Control of Permeation of Lanthanide Ions Across Phosphate-Functionalized Liposomal Membranes

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Abstract: Unilamellar vesicles of anionic, phosphate-functionalized lipid **1** (DPGPNP, 1,2-di-*O*-hexadecanoyl-*rac*-glyceryl *p*-nitrophenyl phosphate, sodium salt) have been prepared both by sonication and by extrusion. The phosphate head groups are hydrolytically stable at pH 7 but can be specifically cleaved, at 25 °C, on the exo surface by adding, to preformed aggregates, lanthanide(III) cations which are unable to permeate across the bilayer. The exovesicular specificity disappears when the cleavage is performed above the phase transition temperature of the vesicles not because of facilitated permeation of the cations but because of the increased rate of flip-flop of the lipid. Addition of lipophilic amines (*N*-hexadecyl-*N*,*N*′,*N*′-trimethylethylenediamine, TMED C_{16}; *N*-hexadecylhistamine, C_{16}-His, hexadecylamine, $C_{16}NH_2$) and, to a lesser extent, the cationic surfactant cetyltrimethylammonium bromide, CTABr, induces the cleavage of the remaining fraction of lipid in exo-cleaved vesicles. The additives affect the permeability of the bilayer, likely creating "leaky patches" in the vesicular membrane and, in the case of the amines, also transporting the cations across the bilayer by forming lipophilic complexes.

Introduction

The permeation of ionic species across biological membranes is of paramount importance because it influences many biological processes essential for the maintenance of life.[1] Typically, biological membranes are impermeable to ions[2] and membrane proteins or simpler peptides modulate ion fluxes across the lipid layers.[1,3] Permeability may also be influenced by the fluidity of the membrane itself or by the vertical movement of lipids (flip-flop) between the leaflets that comprise the membrane. Understanding these phenomena and, even more importantly, controlling them, is a challenging task that is made more difficult in biological membranes because of their structural complexity. Simple model systems, like those constituted by vesicles (liposomes),[4] can be studied more easily, even though their simplicity is often more presumed than real.

Several model membrane systems, including monolayers, planar bilayers, and liposomes, have proven experimentally useful in extending our understanding of biological membranes.[5a] Indeed, Fendler maintains that "much of our chemical understanding of membrane structures has been obtained through the investigation of models", with monolayers, bilayers, and phospholipid vesicles extensively employed.[5b] Synthetic phosphatidylcholines have also been useful in modeling the effects of drugs, cholesterol, peptides, and proteins on the thermotropic phase behavior of biological membranes.[5c] Additionally, these synthetic systems exhibit useful and unique chemistry of their own; they organize substrates, alter microenvironments, act as carriers, and modify "normal" reagent reactivities.[5b]

Many research groups have oriented their endeavors toward the understanding of permeation and mobility in vesicular systems, and toward the construction of synthetic molecules able to affect these phenomena. Effective systems have been reported by Menger,[6] Nolte,[7] Voyer,[8] Fyles,[9] Lehn,[10] Ghadiri,[11] Gokel,[12] and Echegoyen.[13] Scrimin *et al.* recently reported[14] a tripodal ligand functionalized with 10-amino acid peptides that showed tunable, Zn(II)-dependent activity.

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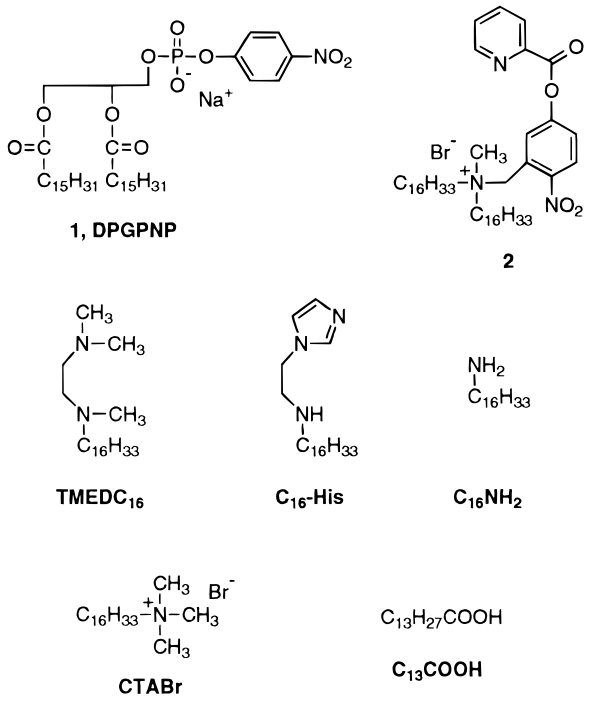
On the other hand, in a series of papers[15] Moss *et al.* highlighted some of the structural parameters that influence transbilayer mobility in vesicles. Under conditions of slow flip-flop and the impermeability of the bilayer to reactants, chemical differentiation of the interior and exterior surfaces of vesicles made of functional lipids was achieved. For instance, only phosphate esters residing on the outer layer of vesicular **1**, prepared at pH 5.5, were hydrolyzed when the external pH was raised to 11.8.[16] However, when excess CTACl (cetyltrimethylammonium chloride) was added to the above, partially-cleaved vesicles, the remaining fraction of lipids was rapidly hydrolyzed. It was suggested that CTACl "inserted" into the vesicles providing regions permeable to OH^-, thus facilitating endovesicular attack. More recently, we have observed[17] that addition of Eu(III) to DPGPNP vesicles also resulted in the hydrolysis of the outer phosphate groups, whereas addition of the lipophilic ligand TMED C_{16} accelerated the hydrolysis of the remaining **1**. The reasons for this behavior were not analyzed. In the present paper, we specifically address the effect of additives on vesicular **1** in the presence of Eu(III) and other lanthanide ions.

Results and Discussion

Vesicle Preparation and Characterization. Unilamellar vesicles of functional lipid **1** were prepared at pH 7 (0.025 M HEPES buffer) by sonication (immersion probe) or by extrusion (0.025 M HEPES buffer, 0.025 M KCl) through polycarbonate filters (two stacked 100/200 nm filters). Note the different ionic strengths for the two preparations. The vesicles prepared by sonication were not stable at higher ionic strength and, hence, could not be prepared under the same conditions as those used in extrusion. The two different methodologies gave aggregates which, although quite different in size, showed very similar phase transition temperatures, T_c. Aggregate dimensions were determined by dynamic light scattering, whereas T_c values were obtained from discontinuities in graphs of fluorescence polarization (P) *vs* temperature by using 1,6-diphenylhexatriene as an internal probe[18] (see Supporting Information) or by differential scanning calorimetry (DSC). The hydrodynamic diameters and T_c were 580 ± 80 Å, 38 °C (by fluorescence polarization), for the sonicated vesicles and 1300 ± 150 Å, 41 °C (by fluorescence polarization), and 41.8−42 °C (duplicate runs, by DSC) for the extruded ones. The size distribution of the sonicated vesicles was slightly broader than that of the extruded ones. However, the light scattering data could be satisfactorily analyzed with a Gaussian analysis and showed normalized standard deviations (or coefficients of variation) of 0.27 and 0.21 for sonicated and extruded vesicles, respectively. These standard deviations, considering the low ionic strengths of the solutions, are consistent with unimodal distributions of the vesicles.[19] The size of the vesicles prepared by sonication and the methodology of preparation for those obtained by extrusion[4] strongly support the formation of unilamellar vesicles made of a single bilayer of lipids. Indirect support of the unilamellar composition is provided by the hydrolysis experiments[16] (see also below). Vesicle preparations were stable for several hours but tended to grow and eventually precipitate after two or more days.

Chart 1



Lanthanide-Catalyzed Hydrolysis of Vesicular 1. The above vesicular preparations are fairly stable from the hydrolytic point of view at pH 7, in line with the hydrolytic inertness of phosphate diesters at neutral pH. However, upon the addition of lanthanide salts, 54−70% of the total phosphate[20] is cleaved with rate constants that depend only weakly on the nature of the lanthanide,[21] but more strongly on the mode of preparation of the aggregate (Table 1). Lipids of the larger aggregates prepared by extrusion appear to react about 5 times faster than those of the smaller, sonicated vesicles. However, the percentage of cleaved lipid is higher with the sonicated vesicles than with the extruded ones. This is consistent with the cleavage of the lipids residing in the outer leaflet of the membrane of both vesicular preparations. The higher curvature of the bilayers of the smaller vesicles affects both the distribution of lipids residing in the outer and inner leaflets of the vesicular membrane and

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(20) The total amount of phosphate present was determined by addition of excess CTABr to an aliquot of the vesicle solution and following the cleavage at pH = 12.[16] The final absorbance was then corrected for the difference in pH.

(21) This is in accord with findings of other authors, see: Morrow, J. R.; Buttery, L. A.; Berback, K. A. *Inorg. Chem.* **1992**, *31*, 16. Breslow, R.; Huang, D.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4080.

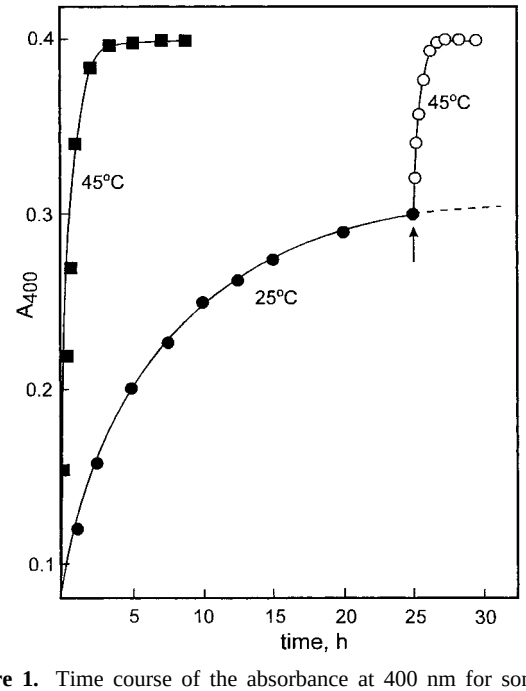

Figure 1. Time course of the absorbance at 400 nm for sonicated vesicles of 5.4×10^{-5} M lipid **1** upon the addition of 1.7×10^{-3} M $EuCl_3$ for an experiment carried out at pH 7, initially at 25 °C (●) and, after 26 h, at 45 °C (○), and also for an experiment carried out at 45 °C from the beginning (■).

the packing of the head groups and, hence, the accessibility to metal cations.[22] In larger, extruded vesicles the number of lipids residing in the outer or inner layers is very similar whereas in smaller, sonicated vesicles the number of lipids in the outer layer is larger.[23]

Exovesicular (or exoliposomal) phosphates interact with the lanthanide ions that accelerate their hydrolysis while endovesicular substrate groups are not exposed to them. This implies that neither the permeation of the metal ions nor the transbilayer movement of the lipids are fast (relative to exovesicular cleavage) under these conditions. Residual (endovesicular) phosphate is cleaved at a much slower rate ($k_\psi \approx 4.4 \times 10^{-6}$ s^{-1} for sonicated vesicles, see below) at 25 °C, or in a much faster process when the temperature is raised above T_c. Quite important is the observation that raising the reaction temperature above the T_c leads to the total disappearance of the biphasic kinetic behavior, while 100% of the total phosphate[20] is cleaved in a single monoexponential kinetic process upon the addition of the lanthanide salt.

Figure 1 reports biphasic and monophasic kinetic profiles observed below and above the T_c for the Eu(III) cleavage of small sonicated vesicles of **1**. An Arrhenius-type plot showing the dependence of the rate constants for the hydrolysis of **1** in the presence of 1.7×10^{-3} M Eu(III) as a function of the temperature for sonicated vesicles is shown in Figure 2. At $T \approx T_c$, the biphasic behavior not only disappears but there is also a marked diffference in the slope of the correlation line, indicative of a higher activation energy, E_a, for the hydrolytic process. Changes of E_a for reactions of functional vesicles as a function of the fluidity of their bilayer appear to be quite common.[24]

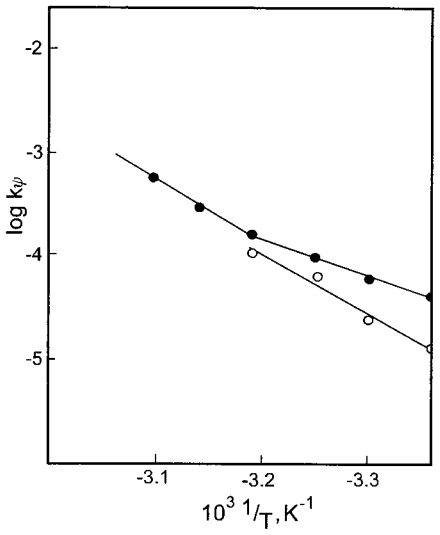

Figure 2. Dependence of the observed rate constant, k_ψ, for the cleavage of **1** at pH 7 on the inverse temperature. (●) Exovesicular (below T_c) or total (above T_c) cleavage upon the addition of 1.7×10^{-3} M $EuCl_3$ to sonicated vesicles of 5.4×10^{-5} M lipid **1**; (○) endovescicular process in exo-cleaved vesicles upon the addition of 1.6×10^{-5} M TMED C_{16}.

The disappearance of the biphasic behavior above T_c may be due to two independent phenomena: (a) an increased rate of flip-flop of lipid **1** and (b) permeation of Eu(III) ions across the bilayer. In the first case, despite the increased fluidity of the membrane, the metal ions would be unable to cross the membrane so that the internal water pool of the vesicles would remain devoid of metal ions.

To check which process is responsible for the disappearance of the biphasic behavior, we ran the following experiment, illustrated in Figure 3. To a solution of vesicular **1** kept at 45 °C *(i.e.* above T_c), was added $EuCl_3$ (1.7×10^{-3} M) to start the cleavage process. After 20 min, when *ca.* half of lipid **1** was hydrolyzed, the temperature was quickly lowered to 25 °C (arrow), and the kinetics of the hydrolysis were monitored at this temperature. At 25 °C the hydrolysis proceeds with a rate constant $k_\psi = 5 \times 10^{-5}$ s^{-1} (very similar to that measured independently, see Table 1). Significantly, not all of the residual **1** is cleaved at this rate. In fact, only when the temperature is again raised to 45 °C is all of the phosphate eventually cleaved. This last fraction of **1** amounts to ca. 30% of the remaining phosphate, in line with the relative interior/exterior distribution of the lipid in sonicated vesicles.

The experiment clearly suggests that raising the temperature above T_c did not alter the impermeability of the bilayer to Eu(III) ions, but only increased the rate of flip-flop of the lipids. These results parallel those we have obtain previously with cationic lipid **2**.[25] In that case too, the temperature did not alter the impermeability of the vesicular membrane to Cu(II) ions. The present results also indicate that the anionic phosphate head

(22) Note, however that Cuccovia has observed opposite behavior in comparing the reactivity of small and large vesicles (see: Kawamuro, M. K.; Chaimovich, H.; Abuin, E. B.; Lissi, E. A.; Cuccovia, I. M. *J. Phys. Chem.* **1991**, *95*, 1458). However, these authors used nonfunctional cationic vesicles and attribute their results, at least in part, to differences in the binding of the substrates. The fact that the substrate is part of the lipid as well as the difference in charge of the present system does not allow a direct comparison with the previously described one.

(23) Assuming a thickness of the bilayer of 37 Å and an identical packing of the head groups in both halves of the bilayer the expected lipid distribution for vesicles of these dimensions is 61/39 and 57/43 for the sonicated and extruded preparations, respectively (see ref 5b, pp 129−130). The fact that we find a 70/30 ratio for the lipid distribution in the smaller, sonicated vesicles may be associated with the bulkiness of the *p*-nitrophenyl group bound to the phosphate, which alters the theoretical distribution in favor of the outer layer.

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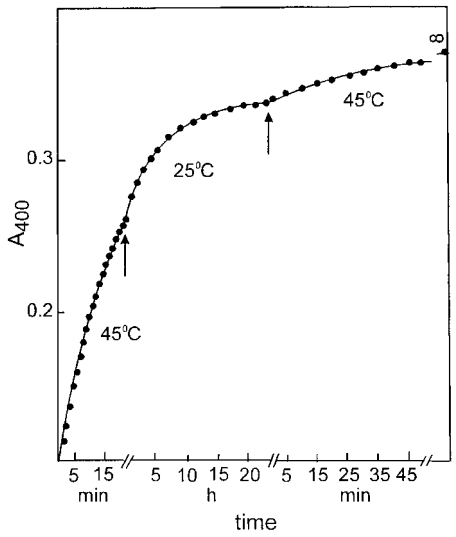

Figure 3. Time course of the absorbance at 400 nm of an experiment carried out at different temperatures with sonicated vesicles of 5.4×10^{-5} M lipid **1** and 1.7×10^{-3} M $EuCl_3$. The arrows denote the times of changes of temperature. (Note the changes in the time scale.)

Table 1. Observed Rate Constants, k_ψ for the Exovesicular Cleavage of Phosphate Lipid **1** at 25 °C with Sonicated or Extruded Vesicular Preparations[a]

	$10^5 k_\psi$, s^{-1} (% cleaved)	
lanthanide(III)	sonicated vesicles	extruded vesicles
Eu	4.1 (70)	20 (61)
Tb	3.8 (68)	17.7(57)
Tm	4.4 (70)	21.8 (55)
Yb		16 (55)
Lu		18.1 (55)

[a] Conditions for extruded vesicles: $[\mathbf{1}] = 5.8 \times 10^{-5}$ M; [lanthanide(III)] $= 1 \times 10^{-3}$ M; pH 7, 0.025 M HEPES, 0.025 M KC1. Conditions for sonicated vesicles: $[\mathbf{1}] = 5.4 \times 10^{-5}$ M; [lanthanide(III)] $= 1.7 \times 10^{-3}$ M; pH 7, 0.025 M HEPES.

groups of **1** do not act as carriers of Eu(III) ions when lipid **1** flips inside. Probably this reflects a weak interaction of the phosphate groups with the metal ions insufficient to desolvate them from water molecules.

Effect of Additives on the Kinetic Behavior of Vesicular 1. In the previous section we showed that total cleavage of vesicular **1** could be obtained by raising the reaction temperature above that of the phase transition. The same result occurs if, to the partially cleaved vesicles, we add the lipophilic ligand TMED C_{16}. Figure 4 shows the kinetic profiles observed after increasing amounts of an ethanolic solution of TMED C_{l6} are added to a solution of sonicated vesicles of **1** incubated for 26 h at 25 °C.[26] It may be immediately appreciated that the rate constant of the slow kinetic process increases steadily as the amount of additive increases. This fact is quantified in the inset to the same Figure where the linear dependence of k_ψ *vs* [TMED C_{16}] is highlighted.

From this plot we estimate a rate constant at zero additive concentration of 4.4×10^{-6} s^{-1} ($t_{1/2} = 44$ h), which sets an upper value of the rate constant for the permeation of Eu(III)

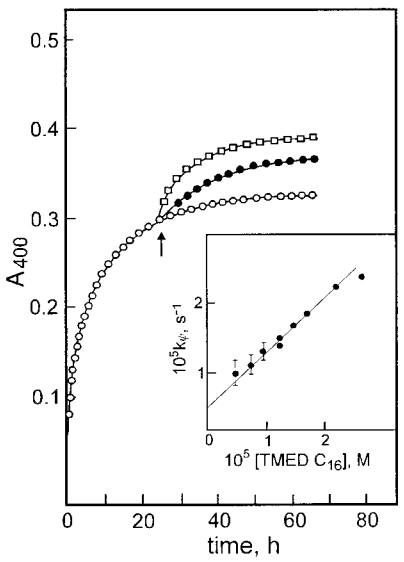

Figure 4. Time course of the absorbance at 400 nm for the cleavage of sonicated vesicles of 5.4×10^{-5} M lipid **1** upon the addition of 1.7×10^{-3} M $EuCl_3$ (25 °C, pH 7) (○); upon the addition of 1.6×10^{-5} M TMED C_{16} (●); and upon the addition of 2.1×10^{-5} M TMED C_{16} (□). Inset: Dependence of the rate constant for the endovesicular cleavage of **1** on the concentration of TMED C_{16} added to the exo-cleaved vesicles (conditions as above). Points at lower [TMED C_{16}] have been determined assuming the infinity absorbance observed for faster reactions.

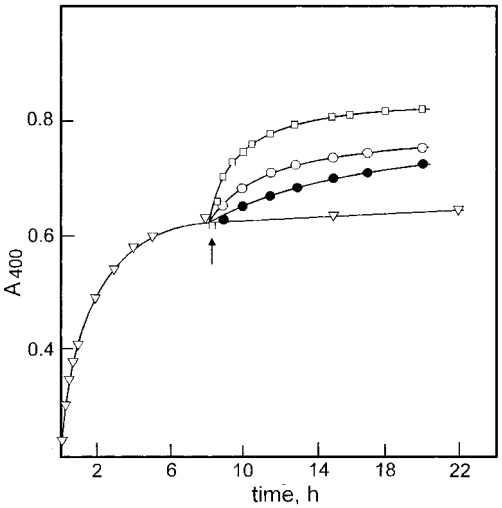

Figure 5. Time course of the absorbance at 400 nm for the cleavage of extruded vesicles of 7.0×10^{-5} M lipid **1** upon the addition of 1×10^{-3} M $TmCl_3$ (25 °C, pH 7.2, 0.025 M HEPES buffer, 0.025 M KCl). After 8 h (arrow), 2.5×10^{-5}, (●), 5.0×10^{-5}, (○), 1.0×10^{-4} (□), or no TMED C_{16} (▽) was added.

across these vesicles or that of flip-flop (depending on which process is faster). The possibility of following this experiment directly is hampered by the instability of the vesicle preparation over the long period of time it requires.

Similar data were obtained by using large, extruded vesicles and Tm(III) ions instead of Eu(III); see Figure 5. For instance, with these vesicular preparations, increasing the concentration of TMED C_{16} from 2.5×10^{-5} to 1×10^{-4} M increased the rate constant of the endo cleavage process from 8.9×10^{-5} to 1.1×10^{-4} s^{-1}. The Tm(III) results also demonstrate that the initial exo-specific, metal-catalyzed phosphate cleavage is not dependent on vesicle size or mode of preparation, or on the identity of the lanthanide cation.

(25) Ghirlanda, G.; Scrimin, P.; Tecilla, P.; Tonellato, U. *J. Org Chem.* **1993**, *58*, 3025. For an extended series of investigations relating the dynamics of flip-flop to lipid molecular structure, see refs 15, especially 15j.

(26) Control experiments demonstrated that the small amount of ethanol added with the additive did not affect the permeability of the membrane. Note, however, that higher ethanol concentrations alter the permeability, see: Hunt, G. R. A.; Jones, I. C. *Biochim. Biophys. Acta* **1983**, *736*, 1.

Table 2. Observed Rate Constants for the Cleavage of Residual (Endovesicular) Lipid Phosphate in Sonicated, Exocleaved Vesicles of **1**[a]

additive[b]	$10^5 k_\psi$, s^{-1}	additive[b]	$10^5 k_\psi$, s^{-1}
none	(0.44)[c]	$C_{16}NH_2$	1.75
C_{16}-His	4.18	CTABr	1.0
TMED C_{16}	2.28	$C_{13}COOH$	(0.5)[d]

[a] Conditions: 25 °C, $[EuCl_3] = 1.7 \times 10^{-3}$ M, [**1**] $= 5.4 \times 10^{-5}$ M. [b] [Additive] $= 2.0 \times 10^{-5}$ M; see Chart 1 for additive structure. [c] Estimated from the plot of Figure 4. [d] The effect is almost indistinguishible from that of the solution without additive; the rate constant is estimated.

A possible explanation for the effect of the added TMED C_{16} is that it chelates the lanthanides,[27] acts as a carrier of the ions, and allows their effective transport across the vesicular membrane where they initiate the endoliposomal cleavage of the phosphate. Alternatively, the single-chain diamine molecules could insert into the vesicles, affording leaky "patches" that facilitate the permeation of the ions. It is also possible that instead of influencing the permeation of the ions, the additive affects the rate of flip-flop of the lipids. It should be noted that the T_c (as measured from fluorescence polarization experiments, see Supporting Information) of sonicated vesicles is practically not affected by the addition of 1.6×10^{-5} M TMED C_{16}. This tends to support a mechanism that involves sorting of the additive with the formation of "patches" in the membrane. In any event, the first (carrier) hypothesis cannot be the only explanation for the observed phenomenon, because increased endoliposomal cleavage is also observed when the cationic surfactant CTABr is added to the exo-cleaved vesicles. Note that the effect of CTABr, as well as that of the additives, occurs at a concentration well below that required for the destruction of the liposomes as is clearly shown by light-scattering experiments.

The effect of various additives on the rate constant for endovesicular cleavage is reported in Table 2, which refers to 5.4×10^{-5} M exo-cleaved vesicular solutions to which 2.0×10^{-5} M additive is added. Larger effects are observed with lipophilic amines like C_{16}-His and TMED C_{16}, which can chelate the lanthanide ions, or $C_{16}NH_2$, which, in principle, can also coordinate to the ions, although to a lesser extent. These amines are likely to be involved in transport of the lanthanide ions as lipophilic complexes. Enhanced rate of transport of transition metal and lanthanide ions across model membranes by lipophilic ligands is a known process. Since, however, the effect of CTABr cannot be ascribed to transport of the ions across the membrane, it appears that in these systems the increased rate of endovesicular phosphate cleavage induced by lipophilic additives may also be due, as suggested above, to (a) their ability to alter the permeability of the membrane, forming regions that are more fluid and, hence, more permeable to the ions, or (b) to the acceleration of the flip-flop of **1** (flippase-like effect).

The failure of $C_{13}COOH$ to influence permeability is rather surprising, particularly in the light of the reported fast movement of fatty acids across phosphatidylcholine bilayers.[28] However, considering that phosphatidylcholine is a zwitterionic lipid, it is quite possible that the electrostatic repulsion due to the anionic nature of vesicular **1** alters both the partition of the fatty acid in the vesicular membrane and/or its permeation across the bilayer. Alternatively, in analogy with what was suggested above for the phosphate head groups of **1**, the carboxylate may not provide sufficient desolvation of the cations from water.

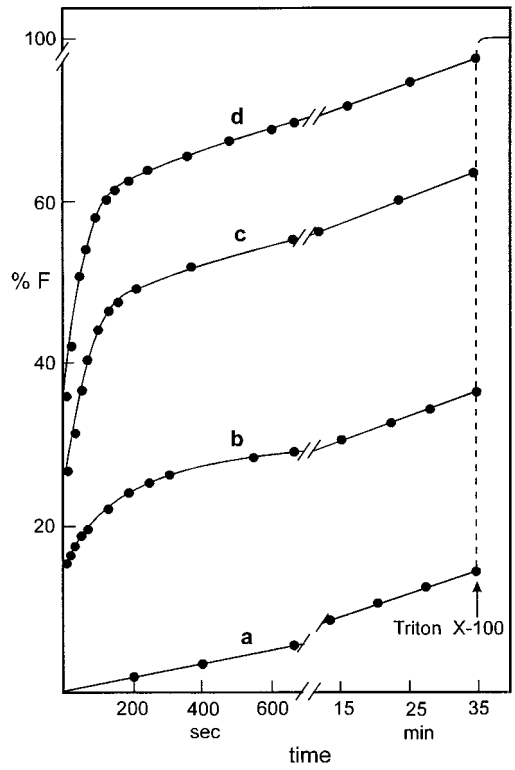

Figure 6. Time course of the fluorescence increase when CF-loaded vesicles of sonicated **1** ([**1**] $= 1.1 \times 10^{-4}$ M, pH 7, 25 °C) are exposed to different concentrations of added TMED C_{16}. The arrow indicates when an excess of Triton X-100 was added. ([TMED C_{16}] = 0 (a), 1.3×10^{-5} (b), 2.7×10^{-5} (c), and 3.3×10^{-5} M (d).

To discriminate between the enhanced permeability or the enhanced flip-flop rate hypotheses, we ran fluorescence experiments with vesicle-entrapped carboxyfluorescein (CF).[29] In these experiments, vesicles of **1** were obtained by sonication in the presence of highly concentrated (5×10^{-2} M) CF. Subsequent elution on a Sephadex G-75 column with an aqueous solution (0.1 M in NaCl, to compensate for the osmotic pressure, and HEPES buffer, 0.025 M, pH 7) allowed us to separate free CF from CF-loaded vesicles. The final concentration of vesicular **1** was adjusted to 1.1×10^{-4} M. This concentration, which is twice that used for the hydrolysis experiments, is the lowest possible one that still represents a concentration of CF high enough to detect its increase of fluorescence after its release and dilution in the bulk solvent upon inducing leakage of the vesicles.

Leakage experiments were performed by adding various amounts of additives and following the increase of fluorescence with time. Fluorescence due to leakage is reported as a percentage of the total fluorescence determined by destroying the vesicles by the addition of excess Triton X-100 at the end of the experiment (see the Experimental Section for details). Figure 6 reports the time course of experiments carried out in the presence of different concentrations of added TMED C_{16}. Under the conditions used for these experiments (higher lipid concentration and ionic strength than those employed for the kinetics) these vesicles are rather leaky and release *ca.* 10% of their CF content in 20 min. However, the addition of the additive greatly increases the rate of release of trapped CF.

(27) A log $K \approx 10$ is reported for the binding of 1,2-aminoethane to Eu(III) in anhydrous acetonitrile, see: Forsberg, J. H.; Moeller, T. *Inorg. Chem.* **1969**, *8*, 889.

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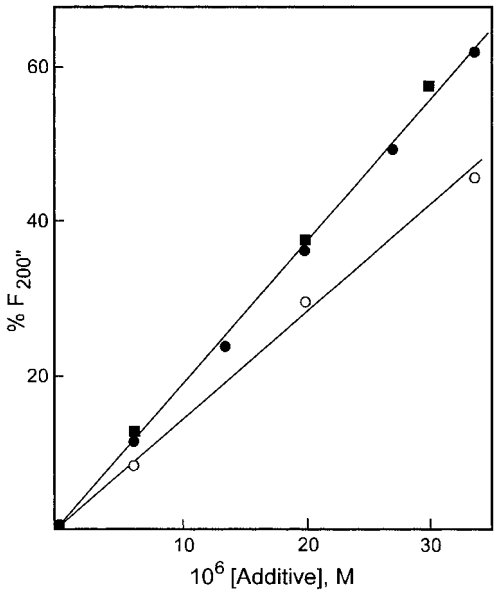

Figure 7. Relative amount of released CF after 200 s from CF-loaded vesicles of **1** ([**1**] 1.1×10^{-4} M, pH 7, 25 °C) as a function of the concentration of added TMED C_{16} (●),CTABr (■), or $C_{16}NH_2$ (○).

The time dependence of the process does not follow a simple kinetic law.[29b] Upon mixing, there is a burst of fluorescence, followed by a relatively fast process and, after *ca.* 200 s, by a slow one. Due to this complexity, we have compared the effectiveness of the various additives by reporting the percent fluorescence observed 200 s after the addition of the additive. These results are reported graphically in Figure 7. They reveal the effects to be quite similar for the three additives studied. Increasing only the rate of flip-flop of lipid **1** would not affect the rate of leakage of the dye from the vesicles. Therefore, we infer that the additives increase the permeability of the bilayer membrane.

We can now compare these results with those obtained from the hydrolysis experiments (Table 2). This comparison should be made with some caution because, as said above, the higher ionic strength required for the leakage experiments makes these vesicles much more leaky than those studied in the hydrolysis. Accordingly, we only compare the effects of the additives within the same vesicular preparation. We notice a higher efficiency of TMED C_{16} and $C_{16}NH_2$ relative to CTABr in the case of the hydrolytic system, whereas only very minor differences are present in the CF leakage studies. This supports our hypothesis that these amines coordinate to Eu(III) and that the resulting lipophilic metal complexes rapidly permeate across the liposomal membrane. This does not exclude the simultaneous participation of a "leaky patch" passive permeation mechanism. Although no information can be obtained from these experiments on possible effects of the additives on the rate of flip-flop of the functional lipid, we may draw the following conclusions: (a) the amine and ammonium-based additives alter the permeability of the liposomal membrane, increasing the permeation of ions (Eu(III)) or CF,[30] and (b) the amines, able to coordinate to the metal ions, may also act as carriers of the lanthanides across the bilayers.

Conclusion

In this study we have shown that unilamellar vesicles of anionic, phosphate-functionalized lipid **1** which are hydrolytically stable at pH 7 can be specifically cleaved on the exo surface by adding, to preformed aggregates, lanthanide(III) cations. The hydrolytic process depends very little on the nature of the cations but appears to be slower with smaller, sonicated vesicles than with larger, extruded preparations. The exovesicular specificity disappears when the cleavage is performed above the phase transition temperature of the vesicles. With variable-temperature experiments we are able to demonstrate that this result is not associated with the permeation of the cations across the bilayer but, rather, with the increased rate of flip-flop of the lipid. This behavior of the vesicles made of the *anionic* lipid **1** is very similar to that found with vesicles made of *cationic* lipid **2** (with Cu(II) cations), suggesting that the charge of the aggregate hardly influences its permeability to metal cations in spite of the electrostatic interaction between the anionic aggregate and the lanthanide cations in the present case.

Addition of lipophilic amines or, to a lesser extent, cationic surfactant CTABr, at concentrations that do not destroy the vesicles, induced the cleavage of the remaining fraction of lipid in exo-cleaved vesicles whereas a fatty acid was inactive in this regard. By means of CF-leakage experiments, we proved that the additives affect the permeability of the bilayer likely creating "leaky patches" in the vesicular membrane. By comparing the fluorescence with the cleavage experiments we found that the amines were also involved in the transport of the cations, through the formation of lipophilic complexes able to cross the bilayers. This made them more active than the cationic surfactant in inducing the cleavage of the remaining fraction of intact **1**. Our experiment gave no clues about the possibility of these additives acting as flippases to accelerate the rate of flip-flop of the lipids.

This study of vesicular **1** has allowed us to examine several parameters that influence the permeability of (lanthanide) cations across the vesicular bilayer and to learn how the permeability can be controlled by using very simple additives. It has also been shown that the fluidity of the membrane does not influence the permeation of the (lanthanide) cations in spite of their interaction with the phosphate head groups at the aggregate/aqueous solution interface which is at the root of the observed acceleration of the cleavage of this functional group.

In conclusion, we note that there is a continuing increase of interest in lanthanides: they are currently used as contrast agents for NMR imaging[31] and several recent reports have highlighted their ability, as aquo ions or complexed to appropriate ligands, to accelerate the hydrolysis of DNA and RNA.[32] In this context the knowledge of parameters affecting metal cation permeation across biomembranes becomes important. With our model system we have shown that although Eu(III) cannot permeate a vesicular membrane, the addition of very simple lipophilic amines may greatly alter its permeability across the bilayer. Models should not be confused with real systems, but they can provide relevant mechanistic information useful in manipulating the chemistry of more complex natural systems.

Experimental Section

General Methods and Materials. Chemical shifts of ^{1}H-NMR spectra are reported in ppm relative to internal Me_4Si. The buffer used

(30) It has been reported that premicellar concentrations of certain surfactants alter the permeability of phospholipid bilayers, see: (a) Hunt, G. R. A. *FEBS Lett.* **1980**, *119*, 132. (b) Naka, K.; Sadownik, A.; Regen, S. L. *J. Am. Chem. Soc.* **1993**, *115*, 2278.

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throughout was HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. $EuCl_3$, $TmCl_3$, $TbCl_3$, $LuCl_3$, and $YbCl_3$ were analytical grade materials used as received. Stock solutions were titrated against EDTA following standard procedures.[33] *N*-Hexadecyl-*N*,*N*,*N*′-trimethylamine (TMED C_{16})[34] and *N*-hexadecylhistamine (C_{16}-His)[35] were prepared following reported procedures.

1,2-Di-*O*-hexadecanoyl-*rac*-glyceryl *p*-Nitrophenyl Phosphate, Sodium Salt (1, DPGPNP). *p*-Nitrophenylphosphodichloridate (0.5 g, 1.9 mmol) was dissolved in 20 mL of methylene chloride and cooled in an ice bath. To the solution was added, over 20 min, *via* a dropping funnel, 500 mg (0.88 mmol) of 1,2-di-*O*-hexadecanoyl-*rac*-glycerol and 0.25 mL (1.9 mmol) of triethylamine dissolved in 50 mL of methylene chloride. After the addition, the ice bath was removed and the reaction mixture was stirred for an additional 2 h at room temperature, then warmed to 35 °C for 10 min. After the solution was cooled to room temperature, 30 mL of 5% aqueous $NaHSO_3$ was added, the pH was adjusted to 1-2 with HC1, and the mixture was stirred at room temperature for 10 min. The methylene chloride phase was separated and dried over Na_2SO_4, and the solvent was stripped off. The oily residue was crystallized from ethyl acetate to give 0.46 g (68%) of the title compound, mp 44−46 °C. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.9 (brt, 6H, $2CH_3$), 1.3 (m, 48H, $2(CH_2)_{12}$), 1.6 (brt, 4H, $2CH_2$), 2.3 (brt, 4H, $2CH_2C{=}O$), 4.1−4.4 (m, 2H) and 4.4−4.6 (m, 2H, $C(=O)OCH_2$ and CH_2OP), 5.3 (m, 1H, CHO), 7.4 and 8.3 (AA′BB′, 4H, aryl). Anal. Calcd for $C_{41}H_{71}NO_{10}PNa$ (791.996): C, 62.18; H, 9.04; N, 1.77. Found: C, 62.02; H, 8.91; N, 1.81.

Vesicle Preparation. (a) By Sonication. DPGPNP (1.5 mg) was dissolved in 1 mL of $CHCl_3$ and the solution was slowly evaporated with a gentle nitrogen stream. The resulting film was kept under vacuum for *ca.* 1 h, 7.5 mL of 0.025 M pH 7 HEPES buffer was then added, and the mixture was sonicated for 10 min at 35 °C (Branson B15 sonifier, immersion probe, 50% power output). The solution was allowed to cool to room temperature and then filtered through a 0.45 μm Millipore filter. **(b) By Extrusion.** A thin film of DPGPNP (**1**) was prepared in a 200-mL round-bottomed flask by rotary evaporation of 6 mg of lipid dissolved in <2 mL of chloroform. The film was further dried for 1 h with a vacuum pump. It was then thoroughly hydrated with 15 mL of 0.025 M aqueous HEPES buffer (0.025 M in KCl). The resulting preparation was prefiltered through a 0.8 μm syringe filter. Next, the filtrate was subjected to 5 cycles of slow warming to 45−50 °C (water bath), followed by mechanical vortexing (3 min). The solution of multilamellar vesicles thus formed was then extruded 10 times through two stacked Corning Costar polycarbonate filters (100, 200 nm) under 200 psig of nitrogen at a barrel temperature of 54 °C with use of a Lipex Biomembranes extruder.

Vesicle Characterization. (a) Phase Transition Temperature. To the proper amount of **1** dissolved in $CHCl_3$ (to obtain a final 5.4×10^{-5} M vesicle preparation) was added diphenylhexatriene (DPH) dissolved in tetrahydrofuran (to obtain a 5×10^{-6} M final concentration of DPH). Vesicles were then prepared, after solvent evaporation, following one of the above procedures and the fluorescence polarization (P) was determined at different temperatures. T_c was obtained as the midpoint of the discontinuity in the graph of P *vs* temperature.[18] In the case of extruded preparations, liposome solutions were also scanned from 15 to 50 °C with a Microcal MC-1 scanning calorimeter. A single, sharply defined phase transition was observed on duplicate runs. **(b) Dynamic Light Scattering**. The vesicle preparations were analyzed at a 90° scattering angle with a Spectra-Physics Model 2916-04s Argon laser source, operating at 488 nm, and a NICOMP 370 autocorrelator, analyzing the light scattering data with proprietary software provided with the device.

Kinetic Studies. To 2-3 mL of vesicular **1** prepared as above and kept in a cuvette at 25 °C (or as indicated) was added the proper amount of lanthanide salt stock solution to make the final concentrations reported in the figures and tables. Additives were added as ethanolic solutions, and the maximum amount of ethanol added was 5% of the total volume. This amount does not affect the course of the kinetic processes studied. Kinetics were followed at 400 nm by measuring the increase of absorbance due to the release of *p*-nitrophenolate. Rate constants were obtained by nonlinear regression analysis of the absorbance *vs* time data with the software package Enzfitter.[36] Reproducibility of repeated runs was ±5%. Variable-temperature experiments were performed with use of a glass-jacketed cuvette by switching the temperature of the circulating bath as required at proper times during the experiment.

4(5)-Carboxyfluorescein (CF) Leakage Experiments. Vesicles of lipid **1** (1.1×10^{-4} M) were prepared in the presence of 5×10^{-2} M CF by sonication. Subsequently, the vesicles were eluted on a Sephadex G-75 column with an aqueous solution that was 0.1 M in NaCl and 0.025 M in HEPES buffer, pH 7. The resulting CF-loaded vesicles were analyzed with a fluorescence spectrophotometer ($\lambda_{exc} = 488$ nm, $\lambda_{em} = 520$ nm) following the increase of fluorescence with time[29] after the addition of different amounts of additives as ethanolic solutions, as in the kinetic experiments described above. 100% CF content was determined by complete vesicle disruption with excess Triton X-100 (30 μL of a 0.1 M solution).

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Supporting Information Available: Figure depicting the fluorescence polarization experiments with the different vesicular preparations and in the presence of additives (1 page). See any current masthead page for ordering and Internet access instructions.

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