Permeability Changes of Phospholipid Vesicles Caused by Surfactants

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The partition coefficient at equilibrium of different surfactants between the aqueous phase and the lipid bilayer of small unilamellar vesicle (SUV) liposome has been determined. The release of the fluorescent agent 5-(6) carboxyfluorescein from the interior of liposomes, induced by a nonionic surfactant octylphenol ethoxylated with 10 units of ethylene oxide (Triton X-100), by two anionic surfactants — sodium dodecyl sulphate and sodium dodecyl ether sulphate — and by an amphoteric surfactant dodecyl betaine was studied at sub-solubilizing concentrations. The following increasing order of the partition coefficients obtained for each surfactant can be observed: Triton X-100 > sodium dodecyl ether sulphate > sodium dodecyl sulphate > dodecyl betaine. There was a strong positive association between coefficient of partition and the ability of the different surfactants to modify the permeability of liposomes. The importance of the presence of ethylene oxide units in the molecular structure of the surfactant in relation to alter the partition coefficient in front of SUV liposomes is indicated.

KEY WORDS: Liposome-surfactants interaction, partition coefficients, permeability changes.

Phospholipid vesicles or liposomes are interesting structures which have rapidly come into widespread use as models for biological membranes and as delivery systems where encapsulation and protection of substances are required, such as drug delivery (1,2).

Water soluble amphiphiles (surfactants) are commonly in use in membrane research (3). However, certain aspects of surfactant action at sublytic concentrations is poorly understood. The area of liposome-surfactant interaction is interesting for two at least important reasons — membrane reconstitution and membrane fusion. As a consequence, surfactants have been used to prepare large unilamellar vesicles using removal methods so that the physicochemical properties of the lipid surfactant systems have been progressively investigated (4-7). On the other hand, the interactions of surfactants with phospholipid vesicles have been extensively studied as a result of the high interest of the solubilization or permeability changes of biological membranes (8-14).

At sub-solubilizing concentrations, surfactants incorporate into the phospholipidic bilayers, where they cause changes in its physical properties (15,16). An obvious consequence of such perturbations could be a change in membrane permeability. At such concentrations it is generally accepted that equilibrium partition of the surfactant between the bilayer and the aqueous medium governs the incorporation of surfactant into the bilayer (17). For a system containing PL (mM phospholipid) and S_T (mM surfactant), a partition coefficient can be defined as:

$$K = \frac{S_B/PL}{S_w}$$

where S_W and S_B are concentrations of surfactant in the aqueous and bilayer, respectively. From the definition of an effective surfactant to phospholipid ratio, R_{eff} as:

$$R_{eff} = \frac{S_B}{PL}$$

It follows that:

$$K = \frac{R_{eff}}{S_w}$$

In the present work, the determination of the partition coefficients of different surfactants between lipid bilayer and aqueous medium in small unilamellar vesicles, directly related with its ability to modify the permeability of liposomes, has been carried out through a series of experiments based on the measurements of 5-(6) carboxyfluorescein release from the interior of liposome vesicles.

Fluorescence self-quenching (FSQ) methods are based on the loss of fluorescence efficiency when fluorophore molecules are present at high concentration (18). Thus, these molecules entrapped inside a liposome may emit only a few percent of the fluorescence that it would if released and diluted into the surrounding medium. The approach, then, is to monitor the fluorescence of liposomes containing a concentrated 5-(6) carboxyfluorescein solution. Almost all of the fluorescence detected can be ascribed to this fluorescent dye released from liposomes, and total fluorescence is then determined after breaking up the remaining vesicles normally with Triton X-100 (19,20).

The selected surfactants were sodium dodecyl sulphate as a typical anionic surfactant widely used both in theoretical studies and practical applications; a sodium dodecyl ether sulphate to find the influence of the ethylene oxide groups on the anionic surfactant behavior; octyl phenolethoxilated with 10 units of ethylene oxide (Triton X-100) as a representative nonionic surfactant, used in the solubilization of phospholipid membranes (21-25); and dodecyl betaine as a representative of amphoteric surfactant frequently used in cosmetic formulations (26). When the surfactant concentrations that promote the half release of carboxyfluorescein from liposomes are plotted on the ordinate against the phospholipid concentrations on abscise, a linear relationship is observed. This linear dependence could be described by the following equation:

$$S_T = S_W + R_{eff} \cdot (PL)$$

The K values obtained by dividing the slope R_{eff} by the ordinate value S_W could allow establishment of a criterium for the evaluation of surfactant activity on phospholipid vesicles.

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MATERIALS AND METHODS

Phosphatidylcholine (PC) was purified from egg lecitin (Merck) according to the method of Singleton (27), and shown to be pure by thin-layer chromatography (TLC). The fatty acid composition of the PC, determined by gas-liquid chromatography (GLC), was as follows: palmitic acid (16:0), 37.7%; stearic acid, (18:0) 7.0%; oleic acid, (18:1) 36.4%; and linoleic acid (18:2), 17.5%. Phosphatidic acid (PA) from egg yolk lecitin was purchased from Sigma Chemical Co. (St. Louis, MO). Both lipids were stored in chloroform under nitrogen at -20°C until use.

Sodium dodecyl sulphate (SDS) was obtained from Merck and sodium dodecyl ether sulphate (SDES) was supplied by Tenneco SA (Barcelona, Spain). The latter was a commercial grade product with an active matter of 28.8%, 2.5 average in ethylene oxide units and the following average in alkyl chain: C-10, 3.9%; C-12, 68.1%; C-14, 22.2%; and C-16, 4.9%. Nonionic surfactant Triton X-100 (OP-10EO), octylphenol ethoxylated with 10 units of ethylene oxide and an active matter of 100% was used. The amphoteric surfactant dodecyl betaine (D-Bet) was specially prepared by Albright and Wilson Ltd. (Warley, West Midlands, U.K.); the active matter was 30% in aqueous solution and the amino free contents was 0.20%.

Piperazine-1,4-*bis*(2-ethanesulphonic acid) (PIPES buffer) obtained from Merck was prepared as 20 mM PIPES adjusted to pH 7.2 with NaOH, containing 110 mM Na_2SO_4. Polycarbonate membranes and membrane holders were purchased from Nucleopore. 5-(6) Carboxyfluorescein (CF), was obtained from Eastman Kodak (Rochester, NY) and further purified by a column chromatographic method (28).

Liposome preparation. Small unilamellar vesicles (SUV) were prepared by extrusion of large unilamellar vesicles, previously obtained by the reverse phase evaporation method (29,30) which was based on the earlier protocol described by Szoka and Papahadjopoulos (31). Briefly a chloroform solution containing egg phosphatidylcholine and phosphatidic acid with a molar ratio of 9:1 was evaporated. Then, a 3:1 v/v mixture of ethyl ether/PIPES buffer containing 10 mM CF was added. Gentle sonication led to the formation of a W/O type emulsion. After evaporating the ethyl ether under reduced pressure, a viscous gel was formed. The elimination of the final traces of the organic solvent transformed the gel into a liposome suspension. Small unilamellar vesicles were obtained by extrusion of vesicle suspensions through 0.8, 0.4, 0.2 and 0.1 μm polycarbonate membranes (Nucleopore, Pleasanton, CA) to obtain an uniform size distribution (32). Vesicles were freed of unencapsulated material by separation through Sephadex G-50 (Pharmacia, Uppsala, Sweden) by column chromatography. The range of phospholipid concentration in liposome suspension studied was 0.1-1.0 mM.

Phosphorus estimation. Phospholipid concentration of the liposome vesicles was determined by Allen's method (33).

Surface tension measurements. Surface tension values were measured by the ring method (34) with a Lauda tensiometer 7201. The apparent values of surface tension were corrected using the Harking-Jordan factors. The critical micelle concentration (CMC) of the surfactants in water and PIPES buffer was determined, plotting these corrected surface tension values vs concentration.

Quasielastic light scattering. The mean size and polydispersity of the liposome preparations obtained by the combination of reverse phase evaporation and extrusion through polycarbonate membranes were determined by a Photon Correlator Spectrometer (Malvern Autosizer IIc). The instrument consists of an optical unit with a 5 mW Laser of He-Ne (λ=623.8 nm), a temperature-controlled cell holder, a digital autocorrelator (Multi-8 of 72 channel model 7032) and "on line" data analysis performed by a computer. Samples were adjusted to the adequate concentration range with PIPES buffer. The measurements were made at 25°C, lecture angle of 90°.

Monitoring the release of CF from liposomes. A suspension of SUV liposomes containing concentrated CF in the interior hardly fluoresces, but fluorescence strongly increases upon liberation from the concentration quenching when CF is released from the interior to the bulk aqueous phase. Therefore, permeability changes of liposomal bilayers induced by surfactants can be determined quantitatively by monitoring the increase in the fluorescence intensity of CF (8,19).

Fluorescence measurements were run on a Shimadzu RF-540 spectrofluorophotometer (Shimadzu, Columbia, MD) equipped with a thermoregulated cell compartment using an excitation wavelength at 495 nm and emission at 515.4 nm. Small amounts of the buffered solutions, containing different surfactant concentrations, were added to quartz cuvettes filled with a liposome suspension. The fluorescence intensity measurements were made at 25°C. The total amount of CF encapsulated in liposomes was determined by completely destroying the liposomes by the addition of 60 μL of 10% v/v Triton X-100 aqueous solution to 2.0 mL of liposome suspension (19).

The amount of released CF was calculated by means of the following equation (8):

$$\% \text{ CF release} = \frac{I_t - I_o}{I_\infty - I_o} \times 100$$

where I_o is the fluorescence intensity of CF-loaded liposome suspension at 515.4 nm in the absence of any surfactant at initial time, and I_∞ is the fluorescence intensity at 515.4 nm after destroying the liposomes by addition of Triton X-100, as mentioned above. I_t corresponds to the fluorescence intensity at the same wavelength measured at 40 min after adding the surfactant solution to a liposome suspension.

RESULTS AND DISCUSSION

Determination of particle size distribution of liposomes. The determination of particle size distribution of liposomes suspensions was carried out using a Malvern Autosizer IIc, as described earlier (35).

Particle size of liposome suspension in the range of phospholipid concentrations from 0.165 mM to 0.990 mM varied little (Table 1). The index of polydispersity value is indicated for each liposome suspension. It can be observed that in all cases the particle size distribution shows a similar value around 100 mn, confirming that these liposomes were SUV. In addition, the polydispersity index values were lower than 0.1, indicating that the size distribution was very homogeneous.

Determination of the critical micelle concentration. In the study of permeability changes caused by surfactants, it is useful to know the values of the CMC of these

TABLE 1

Particle Size Distribution of Liposome Suspensions and Polydispersity Index Values for Different Phospholipid Concentrations

(PL)	0.165 mM	0.330 mM	0.495 mM	0.660 mM	0.825 mM	0.990 mM
Mean vesicle size (nm)	101	102	104	102	104	103
Polydispersity index	0.091	0.091	0.090	0.087	0.088	0.090

surfactants in the aqueous working medium. The CMC results obtained for each surfactant in the buffered medium and in water are shown in Table 2. The CMC values for the studied surfactants differ in the PIPES buffer from those obtained in water (except for the nonionic surfactant OP-10EO), probably due to the higher ionic strength.

TABLE 2

CMC Values Obtained at 25°C for OP-10EO, SDS, SDES and D-Bet in Water and PIPES Buffer

	CMC (mM)	
Surfactant	Water	PIPES buffer
OP-10EO	0.18	0.15
SDS	7.5	0.50
SDES	2.0	0.12
D-Bet	2.0	1.25

Permeability studies. In order to determine the rate at which the liposome membranes were permeabilized by surfactants a time curve of the changes in CF fluorescence were carried out with OP-10EO, SDS, SDES, and D-Bet. For these tests SUV liposome suspensions at two concentrations of phospholipid (0.1 mM and 1.0 mM) were treated with surfactant (0.3mM), and subsequent changes in permeability were studied as a function of time. Measurements of CF release from liposomes were made both in presence and in absence of surfactants.

The CF release values shown in Figures 1 and 2 are given as a difference between CF released in presence and in absence of surfactants, with the purpose to shows only the real increase of CF release caused by surfactants. The CF release of liposome suspensions in absence of surfactants after 40 min shows values between 0.5 and 1.2%.

The permeability kinetics for each surfactant follows different ways (Fig. 1). The surfactant that stabilized the permeability rate is OP-10EO for both phospholipid concentrations. It requires 20 min to obtain a release equilibrium. On the contrary, the anionic surfactants SDS and SDES need 40 min to reach the equilibrium, especially at higher lipid concentration in liposomes (Fig. 1B). It can be seen that for a given surfactant, CF release is higher when phospholipid vesicle concentration is lower (Fig. 1A). As a consequence, the changes in permeability were studied in all cases 40 min after the addition of surfactants to liposomes at 25°C.

Considering the permeability changes caused by anionic surfactants, a ratio could be established between the time necessary to obtain a constant value of permeability and the presence of phosphatidic acid in phospholipid bilayers (molar ratio 9:1). Phosphatidic acid promotes some electrostatic repulsion between both compounds which partially inhibit the interaction, mainly in the early stage of process.

In order to know the partition coefficient of surfactants between aqueous media and lipid bilayers, a systematic investigation of SUV liposome permeability against each surfactant was carried out studying the changes in CF release from 0.1 to 1.0 mM phospholipid concentration

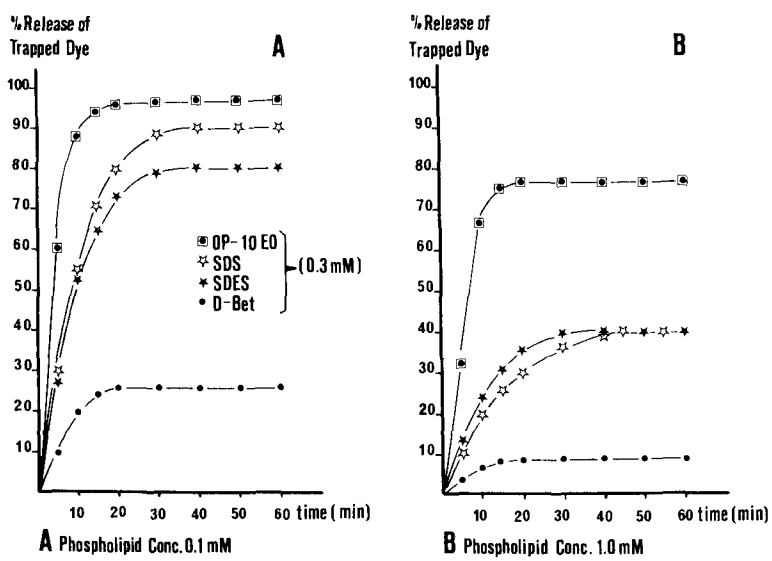

FIG. 1. Time curve of the release of CF trapped in SUV liposomes caused by OP-10EO, SDS, SDES, and D-Bet surfactants (0.3 mM). The phospholipid concentrations of liposome were 0.1 mM (A) and 1.0 mM (B).

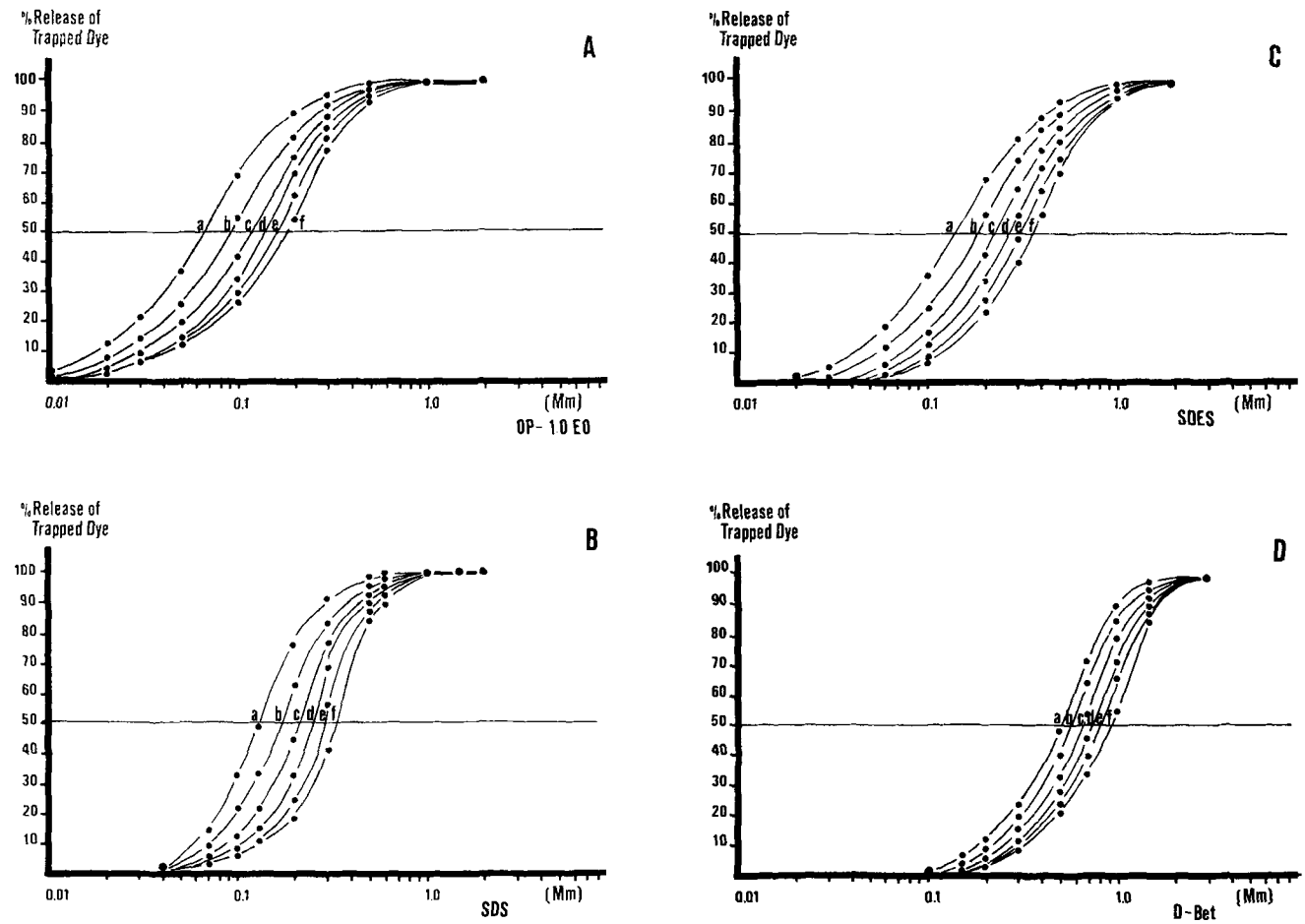

FIG. 2. CF release caused by surfactants (A, OP-10EO, B, SDS, C, SDES, and D, D-Bet), for suspensions of SUV liposomes at different phospholipid concentrations (a, 0.165 mM; b, 0.330 mM; c, 0.495 mM; d, 0.660 mM; e, 0.825 mM; and f, 0.990 mM).

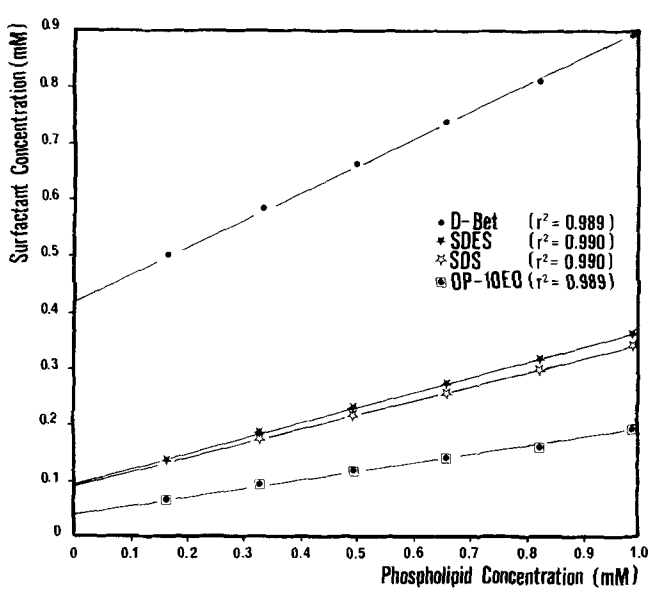

FIG. 3. Surfactant concentrations that promote half maximal value of CF release *vs.* phospholipid concentration. The regression coefficients of the straight lines of each surfactant are given.

range. Percentage of CF release as a function of surfactant concentration for different SUV liposome suspensions are plotted in Figure 2. From these data, the surfactant concentration that promote half-maximal value of CF release have been determined and represented vs phospholipid concentration in Figure 3. A quite acceptable linear relationship is established in each case, the graphs corresponding, as previously stated in the equation:

$$S_T = S_W + R_{eff}\,(PL)$$

where the effective surfactant to phospholipid molar ratio R_{eff} and the aqueous concentration of surfactant S_W are the slope and the ordinate at the origin (zero phospholipid concentration), respectively. These results are shown in Table 3.

Table 2 shows that all S_W values are always smaller than the corresponding CMC values. Thus, SDS has a S_W value of 17.8% in respect to its CMC OP-10EO presents a S_W value of 27.3% in respect to its CMC, and D-Bet and SDES show S_W values of 33.6% and 75% of their corresponding CMCs, respectively.

These results suggest that surfactant-liposome interactions must be ruled mainly by the action of surfactant monomers on the lipid bilayers, unlike the behavior of the surfactants in solubilization processes (3,11), where micelle formation plays a very important role.

TABLE 3

Partition Coefficients (K) of OP-1OEO, SDS, SDES, and D-Bet Between Liposomes and Aqueous Medium

Surfactant	S_w (mM)	R_{eff} (mole/mole)	K (mM^{-1})
OP-1OEO	0.041	0.050	3.65
SDS	0.089	0.253	2.84
SDES	0.090	0.272	3.03
D-Bet	0.420	0.484	1.15

Considering the R_{eff} values, it can be seen that D-Bet is the surfactant whose monomers show the greatest tendency to incorporate themselves onto the phospholipidic bilayers (0.484 mole/mole), while the nonionic surfactant OP-10EO (0.150 mole/mole) shows the smallest tendency. When these tendencies are reported at a given concentration of surfactant monomers in equilibrium with those incorporated in the bilayer it can be obtained the partition coefficient K for each surfactant. From these data the surfactant that shows a high K value is the nonionic surfactant OP-10EO (3.65 mM^{-1}) followed by the anionic surfactants SDES (3.03 mM^{-1}) and SDS (2.84 mM^{-1}). The lower K value of the surfactant tested corresponds to the amphoteric surfactant D-Bet (1.15 mM^{-1}). Comparing the results of Figure 1 with the values given in Table 3 a positive association between coefficient of partition and the ability of the different surfactants to modify the permeability of liposomes can be established.

In general terms, the relative importance of the presence of ethylene oxide units in the molecular structure of surfactants in relation to the changes in the partition coefficient values can be assigned. In this sense, comparing the K values of the two anionic surfactant considered (SDS and SDES), the increase of the K value of the SDES in front of SDS could be attributable to the presence of 2.5 ethylene oxide units in its molecular structure, because it is the unique structural difference existing between both anionic surfactants. Those ethylene oxide units increase the hydrophilic character of the surfactant. This fact could be responsible for the changes in K values. On the other hand, the K value obtained for OP-10EO is comparable with that reported in the literature (36), confirming the effectiveness of this nonionic surfactant in the interaction with lipidic bilayers.

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