0.180
0	M	57	23.6	—	—	57	0.154

[a]PC, phosphatidylcholine; OG, octyl glucoside; CF, 5(6)-carboxyfluorescein.
[b]M, monomodal; B, bimodal.

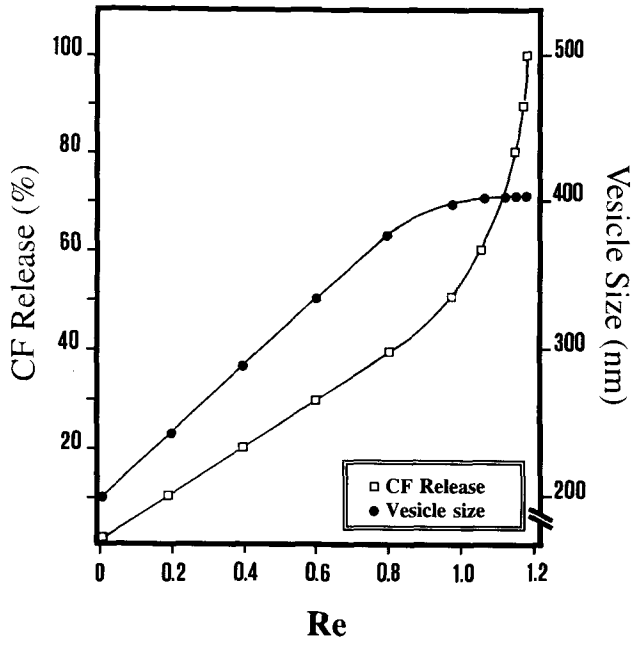

FIG. 5. Variation in the percentage of CF release and vesicle size of liposomes vs. Re at subsolubilizing level. See Figures 1 and 4 for abbreviations.

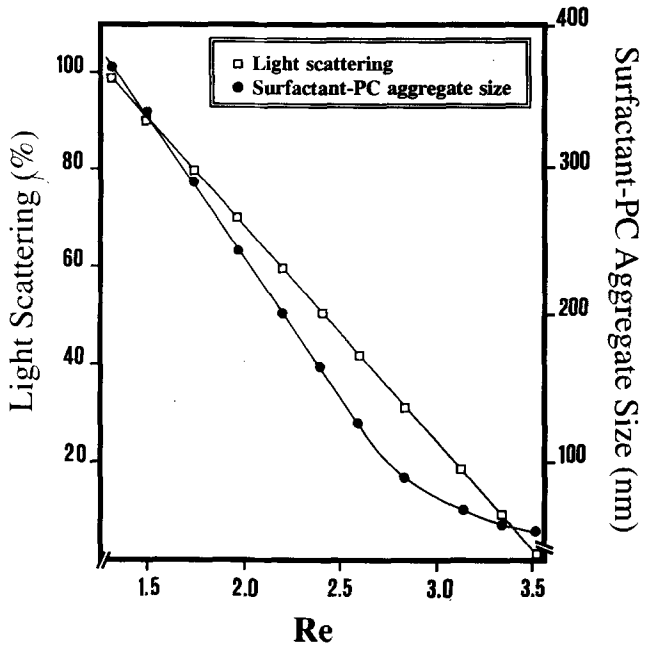

FIG. 6. Variation in the percentage of static light scattering and surfactant-phosphatidylcholine aggregate size vs. Re at solubilizing level. See Figure 4 for abbreviation.

Re values exceeding 1.0 resulted in a lower growth of vesicles, which achieved a maximum for 70–80% CF. This finding provides new information with respect to that reported by Vinson *et al.* (24), who studied this interaction by the Cryo-TEM technique. Thus, whereas Vinson *et al.* (24) reported a drastic decrease in the vesicle size for approximately 40–50% CF release, we detected a growth of the vesicles in this interaction step (Table 1).

Given that more than 80% of the permeability changes occurred in the initial interaction steps (Fig. 1) and that the vesicle growth took place in a few seconds after OG addition, we may assume that the growth of vesicles was related to the leakage of entrapped CF for Re values below 1.0. These two physicochemical properties were also dependent on the bilayer composition (Re). These findings are in agreement with those reported by Almog *et al.* (7). In the interval of CF release percentages between 60–100 (Re values between 1.05 and 1.18), an abrupt increase in bilayer permeability occurs, with only a slight change in the size of vesicles. These findings may be explained by bearing in mind that, at low Re (Re up to 1.0), possibly only the outer vesicle leaflet was available for interaction with surfactant molecules (linear increases in % of CF released and in the size of vesicles). However, increasing Re (Re higher than 1.0) leads to an increased rate of flip-flop of the surfactant molecules (or permeabilization of the bilayers to surfactant); thus it also makes the inner monolayer available for interaction with added surfactant. These findings are in agreement with the results previously reported by Schubert *et al.* (25) for the interaction of sodium cholate with PC liposomes.

Figure 6 shows the variation in the percentage of static light scattering and the surfactant-PC aggregate size (average mean) vs. Re at solubilizing levels. The increase in Re produced a linear decrease in both parameters, except for the surfactant-PC aggregate size in the range of static light-scattering values between 30 and 10% (Re values higher than 3.0). This means that, in the range of Re values between 1.3 and 3.0 (corresponding to the mixed micelle formation), a direct correlation between both parameters was established, as well as with the composition of surfactant-PC aggregates and, consequently, with the Re parameter.

From these findings we may conclude that a direct relationship was established in the initial interaction steps (10–50% CF release) between the growth of vesicles, the leakage of entrapped CF, and the Re. However, in the interval of CF release percentages between 60–100 (Re values between 1.05 and 1.18), an abrupt increase in bilayer permeability occurs with only a slight change in the size of vesicles. A similar relationship was established at solubilizing levels in the range of Re values between 1.3 and 3.0, where decreases in the surfactant-PC aggregate size and in the static light-scattering also depended linearly on Re and, hence, on the composition of these aggregates.

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