Control over Vesicle Rupture and Leakage by Membrane Packing and by the Aggregation State of an Attacking Surfactant[1]

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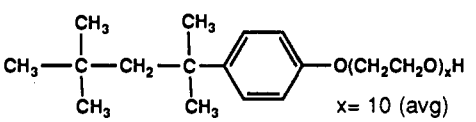
Abstract: Triton X-100 (**T**) in its monomeric form has been found to induce the *leakage* of 5(6)-carboxyfluorescein (**CF**) from tightly packed gel-phase vesicles derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 25 °C). In contrast, micellar solutions of **T** were found to promote the catastrophic *rupture* of the membrane. These conclusions are based on an analysis of the fluorescence self-quenching efficiency of recovered vesicular **CF** after incubation with **T**, below and above its critical micelle concentration. Analogous fluid-phase vesicles made from DPPC (50 °C) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, 25 °C) were found to release **CF** by a leakage mechanism, regardless of whether the attack came from a micellar or monomeric form of **T**. Increasing the compactness of POPC vesicle bilayers by incorporation of 45 mol % cholesterol resulted in a membrane that was susceptible toward rupture. The synergistic control over vesicle rupture and leakage by the packing properties of the target membrane and by the aggregation state of the attacking surfactant represents a fundamental and heretofore unrecognized feature of surfactant–lipid bilayer interactions. The implications of these findings for the rational design of membrane-disrupting drugs are briefly discussed.

Introduction

The rational design of antimicrobial drugs that function via membrane disruption requires a detailed understanding of how lipid bilayers respond toward perturbing agents. At present, such interactions remain poorly defined at the molecular as well as the supramolecular level.[2-6] Recently, we have shown that one of the most commonly used membrane-disrupting agents, Triton X-100 (**T**), can 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 release none.[7] We have also demonstrated that the packing density of the membrane plays a critical role in defining the pathway for release, i.e., loosely packed bilayers favor leakage, and tightly packed membranes favor rupture.

In this paper, we show that *it is the combination of membrane packing and the supramolecular state of the attacking agent which controls rupture/leakage pathways*. Specifically, we show that **T**, in its monomeric form, induces the *leakage* of **CF** from tightly packed gel-phase vesicles derived from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 25 °C) and that corresponding micellar solutions promote the release via a *rupture* pathway. We further show that analogous fluid-phase vesicles made from DPPC (50 °C) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, 25 °C) release **CF** by a leakage mechanism, regardless of whether the attack is made by a micellar or monomeric form of **T**, and that incorporation of 45 mol % cholesterol into POPC bilayers leads to a rupture-sensitive membrane. Such supramolecular control over bilayer disruption represents a fundamental and heretofore unrecognized feature of surfactant–lipid bilayer interactions, and one that has broad implications for the rational design of drugs that function via membrane-disruption.

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids (Birmingham, AL) as a chloroform solution and used directly. 5(6)-Carboxyfluorescein was obtained from Eastman Kodak and purified according to literature methods.[8,9] Triton X-100 was purchased from Sigma and used directly. House-deionized water was purified using a Millipore Milli-Q filtering system containing one carbon stage and two ion-exchange stages. Vesicle dispersions were prepared using a 10 mM borate buffer (pH 7.4) containing 140 mM NaCl and 2 mM NaN_3 (designated throughout this paper as "borate buffer"). Vesicle extrusions were carried out with a Lipex Biomembrane apparatus (Vancouver, BC). All fluorescence measurements were made using a Perkin-Elmer LS 50 luminescence spectrometer. Excitation of **CF** was at 491 nm; the observed emission was at 521 nm. Surface tension measurements were made using a Nima Model ST tensiometer. 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. Polymerized vesicles (1000-Å diameter) were made from **I** (see Results and Discussion) using procedures similar to those previously described.[10] Samples for electron microscopy (Philips 300) were prepared by depositing appropriate dispersions onto a carbon-coated copper grid for 30 s, followed by removal of excess dispersion by blotting the grid with filter paper. Staining was made by applying one drop of an aqueous solution of uranyl acetate (2%, w/w) to the grid and removing the excess solution (after 30-s contact) with filter paper. Micrographs were recorded at magnifications between 20 000× and 80 000× with accelerating voltages of 80 and 100 kV. All large unilamellar vesicles were prepared by standard extrusion methods.[10]

Percentage of CF Release after Incubation with T. Typically, a 50-μL aliquot of a vesicle dispersion (equilibrated at 50 °C for 1 min for fluid-phase DPPC experiments) was added to each of a series of test tubes that contained 450 μL of buffer plus a given concentration of **T** (equilibrated at 50 °C for fluid-phase DPPC experiments). The resulting

(1) Supported by PHS Grant AI28220 awarded by the National Institute of Health (NIAID).

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dispersion was immediately vortex-mixed for ca. 10 s. After the mixture was allowed to incubate at 25 °C (or 50 °C) for 30 min, a 50-μL aliquot was withdrawn and diluted with 3.0 mL of borate buffer prior to measurement of the fluorescence. A blank value was obtained by carrying out a similar experiment in the absence of **T**. The 100% fluorescence intensity values were determined by addition of 45 μL of a 0.16 M solution of **T** to each sample. 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 value; I_a and I_b are the fluorescence intensities after incubation with and without surfactant, respectively. Specific procedures that were used for kinetic measurements were similar to those that were previously described.[7]

Self-Quenching Efficiency after Partial Release of CF. Typically, a 50-μL aliquot of a vesicle dispersion (equilibrated at 50 °C for 1 min for fluid-phase DPPC experiments) was added to each of a series of test tubes that contained 450 μL of buffer plus a given concentration of **T** (equilibrated at 50 °C for fluid-phase DPPC experiments). The resulting suspension was immediately vortex-mixed for ca. 10 s. After 30 min of incubation at 25 °C (or 50 °C), the dispersions were applied to Sephadex G-50 columns (0.7- × 17-cm), which had been equilibrated with borate buffer containing 50 μM of **CF**-free corresponding vesicles (1000-Å diameter). The columns were then eluted at room temperature with the same buffer, and the vesicles were recovered in the void volume (determined by use of blue dextran, having an average molecular weight of 2000 kDa). The vesicular dispersion (1.6 mL) was collected and diluted to approximately 0.8 μM (final volume equaling 3 mL) with buffer for the analysis of self-quenching efficiency. The resulting samples were then measured before and after treatment with an excess of **T** (45 μL of a 0.16 M solution of **T**) for the determination of total fluorescence. Self-quenching efficiency values Q were calculated by use of the relationship $Q = (1 - \alpha)100$, where α is the observed fluorescence intensity of the vesicular **CF** divided by the total fluorescence that is observed by this same dispersion after complete release by use of excess **T**. The percentage of **CF** that remains encapsulated was determined by dividing the total fluorescence (after complete release) by the total fluorescence of a similar sample that was not incubated with **T** prior to gel filtration. Additional control experiments, carried out with DPPC vesicles containing a trace amount of ^{3}H-labeled DPPC, established that the recovery of lipid in the void volume (under rupture and leakage conditions) was quantitative.

Differential Scanning Calorimetry (DSC). Extruded vesicles (1000-Å diameter) that were prepared from DPPC in borate buffer were incubated with **T** under varying rupture conditions and then subjected to DSC analysis, using borate buffer as a reference. Heating scans were recorded between 10 and 50 °C at a scan rate of 30 °C/h. Three DSC runs were performed for each sample; no significant difference was observed among the scans in the absence of **T**. When **T** was present, however, the second scan was slightly broadened; the third scan was identical to the second. Thermograms reported in the figures are for the first scans. A borate buffer base line was also collected and subtracted from each thermogram. The calorimetric data were analyzed to yield phospholipid excess heat capacities as a function of temperature, and the transition enthalpies were calculated by employing software supplied by Microcal. Thermograms were normalized from mcal/min to mcal/°C by dividing by the scan rate. The heat capacity in units of kcal/°C·mol was determined by dividing by the number of moles of lipid present in the vesicle dispersion.

Results and Discussion

Distinguishing between Vesicle Rupture and Leakage via Fluorescence Self-Quenching Measurements. Weinstein and coworkers have devised an elegant method for distinguishing between vesicle rupture and leakage pathways on the basis of the self-quenching efficiency Q of entrapped **CF**.[8,9] At high intravesicular concentrations (≥100 mM), **CF** has negligible fluroescence due to efficient self-quenching. As the fluorophore is diluted within the vesicles, its fluorescence increases and Q decreases. A rupture process is readily established when the quenching efficiency of vesicular **CF** remains constant, after there is *partial release* from the dispersion. In contrast, a leakage occurrence is reflected by the gradual release of **CF** from all of the vesicles and is revealed by a continuous decrease in **Q** that follows a calibration curve made from similiar vesicles that contain decreasing concentrations of fluorophore.

Protocol for Investigating Rupture/Leakage above and below the Critical Micelle Concentration of T. The experimental approach that we have used for studying the rupture/leakage properties of target vesicles above and below the critical micelle concentration of **T** has been (i) to add aliquots of a given vesicle dispersion (1000-Å large unilamellar vesicles loaded with 100 mM **CF**)[11-13] to varying concentrations of the surfactant, (ii) to remove nonentrapped **CF** by gel filtration after a 30-min incubation period, and (iii) to immediately analyze the recovered vesicles for self-quenching efficiency (Scheme I). In order to investigate a wide range of surfactant concentrations, we have found it necessary to employ a broad range of vesicle concentrations. Specifically, vesicle concentrations (indicated throughout this paper as phospholipid concentration) had to be matched with a given concentration range of **T** in order that *partial* release of the fluorophore could be observed. Such experimental conditions were essential in order to measure self-quenching of the encapsulated **CF** as a function of the extent of release. For example, in our study of the interaction of gel-phase DPPC vesicles with submicellar concentrations of **T**, surfactant concentrations that ranged from 0.40×10^{-4} to 0.68×10^{-4} M were matched with a phospholipid concentration of 20 μM. In contrast, micellar solutions that ranged from 2.5×10^{-4} to 3.1×10^{-4} M were matched with a phospholipid concentration equaling 2050 μM. The critical micelle concentration (cmc) of **T**, estimated to be ca. 2.0×10^{-4} M via surface tension, lies between these two concentration ranges of surfactant. Similar adjustments of the lipid concentrations were required for the study of fluid-phase vesicles made from DPPC and POPC. Thus, in carrying out these rupture/leakage investigations, *both the target concentration and the concentration of the attacking surfactant had to be varied.*

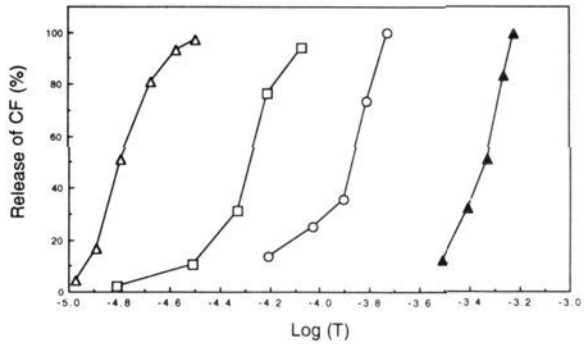
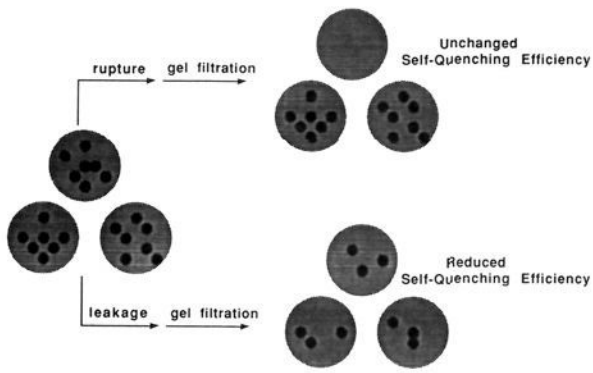


Figure 1. Plot of percentage of release of **CF** from gel-phase DPPC vesicles (1000-Å diameter) as a function of log **T** concentration. Phospholipid concentrations were 5 μM (△), 50 μM (□), 500 μM (○), and 5000 μM (▲). The initial concentration of **CF** in the target vesicles was 100 mM.

Scheme I



Release from Gel-Phase DPPC Vesicles. Release profiles that have been obtained for the disruption of gel-phase DPPC vesicles by **T** at 25 °C are shown in Figure 1. The corresponding self-quenching efficiencies Q that were determined for the recovered

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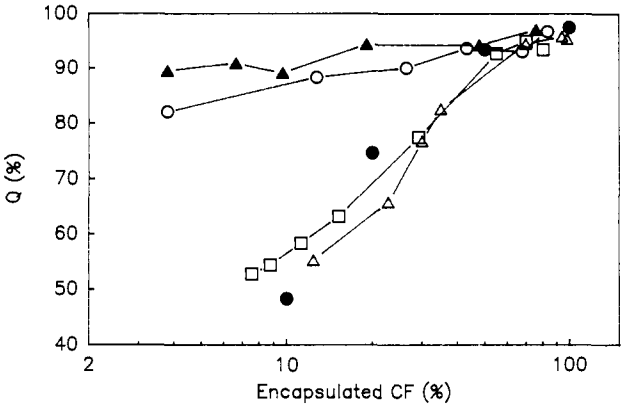

Figure 2. Plot of self-quenching efficiency Q as a function of percentage of CF that remains encapsulated after a 30-min incubation with gel-phase (25 °C) DPPC vesicles. Phospholipid concentrations were 5 μM (△), 50 μM (□), 500 μM (○), and 5000 μM (▲). See Figure 1 for range of T used. The calibration curve (●) was based on internal concentrations of CF equaling 100, 50, 20, and 10 mM.

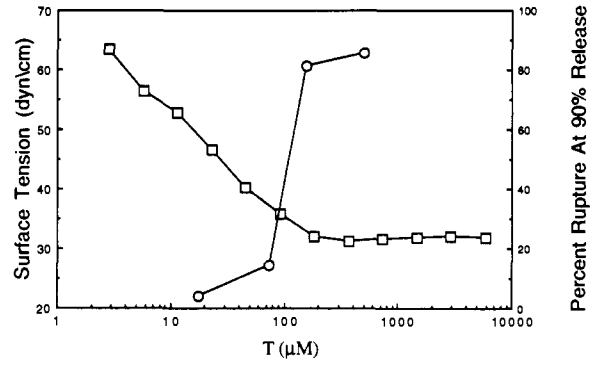

Figure 3. Plot of percent rupture of gel-phase DPPC vesicles at 90% release of CF (i.e., 10% of the original 100 mM CF remains entrapped) (○) and surface tension of buffer (□) as a function of T concentration. Phospholipid concentrations that were used were 5, 50, 500, and 5000 μM. The percent rupture was estimated by interpolation of the data from Figure 2, where 100% rupture and 100% leakage points are defined as those values of Q that were determined for standardized samples containing intravesicular CF concentrations of 100 and 10 mM, respectively. Surface tension measurements were made at 25 °C; no difference was observed at 50 °C.

vesicles are presented in Figure 2. Specific values of Q have been calculated by use of the relationship $Q = (1 - \alpha)100$, where α is the observed fluorescence intensity of the vesicular CF divided by the total fluorescence that is observed by this same dispersion after complete release by use of excess T. Also shown in this figure is a calibration curve that has been constructed from a series of DPPC vesicles containing varying internal concentrations of CF in borate buffer. Inspection of these self-quenching curves reveals that a crossover from leakage to rupture takes place as the concentrations of both the target and the disruptive surfactant are increased. The slight decrease in quenching efficiencies that are observed under rupture conditions at high percentages of release is very similar to that which has been reported by Weinstein.[8,9] This decrease is a likely consequence of free monomer, which is also present in the micellar solutions and which induces the release of CF through a competitive leakage pathway.

In Figure 3, we show a plot of the percentage of rupture that occurs at 90% release (estimated by interpolation of the self-quenching curves presented in Figure 2) as a function of the concentration of T. Also included in this figure are the surface tensions of the buffer as a function of T in the absence of vesicles. From this figure, it is clear that attack by submicellar concentrations of T results in predominant leakage and that when the cmc is approached, a crossover to rupture occurs. Given the polydisperse character of Triton X-100, we regard this correlation between the observed crossover in the mechanism and the onset of micelle formation to be a reasonably good one.

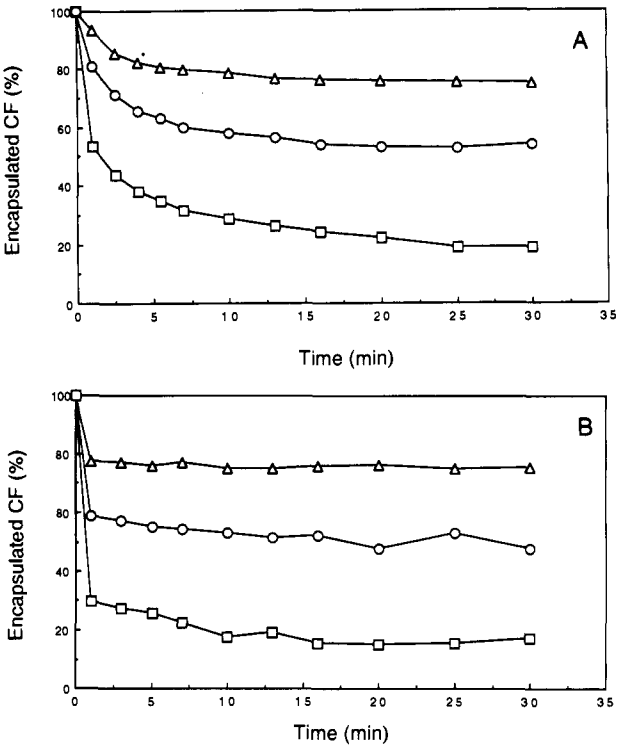

Figure 4. (A) Release of CF as a function of time (25 °C) from 5 μM DPPC vesicles that have been incubated with 0.016 mM T (△), 0.020 mM T (○), and 0.023 mM T (□). (B) Release of CF as a function of time (25 °C) from 5 mM DPPC vesicles that have been incubated with 0.39 mM T (△), 0.47 mM T (○), and 0.54 mM T (□).

Although these rupture/leakage studies have been standardized using a 30-min incubation period, it is noteworthy that the rate of release of CF from these DPPC vesicles was dependent on whether rupture or leakage predominated. Under leakage conditions, the rate of CF release was found to decrease with time; under rupture conditions, the release appeared as a single burst (Figure 5). The latter observation implies that equilibrium considerations are relatively unimportant to this rupture phenomenon and that vesicle rupture has an important kinetic component. For leakage, the situation appears to be more complex.

Examination of gel-phase DPPC vesicles by electron micrscopy has also revealed significant differences between rupture and leakage. Specifically, we have found that unlike a ca. 50%-leaked sample, which showed the presence of normal vesicular structures, a ca. 50%-ruptured dispersion showed gross morphological changes, i.e., the appearance of stacked discoidal particles (Figure 4). Thus, vesicle rupture appears to be a truly catastrophic event which leads to substantial changes in aggregate morphology.

Release from Fluid-Phase Vesicles Made from DPPC and POPC. Analogous rupture/leakage studies have been carried out using micellar and submicellar solutions of T acting on fluid-phase vesicles made from DPPC (50 °C) and POPC (25 °C).[14,15] Specific release and self-quenching efficiency curves that were obtained are shown in Figures 6 and 7, respectively. In contrast to gel-phase DPPC vesicles, these fluid-phase analogs strongly favor leakage pathways *above and below* the critical micelle concentration of T.

Relationship between Vesicle Rupture and Solubilization. The interaction of surfactant molecules with lipid membranes has frequently been considered in terms of a three-stage model, whereby bilayers are ultimately converted into mixed micelles,

(14) The gel-to-liquid crystalline phase transition temperature (T_m) of DPPC is 41.4 °C: 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. The T_m of POPC is −5 °C: DeKruijff, B.; Demel, R. A.; Slotboom, A. J.; Van Deenen, L. L. M.; Rosenthal, A. F. *Biochim. Biophys. Acta* **1973**, *307*, 1.

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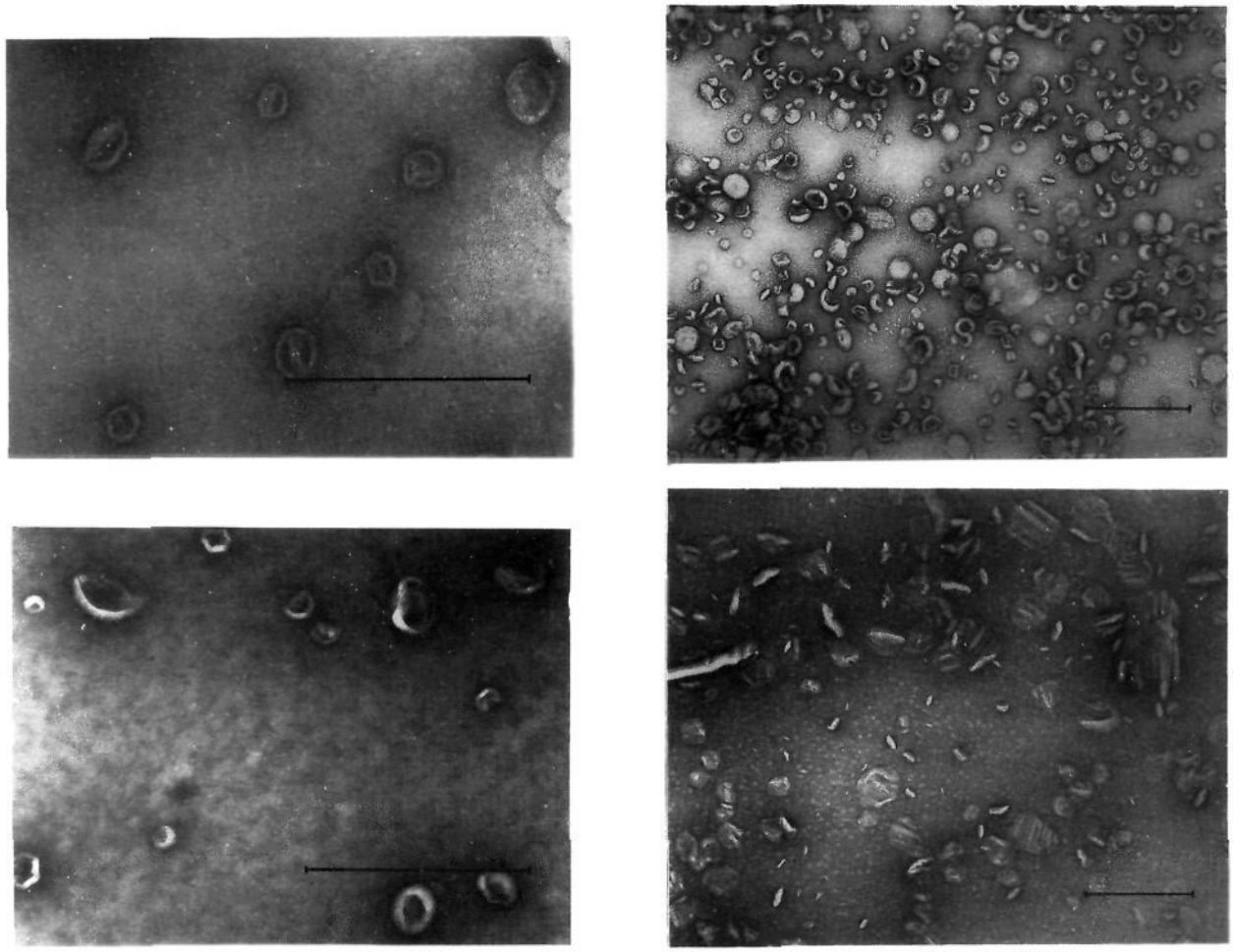

Figure 5. Transmission electron micrographs (2% uranyl acetate) of (top left) 50 μM DPPC vesicles; (bottom left) 50 μM DPPC vesicles in the presence of **T** required for 50% leakage; (top right) 500 μM DPPC vesicles; and (bottom right) 500 μM DPPC vesicles in the presence of **T** required for 50% rupture. Bar represents 0.5 μM.

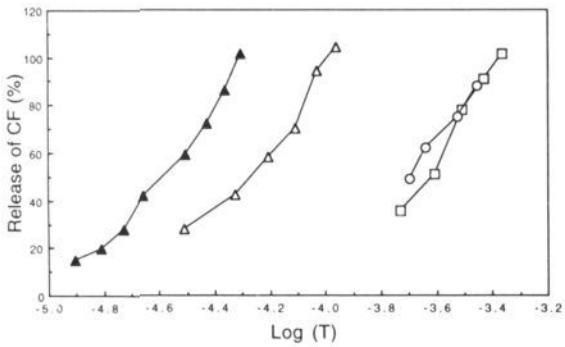

Figure 6. Plot of percentage of release of **CF** from fluid-phase DPPC vesicles (50 °C) and POPC vesicles (25 °C) as a function of **T** concentration. DPPC target concentrations were 100 μM (▲) and 200 μM plus 15 000 μM **CF**-free (empty) vesicles (○); POPC target concentrations were 20 μM (△) and 20 μM plus 1000 μM **CF**-free vesicles (□). A high percentage of empty DPPC vesicles and POPC vesicles were employed under micellar conditions for experimental convenience. The initial concentration of **CF** in all of the target vesicles was 100 mM.

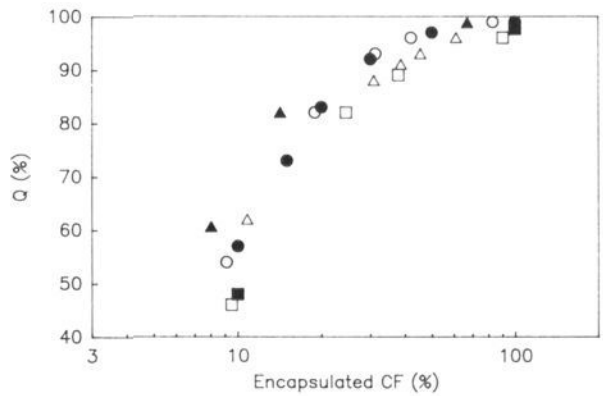

Figure 7. Plot of self-quenching efficiency as a function of percentage of **CF** that remains encapsulated after a 30-min incubation with fluid-phase DPPC vesicles (50 °C) and POPC vesicles (25 °C). DPPC target concentrations were 100 μM (▲) and 200 μM plus 15 000 μM **CF**-free vesicles (○); POPC target concentrations were 20 μM (△) and 20 μM plus 1000 μM **CF**-free vesicles (□). See Figure 6 for range of **T** used. Calibration curves made from DPPC (●) and POPC (■) using internal concentrations of **CF** equaling 100, 50, 30, 20, 15, and 100, 10 mM, respectively.

i.e., the membrane is "solubilized".[4,16-18] In stage I, the surfactant associates with the vesicles by adsorbing onto the outer monolayer leaflet and/or inserting into the hydrocarbon region until a saturation point is reached. Throughout stage I, the integrity of the bilayer phase is fully maintained. When the surfactant concentration outside the membrane exceeds its critical micelle concentration, the system enters a second stage, in which the membrane becomes partially solubilized; i.e., mixed micelles coexist with a lamellar phase. As the surfactant concentration is further increased, the bilayer is fully dissolved and all the phospholipid

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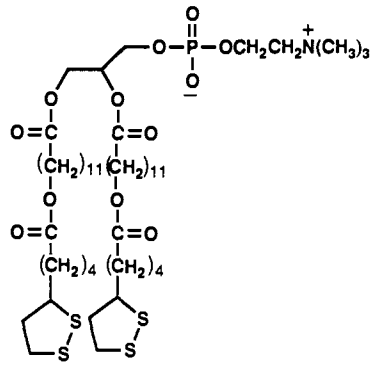
is now present as mixed micelles (stage III).

Previous calorimetric studies have shown that sodium deoxycholate, sodium cholate, and octyl glucoside lower the gel-to-liquid crystalline phase transition temperature T_m of DPPC in stage I.[18] These studies have also indicated that the beginning of stage II can be detected by the onset of a drastic change in the enthalpy of this transition. We have examined our gel-phase DPPC vesicle targets by high-sensitivity differential scanning calorimetry under varying rupture conditions and have found that T_m is shifted to lower temperatures but that the ΔH remains essentially unchanged (6.2 ± 0.2 kcal/mol, Figure 8). These results provide strong evidence that vesicle rupture occurs in stage I. In contrast, similar DSC measurements that were made in the presence of a *large excess* of a micellar solution of T reveal a substantially broadened and complex endotherm as well as a drastic decrease in ΔH (ca. 3.9 kcal/mol). Apparently, the uptake of surfactant by the vesicles (under the experimental conditions used for rupture/leakage determination) is sufficient that its concentration outside the bilayer is reduced to below its cmc, thereby preventing the formation of mixed micelles and the dissolution of the membrane. At much higher surfactant concentrations, however, the bilayer becomes solubilized.[15]

Release from Gel-Phase DPPC Vesicles in the Presence of "Dummy" Vesicles. Evidence for Micelles as the Causative Agent for Rupture. In order to establish that the crossover in mechanism is due to micelle formation and is not a consequence of the requisite increase in vesicle concentration (e.g., vesicle–vesicle fusion being favored at higher lipid concentrations),[19] we sought an experimental method that would allow us to study the rupture/leakage behavior of *one fixed target concentration in the presence of submicellar and micellar concentrations of T*. The technique that we have devised for this purpose employs polymerized (nondisruptable and nonfusogenic) vesicles derived from 1,2-bis[(12-lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**I**).[10]



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In essence, we use such vesicles as "dummy" targets to compete for surfactant binding in order to limit the total amount of T that is available to the real targets. This allows us to expose a relatively low target concentration (required for a submicellar experiment) to micellar concentrations of T without resulting in the *complete* release of the fluorophore. The specific concentration of dummies that is needed for such an experiment is determined empirically. Thus, aliquots of a dispersion containing gel-phase DPPC vesicles plus polymerized vesicles made from I are incubated with varying micellar concentrations of T.

We have found that when 20 μM DPPC targets are employed, a dummy concentration of 2.03 mM is appropriate for observing a partial release of CF, using concentrations of T that range from 2.5×10^{-4} to 3.1×10^{-4} M. Examination of the self-quenching efficiencies of the recovered dispersion clearly shows that the release is now dominated by a *rupture* pathway (Figure 9). This result is in striking contrast to the dominant leakage that is observed when submicellar concentrations of T are used to disrupt similar 20 μM dispersions of DPPC vesicles. In related studies,

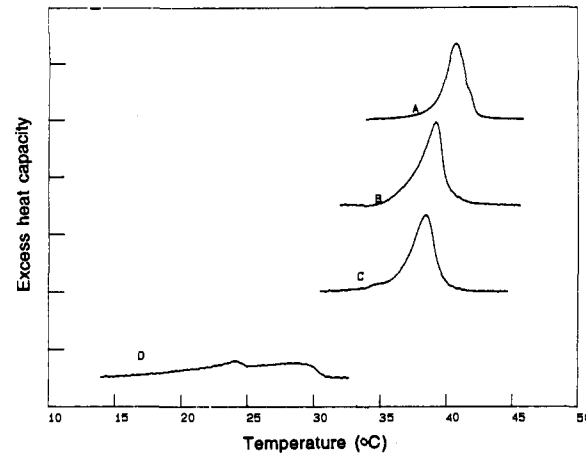

Figure 8. High-sensitivity excess heat capacity profiles of 2.05 μM DPPC vesicles in the presence of (A) 0.0, (B) 0.25, (C) 0.37, and (D) 5.0 mM T. The concentrations of T used in B corresponds to 50% rupture conditions; concentrations for C corresponds to 100% rupture.

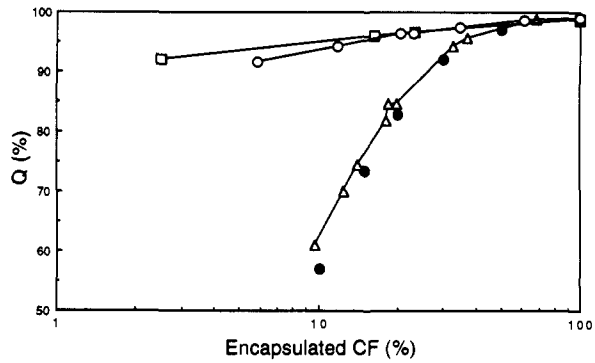

Figure 9. Plot of self-quenching efficiency as a function of percentage of CF that remains encapsulated after a 30-min incubation of vesicle dispersions containing 20 μM of DPPC (△), 20 μM plus 2.03 mM polymerized lipid (□), and 20 μM DPPC plus 2.03 mM CF-free DPPC vesicles (○). Specific concentraton ranges of T that were used for these experiments were $(0.40–0.68) \times 10^{-4}$, $(2.5–3.1) \times 10^{-4}$, and $(2.5–3.4) \times 10^{-4}$ M, respectively. The calibration curve (●) was based on internal concentrations of CF equaling 100, 50, 30, 20, 15, and 10 mM.

we have also found that when 2.03 mM of "empty" DPPC vesicles were used in place of the dummies, very similar rupture behavior was observed (Figure 9). This result demonstrates that the affinity of T toward gel-phase DPPC vesicles is very similar to that of the dummy targets. Taken together, these results establish that micellar aggregates of T are required in order to observe a rupture event.

Model for Vesicle Rupture. The rupture/leakage results that are reported herein clearly show that it is the combination of membrane packing and the supramolecular state of the attacking species which defines the pathway for CF release and that membrane rupture requires both a tightly packed target and an aggregated form of the disruptive agent. On the basis of previous studies of POPC vesicles, we have proposed a *leakage* model in which small numbers of surfactant molecules (i.e., two or three surfactants, which are clustered in adjoining monolayers), are needed to form "escape routes" in the membrane.[7] The fact that *micelles* of T are required for the rupture of gel-phase DPPC vesicles implies that such a process is of a higher "supramolecular order" (i.e., greater numbers of surfactant molecules participate in the disruption process) than vesicle leakage.[20]

Although the intimate details of the rupture mechanism remain to be elucidated, the involvement of micelles leads us to propose the following scenario. We envision that surface micelles are first formed on the outer leaflet of the bilayer. In principle, such an

(19) Wilschut, J. In *Membrane Fusion*; Wilschut, J., Hoekstra, D., Eds.; Marcel Dekker: New York, 1991; Chapter 4.

(20) Micellar aggregates of T consist of ca. 140 surfactant molecules; see ref 4.

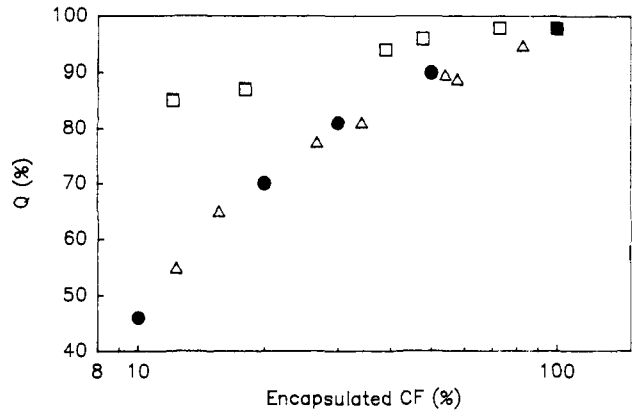

Figure 10. Plot of self-quenching efficiency Q as a function of percentage of CF that remains encapsulated after a 30-min incubation with POPC/cholesterol (55/45) vesicles at 25 °C. Phospholipid concentrations were 5 μM (△) and 1 mM (□). The concentrations ranges of T that were used were 0.047–0.076 mM and 0.22–0.3 mM, respectively. The calibration curve (●) was based on internal concentrations of CF equaling 100, 50, 30, 20, and 10 mM.

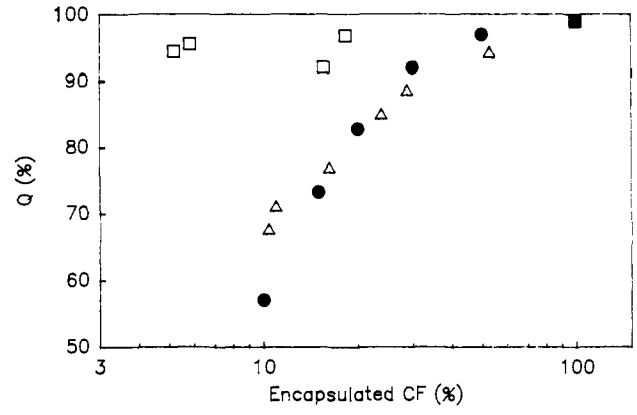

Figure 11. Plot of self-quenching efficiency Q as a function of percentage of CF that remains encapsulated after a 30-min incubation with gel-phase DPPC vesicles (25 °C). Phospholipid concentrations were 20 μM (△) and 2 mM (□). The concentrations ranges of sodium deoxycholate used to release CF were 0.8–1.2 mM and 1.8–2.2 mM, respectively. The calibration curve (●) was based on internal concentrations of CF equaling 100, 50, 30, 20, 15, and 10 mM. The critical micelle concentration of sodium deoxycholate in the buffer system used is estimated to be ca. 2.0 mM.

assembly could result from the direct collision of a micelle from the bulk aqueous phase with the vesicle surface, followed by structural reorganization. Alternatively, since the same forces that lead to aggregation in the bulk phase (micellization) can also lead to aggregation elsewhere, a surface micelle could be assembled spontaneously from a collection of adsorbed T molecules. Subsequent insertion of T into the bilayer (being fed continuously by the surface micelle) then leads to *a high local concentration of the disruptive agent within the membrane* and to the catastrophic loss of vesicle integrity. For loosely packed fluid membranes, we envision that lateral diffusion is sufficiently rapid that T can be dispersed throughout the bilayer on a time frame which avoids the formation of large defects in the lamellar phase.

Implications for Drug Design. From our previous studies, we have shown that rupture processes are not limited to gel-phase membranes. Specifically, we have demonstrated that compact fluid bilayers derived from POPC/cholesterol (1/1) also are prone toward rupture.[7] We have now found that the rupture of such cholesterol-rich membranes requires a micellar solution of T; submicellar concentrations of the surfactant lead to dominant leakage (Figure 10). This finding is important for two reasons. First, it significantly strengthens the connection between a membrane's packing and its rupture/leakage behavior, i.e., cholesterol is known to have a condensing effect on the liquid crystalline phase.[21] Thus, such a crossover from a leakage-prone to a rupture-sensitive membrane by inclusion of cholesterol is fully consistent with our results for gel- and fluid-phase DPPC vesicles as well as for fluid-phase POPC membranes. Second, the fact that human red blood cells (having a similar phospholipid/cholesterol ratio) also require micelles of T for hemolysis suggests that rupture pathways of the type described herein may have relevance to biological membranes.[22] It is noteworthy, in this regard, that membrane rupture could account for some puzzling selectivity features that have previously been found for certain surfactant–drug formulations. It is conceivable, for example, that the "brutal"[6] and relatively unselective action of micellar formulations of Amphotericin B (AmpB) (in vitro and in vivo)[6] as compared with the behavior of liposomal complexes of the drug may derive from competitive rupture/leakage pathways. We hypothesize that liposomal AmpB complexes merely serve as a reservoir of AmpB and that it is attack by AmpB *monomers* that ultimately leads to the more selective lethal attack via channel formation and leakage. If this hypothesis proves to be correct, it should have signfiicant consequences for the design and delivery of drugs that operate via membrane disruption.

Although this study has focused exclusively on Triton X-100 as the membrane-disrupting agent, we note that in preliminary studies, similar supramolecular control over rupture/leakage has been found with sodium deoxycholate. Thus, when submicellar concentrations of this surfactant were employed, a predominant leakage pathway was observed for gel-phase DPPC vesicles; using concentrations that exceeded its cmc (2.0 mM), rupture was dominant (Figure 11). Our finding that two substantially different types of surfactants exhibit this same supramolecular behavior strongly suggests that the present results will have broad generality.

The fact that lipid bilayers can be disrupted by two distinct pathways, depending on their packing properties and on the aggregation state of that attacking agent, should now be taken into account in the design of membrane-disrupting antimicrobial agents. For example, for those microbes that have compact outer membranes (e.g., viral membranes such as HIV),[23] a catastrophic destruction process may be envisioned through the use of highly aggregated species such as polymeric membrane-disrupting agents.[13] Alternatively, monomeric species could provide a leakage pathway for their destruction. For more loosely packed microbial membranes (e.g., cholesterol-deficient bacteria), disruption via leakage may represent the only viable option. Exactly how much biological selectivity can be achieved via rupture and/or leakage processes, however, remains to be determined.

At the very least, our demonstration that micelles and surfactant monomer can disrupt lipid bilayers in fundamentally different ways should open up entirely new avenues for research.

Acknowledgment. We warmly thank Sharon Krisovitch and Yong Chung for technical assistance.

Note Added in Proof. The results of a recent study, involving the Amphotericin B-induced leakage of K^+ from human erythrocytes, provide strong support for our hypothesis that it is the *monomeric* form of the drug that is the selective agent which kills fungal cells over mammalian cells (Legrand, P.; Romero, E. A.; Cohen, E.; Bolard, J. *Antimicrob. Agents Chemother.* **1992**, *36*, 2518).

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(23) Aloia, R. C.; Jensen, F. C.; Curtain, C. C.; Mobley, P. W.; Gordon, L. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 900.