Surfactant-Induced Release from Phosphatidylcholine Vesicles. Regulation of Rupture and Leakage Pathways by Membrane Packing[1]

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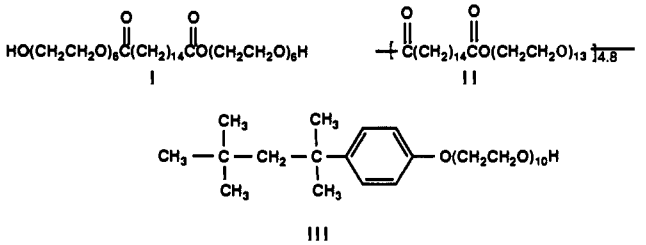
Abstract: A double-headed, single-chain surfactant [$HO(CH_2CH_2O)_6CO(CH_2)_{14}CO_2(CH_2CH_2O)_6H$, I] and Triton X-100 (III) have been found to induce the release of vesicle-encapsulated 5(6)-carboxyfluorescein (CF) by two distinct pathways: (i) a *leakage* process in which there is a gradual release of CF from *all* of the vesicles, and (ii) a catastrophic *rupture* event, whereby a portion of the vesicles rapidly release their entire contents and the remaining fraction of vesicles release none. Evidence for the existence of both pathways, and for their regulation by membrane packing, has been obtained from the measurement of self-quenching efficiencies of vesicular CF before and after incubation with these amphiphiles. Release of CF from fluid vesicles derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), induced by a polymeric analogue of I (i.e., $[CO(CH_2)_{14}CO_2(CH_2CH_2O)_{13}]_{4.8}$, II), has also been found to proceed via a leakage pathway. A kinetic model, which has been developed for the leakage process, implies that small aggregates of membrane-bound surfactant are responsible for the release of CF.

Introduction

Despite numerous investigations that have centered around surfactant–lipid bilayer interactions, the precise mechanism by which a surfactant disrupts a lamellar phase remains poorly understood.[3] One experimental technique that has been extensively used to probe surfactant–lipid membrane interactions involves the measurement of the release of vesicle-entrapped 5(6)-carboxyfluorescein (CF).[4-9] Although this method provides insight into the potency of a disruptive surfactant, and also the lability of the bilayer target, several fundamental issues regarding the nature of this disruption processes remain to be clarified. For example, does the surfactant-induced release of CF occur by a catastrophic *rupture* event, whereby a portion of the vesicles rapidly release their entire contents and the remaining vesicles release none? Alternatively, does the release proceed by a *leakage* process in which there is a gradual release of CF from *all* of the vesicles? Can modest changes in lipid packing significantly affect the pathway for release? Does the disruptive surfactant bind to the vesicular membrane prior to CF release? Does the surfactant act independently in perturbing the bilayer, or does it function in a cooperative manner? If a polymeric surfactant is employed, can it force a rupture process by maintaining clusters of defects within the membrane?

In this paper, we examine the above questions by measuring the fluorescence self-quenching efficiency of vesicle-entrapped CF before and after incubation with three different membrane-disrupting surfactants and also by analyzing the kinetics of the release. Our motivation for this work stems from our hypothesis that a detailed understanding of how surfactants disrupt lipid membranes should help to identify those structural and/or compositional features of biomembranes that may be exploitable from a therapeutic standpoint. A long-range goal of our efforts in this area is to design, rationally, novel classes of antimicrobial agents that can selectively alter the integrity, fusogenic properties, permeability, and recognizability of microbial membranes in the presence of mammalian cells.

Specific targets that have been used in the present study were derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), POPC/cholesterol (2/1), and POPC/cholesterol (1/1). The compactness of bilayers made from DPPC can be readily modified via temperature. In particular, below its gel to liquid-crystalline phase transition temperature (T_m = 41.4 °C), DPPC membranes exist in a highly compact state, where the aliphatic chains are fully extended in an all-trans conformation. At higher temperatures, gauche conformations are introduced into the lipid as the membrane is transformed into a fluid state.[10] Vesicles made from POPC and POPC/cholesterol exist in the physiologically relevant fluid phase at room temperature. The compactness of mixed fluid membranes, in general, tends to increase with increasing mole percentages of cholesterol, as measured by their permeability properties; i.e., at high concentrations of cholesterol, mixed lipid membranes are less permeable toward solutes. The membrane-disrupting agents that have been investigated in this work include a recently introduced double-headed, single-chain surfactant (I),



I II III

a polymeric analogue (II), and one commonly used surfactant for biomembrane disruption, i.e., Triton X-100 (III).[4-6] These surfactants have been shown to cover a broad range of membrane-

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(3) Reviews: Ruiz, J.; Goni, F. M.; Alonso, A. *Biochim. Biophys. Acta* **1988**, *937*, 127. Lichtenberg, D.; Robson, R. J.; Dennis, E. A. *Biochim. Biophys. Acta* **1983**, *737*, 285. Helenius, A.; Simons, K. *Biochim. Biophys. Acta* **1975**, *415*, 29.

(4) Jayasuriya, N.; Bosak, S.; Regen, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 5844.

(5) Jayasuriya, N.; Bosak, S.; Regen, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 5851.

(6) Nagawa, Y.; Regen, S. L. *J. Am. Chem. Soc.* **1991**, *113*, 7237.

(7) Weinstein, J. N.; Ralston, E.; Leserman, L. D.; Klausner, R. D.; Dragsten, P.; Henkart, P.; Blumenthal, R. In *Liposome Technology*; Gregoriadis, G., Ed.; CRC: Boca Raton, FL, 1984; Vol. III, Chapter 13, p 183.

(8) Weinstein, J. N.; Klausner, R. D.; Innerarity, T.; Ralston, E.; Blumenthal, R. *Biochim. Biophys. Acta* **1981**, *647*, 270.

(9) Klausner, R. D.; Kumar, N.; Weinstein, J. N.; Blumenthal, R.; Flavin, M. *J. Biol. Chem.* **1981**, *256*, 5879.

(10) Wilkinson, D. A.; Nagle, J. F. In *Liposomes: From Physical Structure To Therapeutic Applications*; Knight, C. G., Ed.; Elsevier North-Holland Biomedical Press: New York, 1981; p 273.

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disrupting activity as well as selectivity.[4-6]

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were obtained from Avanti Polar Lipids (Birmingham, AL) as chloroform solutions and used directly. 5(6)-Carboxyfluorescein (CF) was obtained from Eastman Kodak and purified according to literature methods.[7] Cholesterol and Triton X-100 were purchased from Aldrich Chemical Co. and used directly. The bolaphile I and supramolecular surfactant II were prepared using procedures similar to those previously described.[4,5] House-deionized water was purified using a Millipore Milli-Q-filtering system containing one carbon and two ion-exchange stages. Vesicle dispersions were normally prepared in a 10 mM borate buffer (pH 7.4) containing 140 mM NaCl and 2 mM NaN_3 (throughout this paper we refer to this buffer as "borate buffer"). Chloroform that was used was of HPLC grade (Burdick and Jackson). Vesicle extrusions were carried out with a Lipex Biomembrane apparatus (Vancouver, BC). Dynamic light scattering measurements were carried out by using a Nicomp 270 submicrometer particle analyzer, equipped with a helium–neon laser (632.8 nm, scattering angle of 90°) and a computing autocorrelator. Samples were filtered by using a 0.45-μm HV4 Millipore filter prior to light scattering measurements. All fluorescence measurements were made using a Turner fluorometer (Model 112). Experimental procedures that were used for measurement of fluorescence self-quenching and rupture/leakage were similar to those described by Weinstein.[7-9]

Preparation of Vesicles from POPC/Cholesterol. Typically, 2 mL of a chloroform solution containing 20 mg (0.026 mmol) of POPC was placed in a test tube (13 × 100 mm) and the solvent then removed under a stream of nitrogen. Cholesterol (10 mg, 0.026 mmol) was added directly to this tube and the mixture redissolved in 1 mL of chloroform. The chloroform was then removed under a stream of nitrogen. After drying [12 h, 23 °C (0.3 mmHg)], the resulting film was dispersed in 1 mL of 100 mM in CF, via vortex mixing. The resulting multilamellar vesicle dispersion was allowed to equilibrate for 0.5 h, subjected to five freeze–thaw cycles (liquid nitrogen), and extruded (10 times) through a 0.1-μm polycarbonate filter (Nuclepore Co.). To remove nonentrapped CF, the resulting large unilamellar vesicles (1000 Å) were purified via gel filtration on a Sephadex G-50 column (1.2 × 40 cm), using a borate buffer as the eluant. Vesicle fractions were collected, and the final volume was adjusted to 5 mL by adding additional buffer. The resulting dispersion was then dialyzed against 200 mL of borate buffer (12 h, 15 °C) and allowed to reach room temperature prior to use.

Procedures that were used for the preparation of other POPC-based vesicles were similar to that which is described above. For vesicles that were prepared from DPPC, vortex mixing and extrusions were carried out at 50 °C. In addition, freeze–thawing cycles were carried out at −196 and 50 °C. Except for calibration curves, the initial vesicular concentration of CF that was used in all release experiments was 100 mM.

Calibration Curves for Fluorescence Self-Quenching Efficiency of Vesicle-Entrapped CF. A series of vesicles (1000 Å) were prepared from POPC (and also from DPPC), using concentrations of CF equaling 10, 25, 50, and 100 mM. An aliquot (5 μL) of each vesicle dispersion was diluted with 4 mL of borate buffer and its fluorescence analyzed to give a value for the vesicle-entrapped CF. A total fluorescence value was then measured after complete disruption of the vesicles, by addition of 50 μL of Triton X-100 (100 mg/mL). The self-quenching efficiency (Q) was determined by use of the following equation: $Q = (1 - \alpha)100$, where α is the fluorescence of vesicle-entrapped CF divided by the total fluorescence after complete disruption of the vesicles by Triton X-100.

Percentage of CF Release after Incubation with Surfactant. An aliquot (10 μL) of an appropriate vesicle dispersion was added to each of a series of test tubes (6 × 50 mm), which contained 90 μL of a given surfactant solution (the final concentration of lipid was ca. 0.5 mM as determined by phosphorus analysis), and the resulting suspension was agitated by vortex mixing for ca. 10 s. After the mixture was allowed to incubate for 0.5 h at 23 °C (or 50 °C for one set of experiments with DPPC), 50-μL aliquots were withdrawn and diluted with 4 mL of borate buffer. The fluorescence was then determined. A blank value was determined by treating 10-μL aliquots of the original vesicle dispersion with 90 μL of buffer, in the absence of detergent. A total fluorescence value was determined by complete disruption of the vesicles, using 90 μL of a buffer solution which was 80 mM in Triton X-100. The percentage of released CF was calculated according to $I(\%) = 100\ (I_a - I_b)/(I_x - I_b)$, where I_x is the 100% fluorescence intensity determined using an excess of Triton X-100 and I_a and I_b are the fluorescence intensities after incubation with and without surfactant, respectively.

The ranges of concentrations of surfactants that were used for these release experiments were as follows: I disrupting POPC; 1.22–2.46 × 10^{-4} M; II disrupting POPC, 0.95–2.14 × 10^{-5} M (repeat unit concentration); III disrupting POPC, 3.21–5.77 × 10^{-4} M; III disrupting POPC/cholesterol (1/1); 3.19–11.5 × 10^{-4} M; III disrupting POPC/cholesterol (2/1), 4.50–5.46 × 10^{-4} M; III disrupting DPPC at 23 °C, 1.28–3.21 × 10^{-4} M; I disrupting DPPC at 23 °C, 1.22–8.85 × 10^{-4} M; III disrupting DPPC at 50 °C, 0.57–1.28 × 10^{-4} M.

Table I. Pseudo-First-Order Rate Constants for Surfactant-Induced Release of CF from POPC-Based Vesicles

surfactant	D_0,[a] M	PL_0,[b] M	k_{exp}, min^{-1}
I	3.68×10^{-5}	4.21×10^{-4}	3.80×10^{-3}
I	6.13×10^{-5}	4.21×10^{-4}	1.07×10^{-2}
I	8.59×10^{-5}	4.21×10^{-4}	2.85×10^{-2}
I	1.23×10^{-4}	4.21×10^{-4}	6.35×10^{-2}
I	8.59×10^{-5}	8.92×10^{-4}	1.18×10^{-2}
I	8.59×10^{-5}	4.09×10^{-4}	2.85×10^{-2}
I	8.59×10^{-5}	1.36×10^{-4}	3.95×10^{-2}
I	8.59×10^{-5}	9.04×10^{-5}	5.64×10^{-2}
II	8.32×10^{-7}	4.20×10^{-4}	5.08×10^{-3}
II	2.38×10^{-6}	4.20×10^{-4}	1.15×10^{-2}
II	3.56×10^{-6}	4.20×10^{-4}	1.84×10^{-2}
II	2.38×10^{-6}	6.24×10^{-4}	8.60×10^{-3}
II	2.38×10^{-6}	4.46×10^{-4}	1.15×10^{-2}
II	2.38×10^{-6}	2.68×10^{-4}	2.02×10^{-2}
II	2.38×10^{-6}	8.92×10^{-5}	4.05×10^{-2}
III	1.56×10^{-4}	4.23×10^{-4}	1.07×10^{-2}
III	1.86×10^{-4}	4.23×10^{-4}	1.29×10^{-2}
III	2.16×10^{-4}	4.23×10^{-4}	1.93×10^{-2}
III	1.86×10^{-4}	9.04×10^{-4}	4.06×10^{-3}
III	1.86×10^{-4}	4.52×10^{-4}	1.29×10^{-2}
III	1.86×10^{-4}	2.26×10^{-4}	3.86×10^{-2}
III	1.86×10^{-4}	1.36×10^{-4}	1.75×10^{-1}

[a] Analytical surfactant concentration; the polymeric surfactant is considered as a repeat unit concentration. [b] Liposome concentration expressed in terms of phospholipid concentration.

Self-Quenching Efficiency after Incubation with Surfactant. A 250-μL aliquot of a 1 mM vesicle dispersion was added to a series of test tubes (6 × 50 mm), which contained 250 μL of a given surfactant solution, and the resulting suspension was agitated by vortex mixing for ca. 10 s. The final lipid concentration was 0.5 mM. After incubating for 0.5 h at 23 °C (or 50 °C for one set of experiments with DPPC), the dispersion was immediately chromatographed using a Sephadex G-50 column (0.7 × 20 cm). The vesicles (1.5 mL) were collected and portions (50 μL) then diluted with 4 mL of borate buffer; the fluorescence was analyzed to give a value for the vesicle-entrapped CF. A total fluorescence value was then measured after complete disruption of the vesicles, via addition of 50 μL of Triton X-100 (100 mg/mL). The concentrations of surfactants that were used for these release experiments were identical with those used for the determination of the percentage of CF release.

Kinetics of Surfactant-Induced Release. Typically, a 12- × 125-mm test tube was equipped with a Teflon-lined magnetic stirring bar and charged with 4.5 mL of borate buffer, containing a desired surfactant concentration. While the contents of the tube were stirred vigorously at 23 °C, 0.5 mL of POPC vesicles (containing 100 mM CF) was rapidly added via pipet. Aliquots (50 μL) were withdrawn as a function of time, diluted with 4 mL of borate buffer, and analyzed for fluorescence. A blank value was determined by treating a portion of the original vesicle dispersion with borate buffer, in the absence of detergent. A total fluorescence value was determined by complete disruption of the vesicles, using 50 μL of Triton X-100 (100 mg/mL). For all kinetic experiments, the total reaction volume used was 5 mL (see Table I for specific concentrations of phospholipid and membrane-disrupting surfactant used).

Results and Discussion

Rupture vs Leakage. To clarify the pathway through which I–III induce the release of CF from vesicular targets, we have employed a fluorescence self-quenching technique that has been introduced by Weinstein and co-workers for the study of protein interactions with lipid bilayers. In essence, by measuring the self-quenching efficiency of *vesicle-entrapped* CF before and after incubation with a disruptive agent, one can discern between two distinct processes: (i) one in which there is gradual leaking of CF from all of the vesicles and (ii) one in which only a portion of the vesicles release their entire contents and the remaining vesicles release none.

At high internal concentrations (≥100 mM), CF has negligible fluorescence due to very efficient quenching by neighboring

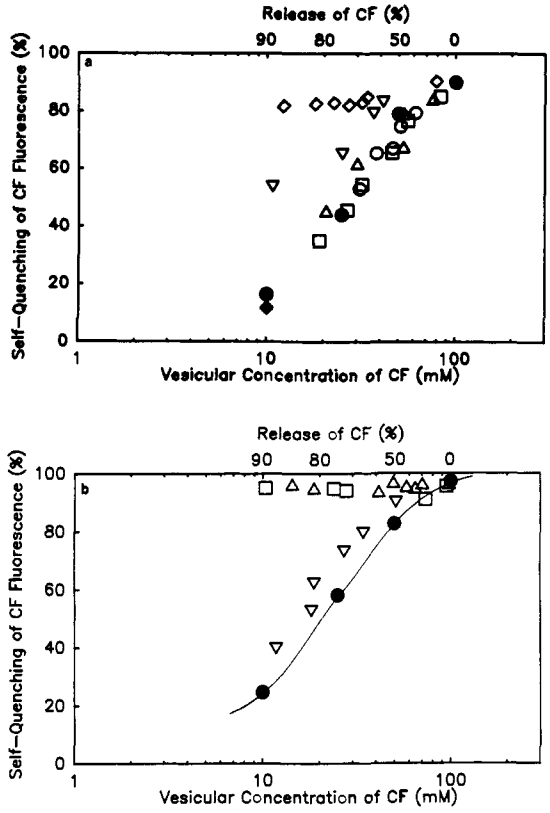

Figure 1. (a) Plot of self-quenching efficiency as a function of vesicular CF concentration (lower x-axis) and percent of CF release (upper x-axis) using 1000-Å-diameter vesicles (ca. 0.5 mM): (●) self-quenching calibration curve for POPC; (◆) self-quenching for POPC/cholesterol (1/1); (□) POPC plus I; (○) POPC plus II; (△) POPC plus III; (◇) POPC/cholesterol (1/1) plus III; (▽) POPC/cholesterol [2/1] plus III. All surfactant-membrane incubations were at 23 °C; the variation in release over the 30-min period was adjusted by the phospholipid/surfactant ratio used. Note: lower x-axis refers only to calibration curves. (b) Plot of self-quenching efficiency as a function of vesicular CF concentration (lower x-axis) and percent of CF release (upper x-axis) using 1000-Å-diameter vesicles (ca. 0.5 mM) made from DPPC: (●) self-quenching calibration curve; (△) plus III at 23 °C; (□) plus I at 23 °C; (▽) plus III at 50 °C. The variation in release over the 30-min period was adjusted by the phospholipid/surfactant ratio used. Note: lower x-axis refers only to calibration curves.

fluorophore molecules; i.e., "self-quenching" is almost complete. As the CF is diluted, however, the self-quenching efficiency decreases and the observed fluorescence increases. Thus, a negligible amount of fluorescence is expected from a phospholipid vesicle dispersion that contains ≥100 mM of entrapped CF. However, if the vesicular concentration of CF is lowered, the self-quenching efficiency is expected to decrease and the observed fluorescence should increase. Illustrations of this effect are clearly seen from self-quenching calibration curves that have been derived using 1000-Å-diameter unilamellar vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (Figure 1 parts a and b, respectively). Here, the self-quenching efficiency of a series of vesicle dispersions has been plotted as a function of entrapped CF concentration. Specific vesicular concentrations of CF that were used were 10, 25, 50, and 100 mM. For purposes of clarity, it should be noted that *the lower x-axis of each graph refers exclusively to these calibration curves.* The upper x-axis, indicating the percentage of CF release, refers to experiments in which a given vesicle dispersion was (i) incubated with varying concentrations of a membrane-disrupting surfactant for 30 min, (ii) purified by gel filtration (to remove nonentrapped CF), and (iii) reanalyzed for self-quenching efficiency. Specific values for self-quenching efficiency, Q, that are reported in these figures have been calculated using the following equation: $Q = (1 - \alpha)100$, where α is the fluorescence that is produced from CF which is entrapped within the vesicles divided by the total fluorescence that is observed by this same dispersion after complete disruption and release by use of excess Triton X-100.[7]

For vesicles that contain an initial dye concentration of ≥100 mM, a rupture process would be indicated by the release of a fraction of the encapsulated CF and an unaltered quenching efficiency for that portion of CF which remains entrapped within the recovered vesicles; i.e., a plot of the percentage of CF release as a function of self-quenching efficiency should correspond, essentially, to a horizontal line that approaches the upper x-axis. In contrast, if CF is released by a leakage process, in which there is partial release of CF from all of the vesicles, then an analogous plot should closely follow a calibration curve, constructed from analogous vesicles made with varying internal CF concentrations.

Large unilamellar vesicles (1000-Å diameter), containing 100 mM CF, were prepared from POPC, POPC/cholesterol (2/1 molar ratio), POPC/cholesterol (1/1), and DPPC and were incubated with varying concentrations of a given surfactant for 30 min at 23 °C (see Experimental Section for specific surfactant concentrations used). Each vesicular dispersion was then passed through a gel filtration column (Sephadex G-50) to remove CF that had been released, and the recovered vesicles were then analyzed for the self-quenching efficiency.

In the case of pure POPC vesicles, the release of CF that is induced by I–III proceeds via a leakage pathway; each of the percent release/self-quenching efficiency plots falls along the calibration curves prepared from POPC. In sharp contrast, when 50 mol % of cholesterol is included in the bilayer, an essentially horizontal plot is obtained with surfactant III. These results show that the self-quenching efficiency of CF in one portion of the vesicles remains high, as additional CF is released from the dispersion (by means of higher concentrations of surfactant), but that it becomes very low in another. In essence, this means that the vesicular concentration of CF remains high for some of the vesicles and approaches zero for others. Such a result clearly reflects a rupture process. Unfortunately, the membrane-disrupting ability of I and II toward bilayers made from POPC/cholesterol (1/1) proved to be too low for rupture/leakage determination.[6] For the vesicles that were made from POPC/cholesterol (2/1), a plot of CF release (induced by III) vs self-quenching efficiency appears to be intermediate between that expected for a leakage and that expected for a rupture process. If it is assumed that cholesterol is randomly distributed throughout the vesicle dispersion, a plausible explanation that would account for this behavior is that some population of vesicles (relatively low in cholesterol content) leaks CF while another population ("cholesterol-rich") releases CF via a rupture pathway. We presume that the contribution of "cholesterol-poor" vesicles in the POPC/cholesterol (1/1) dispersion is sufficiently small, such that it has a negligible contribution to percent release/self-quenching efficiency plot. Finally, we note that with DPPC vesicles in their gel state disruption by I and III also proceeds through a rupture process (Figure 1b). When III is allowed to incubate with these vesicles when they are in their fluid state, however (i.e., 50 °C), the release is converted to a leakage process.

The crossover from leakage to rupture that is observed when the cholesterol content in POPC-based vesicles is increased from 0 to 33 to 50 mol %, together with the fact that DPPC vesicles rupture in the gel phase but are leaky in the liquid-crystalline state, clearly demonstrates that the packing of the bilayer controls the pathway through which CF is released from the vesicles. In addition, the fact that II promotes CF release from POPC vesicles via leakage further indicates that fluid membranes have sufficient resiliency such that they can undergo rapid annealing, even after interaction with a polymeric membrane-disrupting agent.

Kinetics of CF Release. To gain greater insight into the leakage process, we have investigated the kinetics of the release of CF from POPC vesicles. In general, the release is characterized by a rapid burst followed by a slower second phase. For those vesicles which favored a rupture pathway (i.e., DPPC/ and POPC/cholesterol (1/1) vesicles at 23 °C), nearly all of the release appears as a burst. For POPC vesicles, which prefer a leakage pathway, most of the

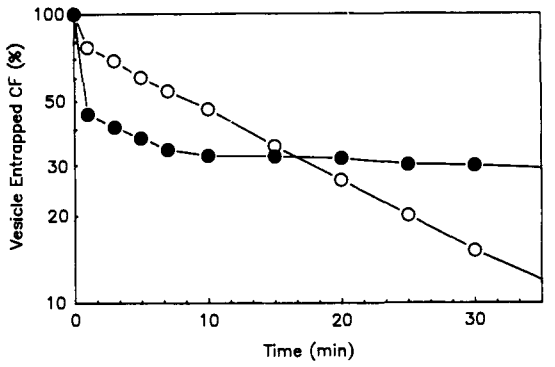

Figure 2. Release of CF as a function of time (23 °C) from 0.5 mM liposomal targets made from (○) POPC and (●) POPC/cholesterol (1/1), induced by 2.56×10^{-4} M III. The first-order release from POPC liposomes extended essentially to completion, i.e., >95% release (data not shown).

fluorophore is gradually released in the second phase; the latter was found to obey simple first-order kinetics (Figure 2). In Table I, we report the observed pseudo-first-order rate constants that have been obtained as a function of vesicle and surfactant concentration.

Kinetic Model for Surfactant-Induced Leakage. In the following section, we develop a theoretical model for surfactant-induced leakage from phospholipid vesicles. We then apply it to our kinetic data and use it as a basis for drawing conclusions regarding the detailed mechanism of release. To simplify our analysis, we have chosen to neglect the burst phase of the kinetics and to consider only the slower second stage.

The first assumption that we make is that membrane disruption requires initial binding of the surfactant to the vesicle's surface and that this binding occurs in a saturable manner, such that the vesicles can be considered, formally, as having a defined number of individual binding sites. For a given phospholipid concentration (PL_0), the concentration of binding sites, B, is then proportional to PL_0, and the proportionality constant, β, may be expressed as $\beta = PL_0/B_0$, where B_0 represents the initial concentration of binding sites. If binding occurs according to

$$B + D \overset{K}{\rightleftharpoons} C$$

then the equilibrium dissociation constant, K, is defined by

$$K = BD/C = (B_0 - C)(D_0 - C)/C$$

or

$$K = (B_0/C - 1)(D_0 - C)$$

where D is the concentration of disruptive surfactant in solution, D_0 is the initial concentration of this surfactant, and C is the concentration of bound surfactant. Substitution for B_0 by PL_0/β then leads to

$$K = [PL_0/(\beta C) - 1](D_0 - C) \qquad (1)$$

Up to this point, all of the above concentrations have been defined in terms of real solution concentrations, i.e., molarity. On the basis of our "site" model, we can also define the surfactant concentration that resides at the membrane surface as the *surface concentration of occupied sites*. Such a concentration would be directly proportional to the *fractional occupancy*, and the latter may be expressed as C/B_0, which is equal to $\beta C/PL_0$.

Finally, if we assume that the rate of leakage of the fluorophore is directly proportional to its vesicular concentration, F, and to the nth power of the surface concentration of surfactant, then the kinetics of the release would be expected to obey the following rate law:

$$-(dF)/(dt) = kF(C/B_0)^n = kF(\beta C/PL_0)^n$$

Here, k represents the intrinsic rate constant which defines the passage of F across the phospholipid bilayer. Thus, for any experiment in which values of PL_0 and D_0 are specified, the release of CF should follow pseudo-first-order kinetics, and the experimental rate constant, k_{exp}, would be given by

$$k_{exp} = k(\beta C/PL_0)^n$$

If we define the symbol k' as

$$k' = \sqrt[n]{k}$$

and then substitute the expression for k_{exp} into eq (1), we obtain

$$K = \left(\frac{k'}{\sqrt[n]{k_{exp}}} - 1\right)\left(D_0 - \frac{PL_0}{\beta k'}\sqrt[n]{k_{exp}}\right) \qquad (2)$$

For those experiments in which PL_0 is held constant and D_0 is varied, D_0 can be expressed as a function of k_{exp} by use of

$$D_0 = \frac{K}{\dfrac{k'}{\sqrt[n]{k_{exp}}} - 1} + \frac{PL_0}{\beta k'}\sqrt[n]{k_{exp}} \qquad (3)$$

Similarly, for those experiments in which D_0 is held constant and PL_0 is varied, PL_0 can be expressed as a function of k_{exp} by use of

$$PL_0 = \left(D_0 - \frac{K}{\dfrac{k'}{\sqrt[n]{k_{exp}}} - 1}\right)\frac{\beta k'}{\sqrt[n]{k_{exp}}} \qquad (4)$$

Since eq 3 and 4 share the same set of parameters, one can carry out a simultaneous nonlinear least-squares fit of the data to verify whether or not they are consistent with our proposed scheme and also to obtain the best value for each constant. Such analysis has been performed using the Simplex method,[11] where all four parameters have been allowed to "float". Comparison of the experimental data with the theoretical curves shows that the release kinetics are, indeed, consistent with our model (Figures 3–5). A summary of the calculated values of K, k', n, and β for I–III is presented in Table II.

Given the limited data that have been used in these analyses, and on the basis of our uncertainty in knowing whether or not global minimums have been reached, we interpret K, k', n, and

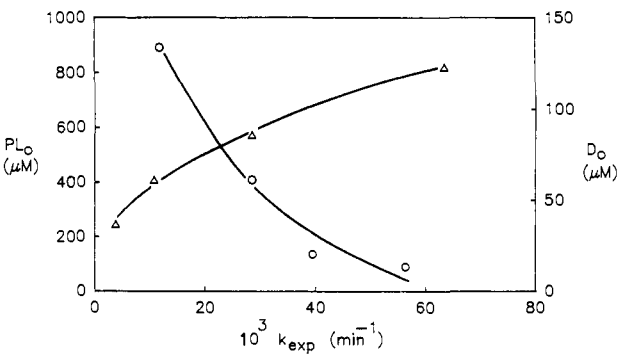

Figure 3. Plot of PL_0 (○, left axis) and D_0 (△, right axis) as a function of $10^3 k_{exp}$ for I. Theoretical curves are given as solid lines.

Table II. Kinetic and Thermodynamic Parameters Calculated from Theoretical Model[a]

surfactant	K, μM	k, min^{-1}	n	β
I	200	3.0	3	3
II	0.4	0.05	1	43
III	13	0.3	2	0.7

[a] Parameters were obtained from a simultaneous nonlinear least-squares fit of the data from Table I, using eqs 3 and 4.

(11) Yamaoka, K.; Tanigawara, Y.; Nakagawa, I.; Uno, T. *J. Pharm. Dyn.* **1981**, *4*, 879.

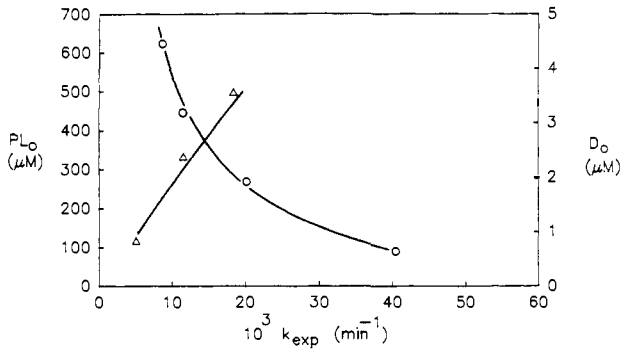

Figure 4. Plot of PL_0 (O, left axis) and D_0 (Δ, right axis) as a function of 10^3k_{exp} for II. Theoretical curves are given as solid lines.

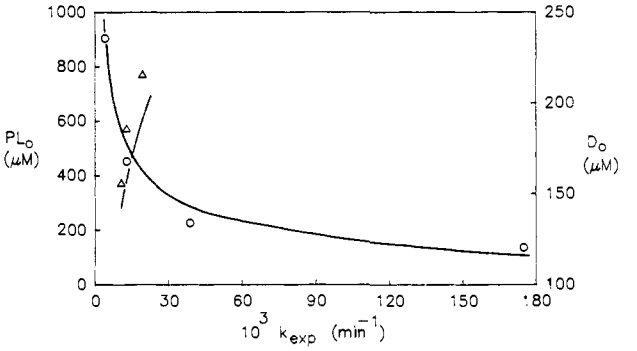

Figure 5. Plot of PL_0 (O, left axis) and D_0 (Δ, right axis) as a function of 10^3k_{exp} for III. Theoretical curves are given as solid lines.

β strictly in *qualitative* terms. Nonetheless, the values that are reported in Table II offer some intriguing clues regarding the disruption process, at the molecular level. First, the n values represent the order of the release with respect to the membrane-bound surfactant. Those values obtained for I–III imply that small aggregates of surfactant are responsible for promoting leakage. The existence of cooperativity among membrane-bound surfactants can be readily accounted for if one considers that the fluorophore must pass through *both* leaflets of the membrane to escape from the vesicle interior. If one assumes that an effective "escape route" must *span the bilayer*, then a minimum of two neighboring surfactants (one buttressed against another in an adjoining monolayer) should be required for leakage. The first-order dependency that is observed for the repeat units of II may also be viewed as a type of "built-in" cooperativity that is an inherent feature of the polymeric agent. Because each repeat unit has at least one covalently attached neighboring unit, every polymer molecule that inserts into the membrane should have the wherewithal for spanning a bilayer and for promoting leakage; i.e., aggregation of polymers within the membrane should not be necessary for constructing an escape route.

The calculated dissociation constants, K, for I and II indicate that the polymeric surfactant is much more tightly bound to POPC than its monomeric analogue. The greater binding of II is a likely consequence of the polymer's ability to associate with the bilayer via multiple sites of attachment. In preliminary experiments, we have found that equilibration of I (74 μM) with *multilamellar* vesicles of POPC (500 μM) results in ca. 80% of the bolaphile being bound and that equilibration of 6.1 μM of II resulted in ca. 95% binding.[12] By use of eq 1, and the values of K and β that are reported in Table II, our model predicts that ca. 40% of the bolaphile and 95% of the polymer should be taken up by the vesicles under these conditions. We regard these experimental results as being in reasonable agreement with our model.

The calculated β values indicate that both I and III should form a densely covered membrane at saturation. In contrast, the maximum surface density of II appears to be significantly less. This result can be explained if it is assumed that not all of the repeat units are inserted into the bilayer. Those that extend outward into the aqueous phase can "spread out" on the vesicular surface, leading to a less densely packed array of bound polymers. While the smaller value of k for II (as compared with that for I) may be also be attributed, in part, to incomplete insertion of the repeat units, we suspect that conformational constraints that are imposed on the hydrophobic units by the polymeric backbone may also limit the effective size of the escape route that is created within the bilayer.

Conclusions

In this paper we have shown that surfactant-induced release of CF from phospholipid vesicles can occur by both rupture and leakage pathways and that it is the packing of the membrane which regulates the release. This finding, in and of itself, demonstrates that surfactant–lipid bilayer interactions are richer in complexity, and potentially more exploitable, than had previously been recognized. A model has been developed for surfactant-induced leakage that is consistent with the observed kinetics for the release of CF from POPC vesicles. This model implies that small aggregates of surfactant are responsible for promoting the leakage of vesicular CF.

Although we can only now speculate on the mechanism of membrane rupture, we currently favor a model which assumes that rupture results from the *autocatalytic* uptake of surfactant by a portion of the vesicles. Specifically, we envision that highly compact bilayers are rendered more pervious to both CF and surfactant, upon sequential insertion of the latter, and that this ultimately leads to two distinct vesicles populations, i.e., one that is rich in the disruptive agent (and ruptures) and one that has a membrane composition which is similar to that of the original bilayer (and fully retains entrapped CF). Studies that are currently in progress are aimed at clarifying the membrane rupture process and also at applying our leakage model to other membrane-disrupting surfactants.

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(12) Specific experimental procedures that were used were similar to those previously described.[4] Multilamellar vesicles were chosen for binding studies for experimental reasons. Unlike the unilamellar vesicles, multilamellar analogues can be readily removed from solution by centrifugation and the supernatant then analyzed for nonbound surfactant. Tacit assumptions that we make in these binding experiments are[4] (i) that the equilibria between membrane-bound and free surfactants for centrifuged (pelleted) dispersions are similar to that found for these same multilamellar vesicles in the dispersed state and (ii) that the surfactants readily diffuse across multibilayers of POPC.