Surface-Differentiated Model Phospholipid Bilayers

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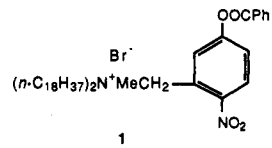
Abstract: Dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC) were synthesized in which the head groups were covalently functionalized with *p*-nitrophenyl benzoate (PNPB) moieties. Liposomes composed of 1 part functional lipid and 7 parts nonfunctional DPPC or DOPC were created at pH 6 and subjected to an exovesicular/endovesicular 12/6 pH gradient. Under these conditions, the exovesicular PNPB groups saponified more rapidly ($k \sim 4.5 \times 10^{-2}$ s^{-1}) than the endovesicular PNPB groups [$k \sim 7 \times 10^{-6}$ s^{-1} (DPPC) or 7×10^{-5} s^{-1} (DOPC)], leading to surface-differentiated liposomes with exovesicular *p*-nitrophenylate labels and intact, endovesicular PNPB groups. The half-times for flip-flop reequilibration of the intact PNPB lipids between the endovesicular and exovesicular leaflets of the liposomal bilayers were (DPPC) ~6 min at 65 °C and (DOPC) ~20 min at 65 °C. The permeabilities of these liposomes to H^+/OH^- were also studied. Comparisons of the DPPC and DOPC flip-flop dynamics to those of analogous, cationic, ammonium ion lipids highlight the relative dynamic stability of the zwitterionic phospholipids in liposomal bilayers.

Within the phospholipid bilayers that provide the framework for nearly all biological membranes,[1,2] a transverse asymmetry of the lipid distribution between the outer (exo) and inner (endo) leaflets of the bilayer is common and of structural and physiological importance.[1-5] For example, the lipid distribution in red blood cells finds phosphatidylcholine and sphingomyelin mainly on the exo surface, whereas phosphatidylethanolamine and phosphatidylserine are largely constrained to the endo surface.[1]

Similar asymmetric distributions occur in small unilamellar liposomes or vesicles constructed of phospholipid mixtures, where the asymmetry is probably due to packing requirements imposed by the curved bilayer membrane: the lipids with larger polar head groups favor the exo surface, which is more gently curved, while lipids with smaller head groups accumulate at the more highly curved, less spacious endo surface.[1] Indeed, the head group "size selectivity", thus imposed, has been cleverly used by Fuhrhop to construct a monolayer vesicle from a macrocyclic bolaamphiphile whose larger thiosuccinic acid head groups assume positions on the outer vesicle surface, while its smaller sulfonic acid head groups are constrained to the inner surface.[6]

The generation of such *surface-differentiated* liposomes is an important step in the modeling of biological membranes.[7] Our approach to this problem focuses on the *chemical* differentiation of the exo from the endovesicular head groups of functionalized lipids using reactions that preferentially occur at the exovesicular surface of the bilayer. For example, bilayer vesicles constructed at pH 4 from the dioctadecylammonium ion surfactant, **1**, possess an acidic inner aqueous pool and bear *p*-nitrophenyl benzoate functionalized head groups at both endo- and exovesicular surfaces. Adjustment of the external pH to 8 and reaction with glutathione (RS^-) rapidly convert the exovesicular functionalities to chromophoric (400 nm) *p*-nitrophenylate moieties. However, the imposed transmembrane exo/endo pH 8/4 gradient decays more slowly than the exovesicular thiolysis reaction occurs. This precludes simultaneous esterolysis at the endovesicular head groups and leads to exo/endo surface-differentiated liposomes.[8]

Similar methodology permitted the construction of surface-differentiated vesicles from the hexadecyl and eicosyl analogues



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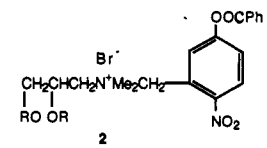

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of **1**,[9] from dialkoxy or diacyloxy pseudoglyceryl ammonium ion lipids **2**,[10] from analogues of **2** with unsaturated acyl residues,[11] and from phytanyl lipids.[12]

A crucial sequel to these surface differentiation experiments is the phenomenon of transbilayer lipid migration ("flip-flop") from one leaflet of the bilayer to the other.[1-4] Clearly, random flip-flop of lipid molecules within a surface-differentiated bilayer will diminish and ultimately destroy the membrane's asymmetry. If we wish to create increasingly sophisticated synthetic membranes, it becomes important to understand the dynamics of lipid flip-flop.

Flip-flop occurs in both natural and model phospholipid bilayers,[1,4] but there has been wide variation both in the methods used to observe the phenomenon and in the reported dynamics. Two examples illustrate these points. McConnell, using a spin-labeled dipalmitoylphosphatidylcholine probe, determined a half-time of 2.6-7 h for its endo to exo migration in egg lecithin liposomes at 30 °C.[13] At the other extreme, Dawidowicz estimated a half-time in excess of 11 days at 37 °C for the flip-flop of a tritiated dioleoylphosphatidylcholine probe in a dioleoylphosphatidylcholine–erythrocyte phosphatidylcholine liposome that had been asymmetrized by means of a phosphatidylcholine-exchange protein.[14]

Obviously, it would be desirable to survey the dynamics of lipid flip-flop in related liposomes, under comparable conditions, using constant methodology and chemically well-defined lipid compositions. One might then be able to closely correlate lipid *molecular structure* with lipid dynamics within the derived liposomes. We initiated a systematic study, first examining the surface-differentiated liposomes derived from lipids **1** and **2**.[9-12] In each case, 9-13% of the functional lipid was combined with 87-91% of the nonfunctional dimethyl (**1**) or trimethyl (**2**) ammonium analogue, so that the liposomes remained cationic throughout the entire experiment.

(1) Gennis, R. B. *Biomembranes: Molecular Structure and Function*; Springer Verlag: New York, 1989; esp. p 151 ff.
(2) Jain, M. K. *Introduction to Biological Membranes*, 2nd ed.; Wiley: New York, 1988; esp. p 105 ff.
(3) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley: New York, 1982; p 113 ff.
(4) Zachowski, A.; Devaux, P. F. *Comments Mol. Cell. Biophys.* **1989**, *6*, 63.
(5) Op den Kamp, J. A. F. *Annu. Rev. Biochem.* **1979**, *48*, 47.
(6) Fuhrhop, J.-H.; Methieu, J. *J. Chem. Soc., Chem. Commun.* **1983**, 144. Interestingly, head group size does not appear to be a major factor in the experiments that we will describe below.
(7) Reviews: (a) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 113. (b) Fuhrhop, J.-H.; Fritsch, D. *Acc. Chem. Res.* **1986**, *19*, 130. (c) Fuhrhop, J.-H.; Mathieu, J. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 100.
(8) Moss, R. A.; Bhattacharya, S.; Chatterjee, S. *J. Am. Chem. Soc.* **1989**, *111*, 3680.
(9) Moss, R. A.; Fujita, T.; Ganguli, S. *Langmuir* **1990**, *6*, 1197.
(10) Moss, R. A.; Ganguli, S.; Okumura, Y.; Fujita, T. *J. Am. Chem. Soc.* **1990**, *112*, 6391.
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(12) Moss, R. A.; Fujita, T. *Tetrahedron Lett.* **1990**, *31*, 7559.
(13) Kornberg, R. D.; McConnell, H. M. *Biochemistry* **1971**, *10*, 1111.
(14) Rothman, J. E.; Dawidowicz, E. A. *Biochemistry* **1975**, *14*, 2809.

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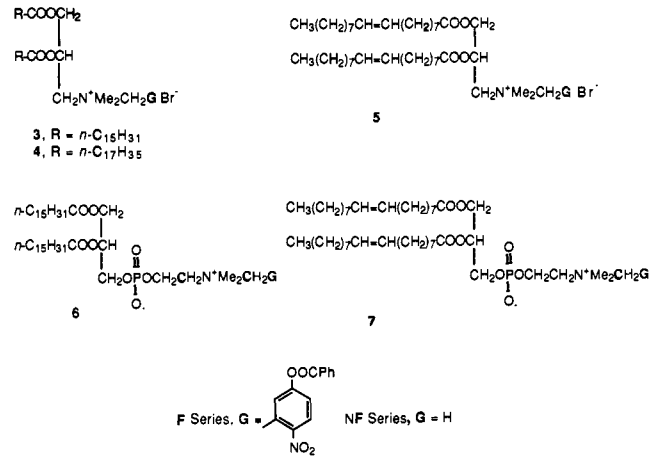
Chart I



Among the elements of molecular structure thus far correlated with lipid flip-flop are chain length[9] and heterogeneity,[15] *gem* vs *vic* chain attachment,[10] ether vs acyloxy linking groups,[10] chain branching,[12] and unsaturation.[11] Longer chains and vicinal attachment (as in **2**) with acyloxy linking groups favor slow flip-flop and the preservation of liposomal asymmetry, whereas geminal attachment, ether linkages, shorter or significantly dissimilar chains, and chain branching or unsaturation are associated with more rapid flip-flop and the decay of asymmetry.

Now we describe the first applications of our methodology to the *zwitterionic* lipids of greater biological relevance. Not only can the new results be directly compared with earlier assessments of lipid dynamics in phosphatidylcholine (PC) liposomes, but we are now able to analyze the comparative effects of head group charge and chain unsaturation in both ammonium ion and PC liposomes. We find dramatic differences between structurally related, saturated or unsaturated, cationic or zwitterionic liposomal aggregates, which appear to parallel the physiological requirements of biological membranes.

Results

Synthesis. The functionalized (F) and nonfunctionalized (NF) lipids central to the present study are **3**–**7**, shown in Chart I. The saturated palmitoyl (**3**-F, **3**-NF) and stearoyl (**4**-F, **4**-NF) and the unsaturated oleoyl (**5**-F, **5**-NF) pseudoglyceryl ammonium ion lipids were available from previous studies.[10,11] The functionalized dipalmitoylphosphatidylcholine (DPPC) lipid, **6**-F, was obtained by quaternization of the corresponding, commercially available, *N*,*N*-dimethylphosphatidylethanolamine, with 4-nitro-3-(bromomethyl)phenyl benzoate (GCH_2Br).[8] DPPC itself (**6**-NF) was, of course, commercially available.

Preparation of the functionalized, unsaturated dioleoyl PC, **7**-F, began with its commercially available, nonfunctionalized analogue, **7**-NF (DOPC) (see Scheme I). Phospholipase D catalyzed exchange[16] with excess *N*,*N*-dimethylethanolamine gave 67% of the (protonated) dimethylaminoethanol–PC, (**8**). After deprotonation, **8** was quaternized with GCH_2Br to afford the functionalized, dioleoyl-PC **7**-F, in 41% overall yield from **7**-NF after purification by preparative TLC. The structures of **6**-F and **7**-F were secured by NMR spectroscopy and elemental analysis.

Phospholipase D is active toward PC's of diverse structure.[1] Coupled with the commercial availability of a wide variety of naturally occurring PC's, the two-step sequence of Scheme I should provide access to many functionalized PC's, suitable for the generation of surface-differentiated liposomes and subsequent studies of flip-flop dynamics.

Liposomes. Liposomes of **6**-F/**6**-NF and **7**-F/**7**-NF were generated by sonication of $CHCl_3$-cast films of 1:7 F/NF surfactant blends in pH 6.0, 10 mM BisTris buffer (μ = 0.01, KCl). Sonications with an immersion probe instrument (55–60 W, 10 min, N_2 atmosphere) were conducted above the gel to liquid crystal transition temperatures (T_c) of the liposomes (see below), i.e., at 55 °C for **6** and 25 °C for **7**. The liposome preparations (cooled to 25 °C in the case of **6**) were filtered through 0.8-μm filters. The final lipid concentrations, employed in all subsequent experiments, were [F] = 5×10^{-5} M and [NF] = 3.5×10^{-4} M.

Dynamic light scattering measurements (Ar laser, 488 nm, 90° scattering angle) gave hydrodynamic diameters of 420 and 350 Å for the DPPC (**6**) and DOPC (**7**) liposomes, respectively (cf. Table I). These values are in good agreement with expectations for small unilamellar vesicles.[17] The DPPC and DOPC liposome preparations remained optically clear for several days upon storage at ambient temperatures. However, *distearoyl*phosphatidylcholine F/NF liposome preparations, analogous to **6**, rapidly became turbid and could not be studied.

The gel to liquid crystal phase transition temperature (T_c) of the 1:7 F/NF DPPC liposome (**6**) was determined as 40 °C from the discontinuity in a plot of the fluorescence polarization of covesicallized 1,6-diphenyl-1,3,5-hexatriene as a function of temperature.[8,18] Details of our experimental method have been described.[8] The T_c we observed for the 1:7 **6**-F/**6**-NF liposomes (40 °C) is similar to values reported for pure **6**-NF (DPPC), which range from 40.6 to 43 °C.[19a] This suggests that the introduction

Scheme I

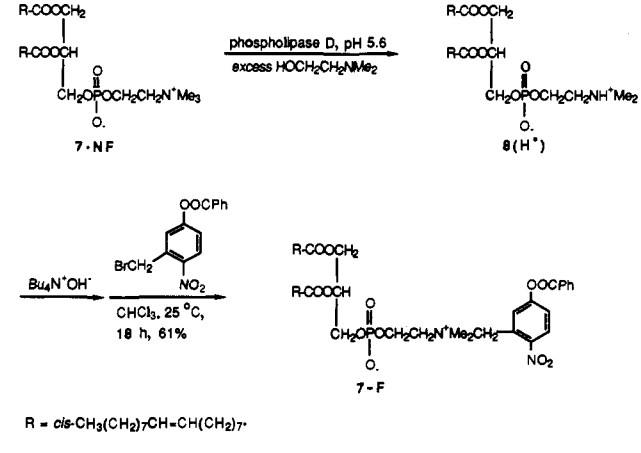


Table I. Dynamics in Liposomal Systems

lipo-some[a]	d,[b] nm	T_c,[c] °C	k_f,[d] s^{-1}	k_s,[e] s^{-1}	phasing[f]	$t_{1/2}$ flip[g]
3[h]	41	44	0.53[i]	0.000 062[i]	70:30	5 min/55 °C; 1 min, 65 °C
4[j]	36	59	0.14[i]	0.000 04[i]	80:20	4 min/55 °C
5[j]	31	<10	0.21[i]	0.002 3[i]	60:40	<1 min/25 °C
6[k]	42	40	0.0044[i]	0.000 007[i]	55:45	20 min/55 °C; 6 min, 65 °C
7[k]	35	<0	0.005[i]	0.000 07[i]	60:40	>20 min/55 °C; 20 min, 65 °C

[a] See text for structures and conditions. The F/NF compositions were 1:7 for **3**, **6**, and **7**, and 1:10 for **4** and **5**. [b] Hydrodynamic diameters from laser light scattering at pH 4 (**3**–**5**), 0.01 M KCl, or pH 6 (**7**, **8**) in BisTris buffer and KCl (both 0.01 M). [BisTris is bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Sigma).] Note: spot checks on several liposome systems showed that their light scattering properties were similar before and after chemical differentiation. [c] Temperature of gel to liquid crystal transition, pH conditions as in note *b*. [d] Rate constants for cleavage of exovesicular *p*-nitrophenyl benzoate moiety at 25 °C; pH as noted. [e] Rate constant for cleavage of endovesicular *p*-nitrophenyl benzoate moiety at 25 °C; pH as noted. [f] Ratio of fast to slow kinetic phases from the corresponding absorbance changes at 400 nm. [g] Approximate half-time for the decay of surface differentiation; see text. [h] Reference 10. [i] At pH 8, 1×10^{-4} M glutathione. [j] Reference 11. [k] This work. [l] Cleavage by OH^- at pH 12.

(15) Fujita, T.; Moss, R. A. *Chem. Lett.* **1991**, 795.
(16) Eibl, H.; Kovatchev, S. *Methods Enzymol.* **1981**, *72*, 632.
(17) See ref 2, p 90.
(18) Andrich, M. P.; Vanderkooi, J. M. *Biochemistry* **1976**, *15*, 1257. Moss, R. A.; Swarup, S. *J. Org. Chem.* **1988**, *53*, 5860.
(19) (a) Marsh, D. *CRC Handbook of Lipid Bilayers*; CRC Press: Boca Raton, FL, 1990; pp 139–140; (b) p 144.

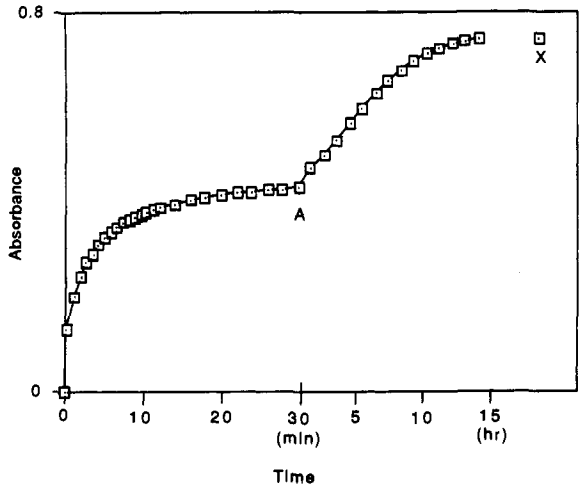


Figure 1. Absorbance at 400 nm vs time for the cleavage of benzoate and the appearance of *p*-nitrophenylate from 1:7 **7**-F/**7**-NF liposomes, prepared at pH 6, at an external pH of 12. Note the discontinuous time axis at point A. Point × marks the infinity titer. See Table I.

of ~13% of functional **6**-F lipid into the predominantly **6**-NF liposome does not greatly perturb the chain packing in the liposomes.

The T_c of the 1:7 **7**-F/**7**-NF DOPC liposomes was not measured but taken as <0 °C because the T_c of pure **7**-NF is variously reported as −14 to −22 °C.[19b] T_c's and hydrodynamic diameters for 1:7 **3**-F/**3**-NF, 1:10 **4**-F/**4**-NF, and 1:10 **5**-F/**5**-NF liposomes were measured previously, in similar fashions,[10,11] and are collected in Table I.

Kinetic Studies. Surface differentiation of 1:7 liposomal **6**-F/**6**-NF and **7**-F/**7**-NF at 25 °C was achieved by raising the external pH of liposomes prepared at pH 6 (in 0.01 M BisTris buffer, μ = 0.01, KCl) to pH 12, using 1 N NaOH. Moderately rapid esterolysis of the *exovesicular p*-nitrophenyl benzoate head groups ensued and was monitored spectrophotometrically at 400 nm. We observed $k_f \sim 4\text{–}5 \times 10^{-3}$ s^{-1} ($\tau_{1/2} \sim$ 2.3–2.8 min), accounting for 55–60% of the total functional head groups present in the liposomes. If the hydrolysis was continued, there followed a very much slower *endovesicular* esterolysis, $k_s = 7 \times 10^{-6}$ s^{-1} for **6** and $k_s = 7 \times 10^{-5}$ s^{-1} for **7**, with $\tau_{1/2} \sim$ 27 or 2.7 h, respectively. These data are collected in Table I.

The observed ~60/40 exovesicular/endovesicular functional head group distributions observed in these kinetic runs are similar to the 65:35 outer/inner lipid distribution estimated in ^{13}C NMR studies of dimyristoyl PC liposomes.[20] Outer/inner lipid ratios in small unilamellar liposomes are generally believed to be in the 60/40 to 70/30 range.[21]

At the conclusion of each kinetic run, infinity titers were secured by lowering the pH to 10 (HCl) and warming to 65 °C to cleave any unsaponified *p*-nitrophenyl benzoate. The total *p*-nitrophenylate absorbance thus obtained was in good agreement with stoichiometric expectations. With **6**, TLC (silica gel, eluent 90:10:1 hexane/ether/acetic acid, developed with iodine) demonstrated the absence of palmitic acid (R_f ~0.3) after the kinetic runs, so that esterolysis of the lipid chains had not occured.

A graphical representation of the head group esterolysis of **7**-F/**7**-NF appears in Figure 1, where there is a very clear demarcation (point A) between k_f and k_s after ~20–30 min. Lowering the pH to 6 (HCl) in this time region afforded the surface-differentiated liposomes required for the flip-flop experiments (see below). The protocol employed to differentiate the **6**-F/**6**-NF liposomes was identical.

In previous studies of *cationic* ammonium ion liposomes (**1**, **3**–**5**), exovesicular cleavage of the *p*-nitrophenyl benzoate was effected by thiolysis with 10^{-4} M glutathione at pH 8. The anionic

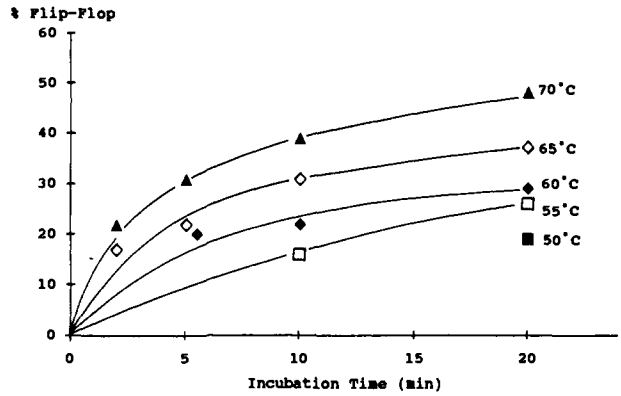


Figure 2. Extent of flip-flop vs time of incubation for surface-differentiated 1:7 liposomes of **6**-F/**6**-NF at various incubation temperatures. See text for the definition of percent flip-flop.

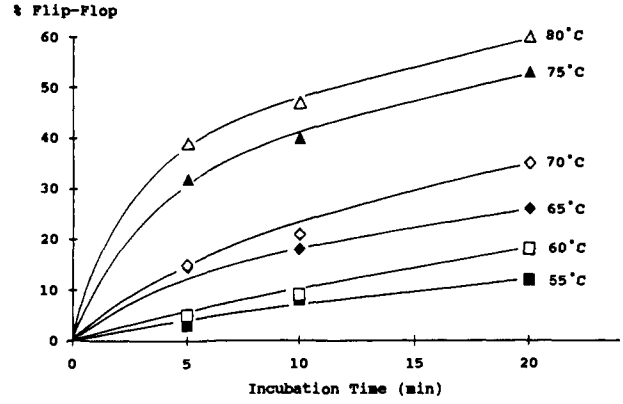


Figure 3. Extent of flip-flop vs time of incubation for surface-differentiated 1:7 liposomes of **7**-F/**7**-NF at various incubation temperatures.

RS^--mediated esterolysis was very rapid ($k > 0.05$ s^{-1}, see Table I) and clearly assisted by the binding of the anionic nucleophiles to the cationic liposomes.[22] With *zwitterionic* liposomes **6** and **7**, electrostatic binding was reduced; glutathione did not accelerate the exovesicular cleavage. However, the requisite surface differentiation could be obtained simply by the action of hydroxide ion, albeit at a higher pH and with slower exovesicular esterolysis.[23]

Flip-Flop Studies. The methodology used in these studies has been previously described.[8–11] Briefly, 1:7 liposomes of **6**-F/**6**-NF or **7**-F/**7**-NF, prepared at pH 6 (HCl) in 0.01 M BisTris buffer, μ = 0.01 (KCl), were surface differentiated by raising the external pH to 12 with NaOH.[24] After completion of the fast exovesicular esterolysis (~30 min, see Figure 1), the pH was lowered to 6 with 1 N HCl, quenching further esterolysis. The acidified, surface-differentiated vesicles were next incubated at a selected temperature for a given time interval, cooled to 25 °C, and readjusted to pH 12 with 1 N NaOH. The extent of lipid flip-flop that had occurred during the incubation was then revealed by a new, fast (k_f) appearance of *p*-nitrophenylate absorbance at 400 nm, representing the cleavage of those functionalized lipid head groups that had flipped from endo- to exovesicular loci. A subsequent k_s reaction was also observed, reporting the slower cleavage of intact, residual endovesicular functional groups.

Samples of surface-differentiated liposomes of **6** or **7** were incubated at five or six different temperatures from 50 to 80 °C. At each temperature, incubation times ranged from 2 to 20 min.

(20) de Kruijff, B.; Wirtz, K. W. A. *Biochim. Biophys. Acta* **1977**, *468*, 318.

(21) Reference 1, p 81; ref 2, p 88.

(22) Kawamuro, M. K.; Chaimovich, H.; Abuin, E. B.; Lissi, E. A.; Cuccovia, I. M. *J. Phys. Chem.* **1991**, *95*, 1458. Cuccovia, I. M.; Quina, F. H.; Chaimovich, H. *Tetrahedron* **1982**, *38*, 917. Moss, R. A.; Hendrickson, T. F.; Bizzigotti, G. O. *J. Am. Chem. Soc.* **1986**, *108*, 5520.

(23) See Discussion for a comparison of H^+/OH^- permeation rates and esterolysis rates for liposomal **6** and **7**.

(24) With cationic ammonium ion liposomes, such as **3**–**5**, the corresponding pH change is 4 to 8. Reference 8 gives a detailed description of the flip-flop protocol.

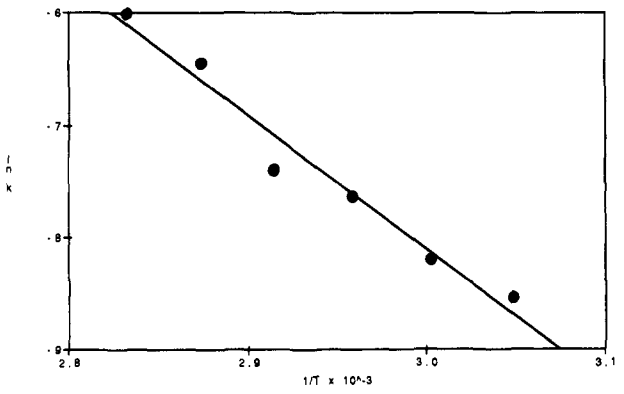


Figure 4. Arrhenius plot for flip-flop reequilibration of surface-differentiated 7-F/7-NF liposomes; the rate constants for reequilibration were obtained from the data in Figure 3 (see text).

Figures 2 and 3 summarize the results of these experiments, and key data appear in Table I. In the figures, percent flip-flop is defined according to

$$\% \text{ flip-flop} = 100D/(F - A) \quad (1)$$

where D is the absorbance change due to the fast, postincubation esterolysis, F is the total absorbance change (total hydrolysis), and A is the absorbance change of the initial fast hydrolysis before incubation. With the DPPC system (Figure 2), **6**-F/**6**-NF, we observed that flip-flop was very slow below 50 °C. Even at 70 °C, reequilibration of the surface-differentiated liposomes to the predifferentiation exo/endo functionalized lipid distribution (55:45) was not quite complete after 20 min. If 55% flip-flop is taken as representative of complete reequilibration of the differentiated liposomes, then Figure 2 affords estimates of the half-times ($t_{1/2}$) for reequilibration of ~20 min at 55 °C or ~6 min at 65 °C.

The behavior of the dioleoyl PC (DOPC) liposomes, 7-F/7-NF, is illustrated in Figure 3. Here, complete reequilibration to an exo/endo distribution of ~60:40 appears to require ~20 min at an incubation temperature of 80 °C. At 65 °C, we estimate $t_{1/2}$ ~ 20 min, indicating that the unsaturated DOPC liposomes are at least as stable to thermally driven flip-flop as the saturated DPPC liposomes (cf. Table I).

The T_c of the DOPC liposomes is <0 °C, so that these aggregates remain in their fluid, liquid crystalline phase down through ambient temperatures. We could therefore construct a rough Arrhenius correlation for the flip-flop in these liposomes so as to extrapolate the rate of this process to lower temperatures. The individual curves of Figure 3 were assumed to reflect first-order time-dependent approaches to reequilibration and were curve-fitted to afford appropriate rate constants. The logarithms of these rate constants were then plotted against $1/T$ to afford the surprisingly good (r = 0.989) correlation of Figure 4, from which $E_a \sim 24$ kcal/mol, $A \sim 1.6 \times 10^{12}$ s^{-1} for flip-flop mediated reequilibration. This affords $t_{1/2} \sim 24$ h at 30 °C.

A related treatment, applied to the data for the **6**-F/**6**-NF liposomes, yields an extrapolated $t_{1/2} \sim 3$ h for reequilibration of the surface-differentiated liquid crystalline aggregates at *40 °C*. We do not extrapolate to lower temperatures because the gel–liquid crystal transition occurs at 40 °C; below this temperature, in the gel phase, the rate of lipid flip-flop would be greatly and discontinuously lowered.[10] The extrapolated flip-flop half-times for the DOPC and DPPC liposomes will be put into context below.

Discussion

Flip-Flop Behavior. The flip-flop dynamics observed with the zwitterionic **6**-F and 7-F PC lipids can be compared with our previous results for cationic ammonium ion lipids **3**–**5** and also with literature data for PC lipids. Let us first consider the "internal comparison" (cf. Table I). The $t_{1/2}$ values for the saturated, pseudoglyceryl ammonium ion liposomes, **3**,[10] compared to those of the DPPC liposomes, **6**, show that the surface-differentiated cationic bilayers reequilibrate by flip-flop 4–6 times more rapidly than the comparable zwitterionic bilayers at 55 or 65 °C. Significantly, the T_c's of liposomal **3** and **6** are similar, suggesting comparable long-chain packing in the liposomes and implicating the change in head groups as the source of the increased resistance to flip-flop of **6**.

This idea is strongly supported by the dramatic contrast in behavior between the *unsaturated* liposomal aggregates **5** and **7**. The unsaturated, cationic, dioleoylammonium ion lipid **5**-F is much less resistant to flip-flop than its saturated analogue **4**-F (or **3**-F) in their respective liposomes; $t_{1/2}$ is <1 min at 25 °C for surface-differentiated liposomes of **5**, whereas liposomal **4** is stable to flip-flop at this temperature. By itself, this difference might be ascribed to a fluidity effect because liposomes of **5** are in the liquid crystalline state at 25 °C but those of **4** (or **3**) are in the gel state. However, a comparison of **5**-F to the unsaturated, zwitterionic lipid 7-F, where *both* liposomal **5** and **7** are liquid crystalline at or above 25 °C, reveals a considerably more profound distinction. PC lipid 7-F not only resists flip-flop at 25 °C but does not significantly display this dynamic property until the temperature exceeds ~50 °C. Indeed, the resistance to flip-flop of unsaturated **7** is comparable to that of saturated **6**.[25]

The resistance of **6** and **7** to flip-flop must be associated with the zwitterionic head groups. The introduction of unsaturation into the lipid chains, which perturbs chain packing[1,2] and completely destroys resistance to flip-flop in the cationic ammonium ion lipids,[11] does not impart a proclivity for flip-flop in the PC liposomes of zwitterionic **7**. An effect of unsaturation on chain packing is, indeed, observed in the PC liposomes; the T_c of liposomal **7** is much lower than that of **6**. However, with the PC liposomes, this is not translated into enhanced lipid flip-flop, as it is with the cationic liposomes. The zwitterionic liposomes are able to maintain surface differentiation at ambient temperatures even in the liquid crystalline state induced by chain unsaturation. Indeed, the combination of surface differentiation *and* membrane fluidity obtained with the zwitterionic PC bilayers is likely to be essential to their biological function.

In contrast, the cationic ammonium lipids maintain long-term surface differentiation only in the gel state, which, at or above 25 °C, requires saturated chains; unsaturation lowers their T_c below 10 °C, induces fluidity, and leads to the rapid loss of surface differentiation via flip-flop.

We suggest that efficient chain packing and the gel state are so essential to resist lipid flip-flop in the cationic liposomes because their head groups strongly repel each other. Once chain packing is perturbed by a cis double bond (as in **5**), the T_c is suppressed, the fluid liquid crystalline state is obtained, and flip-flop rapidly occurs. With the zwitterionic PC liposomes, however, the +/+ head group repulsions are replaced by ±/± electrostatic dipole interactions[26] that may oppose flip-flop even when the bilayer (e.g., **7**) is in the fluid state.

Electrostatic interactions between phospholipid head groups are complex; they might even be net repulsive.[27] However, they must surely be less repulsive than the +/+ interactions of the cationic head groups of liposomal **3** or **5**. In fact, a full comparative analysis of these interactions in cationic or zwitterionic liposomes would have to consider relative head group hydration, as well as the ease of defect formation[28] in each type of liposome. Thus, the primary barrier for the transfer of a lipid molecule from one face of the membrane to the other arises in the passage of

(25) It should be noted that **7** has chains that are two carbons longer than those of **6**. If we had been able to examine F/NF liposomes of distearoyl-PC, the exact saturated analogue of **7**, we might have observed greater resistance to flip-flop in the saturated PC than in **7**. Additionally, we have compared racemic and optically active lipids; e.g., *rac*-**6**-F and *L*-7-F. However, the properties of their derived liposomes should not be significantly biased by this factor. Thus, there were no meaningful differences between racemic or enantiomerically pure **6**-NF in solution surface tension, force-area isotherms of monolayers, differential scanning calorimetry of gels, or NMR spectroscopy of the vesicles: Arnett, E. M.; Gold, J. M. *J. Am. Chem. Soc.* **1982**, *104*, 636.

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the polar head group through the hydrophobic interior of the bilayer.[2] This barrier is expected to require >15 kcal/mol of activation[2] (we find ~24 kcal/mol with **7**; see above) and will include components for head group dehydration and space-creating defect formation.

Comparisons of the flip-flop dynamics of liposomes **3**–**7** are easy to make because the liposomes were similarly prepared and carry identical chemical labels and their dynamics can be assessed in the same way. The results afford clear and sensible correlations between lipid molecular structure and flip-flop dynamics. On the other hand, "external" comparisons of our results for PC liposomes **6** and **7** to literature results are complicated by the variety of labels, monitoring methods, and liposomal constituents employed by the various investigators. Nevertheless, several notable observations can be summarized.

(a) McConnell reported $t_{1/2}$ ~2.6–7 h at 30 °C for flip-flop equilibration of a DPPC head group-labeled spin probe in sonicated egg lecithin liposomes.[13]

(b) Galla employed a chain-labeled pyrene fluorescent probe (1-palmitoyl-2-ω-pyrenedecanoyl-PC) in DPPC liposomes.[29] From Galla's data, we calculate $t_{1/2}$ values of 5.6 h at 41 °C, 3.4 h at 50 °C, and 2.5 h at 60 °C.

(c) de Kruijff used a phospholipid exchange protein to create ^{13}C-labeled, differentiated DOPC and dimyristoyl PC (DMPC) liposomes.[20] Flip-flop equilibration of the DOPC liposomes was 10–17% complete after 32–40 h at 30 °C; with the saturated DMPC liposomes, $t_{1/2}$ values were 25 h at 30 °C (gel phase)[20] and 1 h at 50 °C (liquid crystalline phase).[30]

(d) Thompson employed the phospholipid exchange protein method to create ^3H-labeled, differentiated, large (1000 Å) DMPC liquid crystalline liposomes, and observed $t_{1/2} \leq 9$ h at 30 °C and $t_{1/2} \leq 0.7$ h at 50 °C.[31]

(e) Dawidowicz used the exchange protein methodology to differentiate ^3H-labeled DOPC liposomes and reported that flip-flop equilibration had $t_{1/2} > 11$ days at 37 °C.[14]

Our estimate of $t_{1/2} \sim 24$ h at 30 °C for flip-flop equilibration in differentiated, liquid crystalline DOPC liposomes (**7**) is bracketed by McConnell's finding of $t_{1/2} \sim 2.6$–7 h at 30 °C for flip-flop in egg lecithin liposomes (observation a)[13] and de Kruijff's finding of 10–17% equilibration in DOPC liposomes after 32–40 h at 30 °C (observation c).[20]

Our estimate of $t_{1/2} \sim 3$ h at 40 °C for flip-flop equilibration in differentiated, liquid crystalline DPPC liposomes is about half the value calculated from Galla's data: $t_{1/2} = 5.6$ h at 41 °C (observation b).[29] At 50 °C we estimate $t_{1/2} \sim 1$ h (i.e., from 15% of flip-flop after 20 min; see Figure 2), similar to de Kruijff's observations with DMPC liposomes,[30] but faster than Galla's data of $t_{1/2} = 3.4$ h at 50 °C for DPPC[29] (observations c and b, respectively).

Qualitatively, we conclude that our procedures generate reasonable $t_{1/2}$ values for flip-flop equilibrations in DOPC and DPPC liposomes, especially considering the variations in liposomal components and the experimental methodologies of the available data. Of course, the choice of lipid head group can also influence flip-flop dynamics,[31] and our systems employ unique, *p*-nitrophenyl benzoate labeled lipids. Nevertheless, we believe that the present correlations of molecular structure and liposomal dynamics, carried out within a closely related series of lipids that bear a constant head group, should be valid.

Liposome Permeability. The exovesicular/endovesicular chemical differentiation of liposomal **6** or **7** depends upon the imposition of a 12/6 pH gradient, so that exovesicular *p*-nitrophenyl benzoate (G) groups can be saponified selectively relative to the endovesicular G groups (cf. Figure 1). Therefore, a significant pH gradient must be maintained for some time. Within limits, we have found it possible to directly follow the decay of this gradient at the inner surface of bilayer liposomes.[32]

Specifically, after the complete saponification (pH 12) and subsequent reacidification (pH 6) of the functional head groups in liposomes **3**–**7**, the F lipids carry *p*-nitrophenol (G′) moieties. If we know the pK_a of G′, it can serve as a pH indicator because the *p*-nitrophenol $\rightleftarrows$ *p*-nitrophenylate interconversion is readily monitored by UV spectroscopy; only the *p*-nitrophenylate form absorbs strongly at 400 nm.

The pK_a of a head group-bound, liposomal *p*-nitrophenol group depends on both the charge of the lipid head group and the aggregate charge on the liposomal surface. With the cationic lipids and liposomes, the *p*-nitrophenol acidity is enhanced, and the G′ pK_a is ~4–5.[32] With zwitterionic lipids and liposomes, the pK_a should be closer to 7.2, that of *p*-nitrophenol itself. Indeed, spectrophotometric titration of **6**-F/**6**-NF DPPC liposomes (where F represents the *p*-nitrophenol functionalized head group) gives an apparent pK_a of 7.6, whereas that observed for the corresponding cationic **3**-F/**3**-NF liposomes is 4.8.

We could thus monitor the apparent pH changes at the endo surfaces of liposomal **6**-F or **7**-F when the G′ functionalized liposomes were first equilibrated at pH 6 and then subjected to an exo/endo pH 12/6 gradient at 25 °C. With 1:7 (G′) **6**-F/**6**-NF, we first observed an "instantaneous" rise of absorbance at 400 nm, accounting for about half of the G′ residues, as the exovesicular *p*-nitrophenols were deprotonated at pH 12. There followed a much slower, time-dependent increase in absorbance ($t_{1/2} \sim 30$ min) as the pH at the endovesicular surface rose, and deprotonation of these *p*-nitrophenol residues occurred. However, after 30 min, the pH at the endovesicular surface of the **6**-F/**6**-NF liposomes had only risen from 6 to ~7.6, the pK_a of the G′ residues in liposomal **6**. This result is consistent with the observed, selective *exovesicular* esterolysis of the *p*-nitrophenyl benzoate functionalized **6**-F/**6**-NF liposomes at an external pH of 12, while the internal pH remains considerably lower.

However, in the foregoing experiments, the DPPC liposomes are in the gel state, where H^+/OH^- permeation should be slowed. In contrast, the unsaturated **7**-F/**7**-NF DOPC liposomes are in the more fluid and permeable[33] liquid crystalline state at 25 °C. When these liposomes, bearing *p*-nitrophenol functional head groups, are subjected to the 12/6 pH gradient, their time-dependent, exovesicular deprotonation is characterized by $t_{1/2} \sim 10$ s. How can this be consistent with the successful surface differentiation of the **7**-F/**7**-NF liposomes (Figure 1), where the observed rate constants for esterolyses are k_f (exo) = 5×10^{-3} s^{-1} and k_s (endo) = 7×10^{-5} s^{-1} ($t_{1/2} \sim 2.7$ h)?

We suggest that although the interior pH of liposomal **7** may very rapidly rise from 6 to ~8, it does not continue to increase quickly. Although OH^-/H^+ permeation across the fluid bilayers of **7** may initially be rapid, the resulting decay of the pH gradient is accompanied by the creation of a *counterion gradient* that features (excess) endovesicular Cl^- anions and exovesicular Na^+/K^+ cations. Continued import of OH^- or export of H^+ must increasingly work against the electrical potential caused by the ionic imbalance.[33-37] In contrast to the more permeable H^+ or OH^-, the permeability of chloride, sodium, or potassium ions is low, and the counterion gradient is, normally, only slowly dissipated.

In support of these ideas, we find that when the pH 12/6 gradient esterolysis of 4×10^{-4} M **7**-F/**7**-NF liposomes (i.e., the experiment of Figure 1) is attempted in the presence of 2.5×10^{-6} M valinomycin (a K^+ selective cation carrier[35,36,38]), the esterolytic discrimination between exo- and endovesicular *p*-nitrophenyl benzoate residues is *completely lost* (cf. Figure 5). Now, the entire esterolysis occurs as a quantitative, monoexponential process

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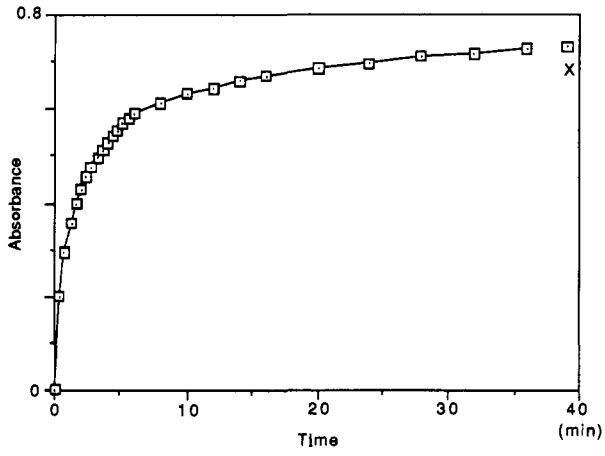

Figure 5. Esterolysis of 7-F/7-NF liposomes, as described in the caption to Figure 1, except that 2.5×10^{-6} M valinomycin was present during the reaction. Point × marks the infinity titer.

with $k \sim 3.3 \times 10^{-3}$ s^{-1} ($r = 0.998$), a rate constant similar to k_f (5×10^{-3} s^{-1}) observed for the *exovesicular* esterolysis of 7-F/7-NF liposomes in the *absence* of valinomycin (Figure 1; Table I).

In the presence of the cation carrier, the initial exo/endo pH 12/6 gradient decays quickly and completely because the counterion imbalance that normally accompanies proton efflux is liquidated by facile K^+ influx. The exo- and endovesicular pH is rapidly equilibrated at 12, and *p*-nitrophenyl benzoate residues on both sides of the membrane saponify at comparable rates, with $k \sim 3\text{-}5 \times 10^{-3}$ s^{-1}.

We conclude that the pH gradient esterolytic surface differentiation of liquid crystalline liposomes, such as **7**, is made possible by slow, rate-limiting, coupled proton/counterion permeation[35-37] that prevents rapid decay of the initial pH 12/6 transmembrane pH gradient much beyond pH 12/8, so that the exovesicular esterolysis can be completed before the endovesicular reaction can significantly proceed.[39]

Conclusions. *p*-Nitrophenyl benzoate head group functionalized liposomes can be conveniently surface differentiated, and the dynamics of reequilibration via lipid flip-flop can be monitored. This facilitates a survey of a wide variety of lipids that differ in molecular structure, so that the relation between lipid molecular structure and intraliposomal lipid dynamics can be elucidated. In particular, the influence of chain length and unsaturation and of head group charge type can now be quantitatively assessed.

Experimental Section

Materials. DL-*N,N*-Dimethyl-1,2-dipalmitoylphosphatidylethanolamine, L-dipalmitoylphosphatidylcholine (**6**-NF), and phospholipase D were obtained from Sigma, and L-dioleoylphosphatidylcholine (**7**-NF) was purchased from Avanti Polar Lipids. Lipids **3**,[10] **4**,[11] and **5**[11] were available from previous studies. 4-Nitro-3-(bromomethyl)phenyl benzoate was prepared as described in ref 8. 1,6-Diphenyl-1,3,5-hexatriene was obtained from Fluka.

Instrumentation. NMR spectra were obtained with a Varian VXR-200 instrument (200 MHz). Sonication was carried out with a Braunsonic Model 2000 probe sonicator. Dynamic light scattering experiments employed a Nicomp Model TC-100 computing autocorrelator, an argon laser light source (488 nm), and a Hazeltine microcomputer that used the cumulant program. Data were collected at 25 °C and a 90° scattering angle; see ref 8 for further experimental details. Kinetic studies were carried out with a Gilford Model 250 spectrophotometer, equipped with a thermostated circulating water bath. For pK_a titrations, absorbance was monitored with a Hewlett-Packard 8451A diode array spectrophotometer; pH was determined with a Radiometer PHM 25 instrument. For T_c measurements, fluorescence polarization was measured with a Perkin-Elmer MPF-3L fluorescence spectrophotometer and Polacoat 4B polarizers for both excitation and emission beams. Experimental details are described in refs 8 and 17. Microanalyses were performed by Robertson Laboratory, Madison, NJ.

***N*-[4-Nitro-1-(benzoyloxy)-3-benzyl]-1,2-dipalmitoylphosphatidylcholine (6-F).** To a solution of 250 mg (347 μmol) of *N,N*-dimethyl-1,2-dipalmitoyl-*rac*-3-phosphatidylethanolamine in 10 mL of chloroform was added 241 mg (717 μmol) of 4-nitro-3-(bromomethyl)phenyl benzoate. The reaction mixture was stirred magnetically for 4 days at room temperature; then, 250 mg of potassium carbonate was added, and the reaction mixture was further stirred overnight. After dilution with chloroform, the reaction mixture was washed with 1 N aqueous HCl and water. The organic layer was separated and dried over anhydrous sodium sulfate, and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in anhydrous diethyl ether, and a white precipitate was formed after standing for ~10 h. The precipitate was washed twice with diethyl ether and then recrystallized from acetone, affording 94 mg (96 μmol, 28%) of **6**-F: mp 120–121 °C; R_f 0.71 (single spot on silica gel TLC, developed by $CHCl_3$/MeOH/H_2O, 65:25:4, detected by I_2 or fluorescence quenching; ^{1}H NMR ($CDCl_3$) δ 0.89 (crude t, 6 H, 2 terminal CH_3's), 1.3 (broad s, 48 H, 2 $(CH_2)_{12}$'s), 1.52 (broad m, 4 H, 2 CH_2's β to carbonyls), 2.23 (t, 4 H, 2 CH_2's α to carbonyls), 3.30 (s, 6 H, $N^+(CH_3)_2$), ca. 4.0 and 4.4 (overlapping m's, 8 H, choline CH_2's and glyceryl CH_2's), 5.18 (m, 1 H, glyceryl CH), 5.45 (s, 2 H, benzyl CH_2), ca. 7.6 and 8.2 (m, 8 H, aromatic). Anal. Calcd for $C_{53}H_{87}N_2O_{12}P{\cdot}0.5H_2O$: C, 64.67; H, 9.03; N, 2.85. Found: C, 64.58; H, 8.70; N, 2.83.

***N,N*-Dimethyl-1,2-dioleoylphosphatidylethanolamine [8(H$^+$), Scheme I].** A solution of 160 mg (204 μmol) of 1,2-dioleoyl-*sn*-3-phosphatidylcholine (**7**-NF) in 5.0 mL of diethyl ester was mixed with 10 mL of 5% (by weight) *N,N*-dimethylethanolamine in 200 mM sodium acetate buffer containing 80 mM of calcium chloride. (The pH of the buffer solution was adjusted to 5.6 with concentrated HCl.) After the addition of 5 mg (ca. 19 units) of partially purified phospholipase D, dissolved in 0.8 mL of distilled water, the resulting emulsion was stored in a sealed glass vial and stirred vigorously, using a magnetic stirrer, at ca. 30 °C for 44 h.

After the addition of 2.0 mL of 500 mM ethylenediaminetetraacetic acid, the pH was adjusted to 8.5 with sodium hydroxide, and the reaction mixture was extracted with a mixture of 10 mL of chloroform and 12 mL of methanol. The aqueous layer was then twice extracted with 5 mL of chloroform, and all of the extracts were combined and washed twice with 20-mL portions of 1 N aqueous HCl. After each washing, the resulting emulsion was broken by the addition of 5 mL of methanol. The combined organic layer was dried over anhydrous magnesium sulfate, and the solvent was removed by evaporation under reduced pressure.

The residue was purified by chromatography over a 6 × 0.8 cm column of silica gel and eluted with chloroform/methanol/concentrated aqueous ammonia, 65:25:4 by volume. The yield was 106 mg (137 μmol), 67% based on dioleoylphosphatidylcholine. The product was one spot on TLC (R_f 0.61 on silica gel, developed with chloroform/methanol/concentrated aqueous ammonia, 65:25:4 by volume, and detected by iodine adsorption): ^{1}H NMR ($CDCl_3$) δ 0.84 (crude t, 6 H, 2 terminal CH_3's), 1.3 (broad s, 40 H, 2 $(CH_2)_4$'s and 2 $(CH_2)_6$'s), 1.55 (broad m, 4 H, 2 CH_2's β to carbonyls), 1.97 (m, 8 H, 4 allylic CH_2's), 2.3 (2 overlapping t's, 4 H, 2 CH_2's α to carbonyls), 2.73 (s, 6 H, $N(CH_3)_2$), 3.12 (broad m, 2 H, CH_2N), 3.94 (t, 2 H, *sn*-3 CH_2), ca. 4.1 and 4.4 (overlapping m's, 4 H, $POCH_2$ and glyceryl *sn*-1 CH_2), 5.20 (m, 1 H, glyceryl CH), 5.30 (m, 4 H, vinyl).

***N*-[4-Nitro-1-(benzoyloxy)-3-benzyl]-1,2-dioleoylphosphatidylcholine (7-F).** To a solution of 135 mg (173 μmol) of protonated **8** in 5 mL of chloroform was added 170 μL (170 μmol) of 1.0 N tetrabutylammonium hydroxide/methanol (Aldrich). After evaporation of the solvent under reduced pressure, the residue was dissolved in 1.0 mL of chloroform, and 80 mg (238 μmol) of 4-nitro-3-(bromomethyl)phenyl benzoate was added. The reaction mixture was stirred at room temperature for 18 h. The solvent was removed by evaporation under reduced pressure, and the residue was dissolved in 50 mL of hexane and washed twice with 40 mL of water that had been made slightly acidic by a drop of 1 N aqueous HCl. After each washing, the resulting emulsion was broken by the addition of 20 mL of methanol. The hexane solution was then dried over anhydrous magnesium sulfate, and the hexane was removed under reduced pressure.

The residue was purified by preparative thin-layer chromatography on a silica gel plate that had been pretreated with 7 mL of 1 N HCl (2 days, sealed plastic bag). The TLC plate was 20 × 20 cm, with a 2-mm layer of silica gel, and was developed with $CHCl_3$/MeOH/1 mM aqueous HCl, 65:25:4 by volume. The spot that contained **7**-F was collected while the plate was still wet and was leached with the above solvent mixture. Filtration of the silica and removal of the solvent under reduced pressure (near 0 °C), gave 108 mg (105 μmol, 61%) of **7**-F as

(39) Mechanisms of the same type must apply in the cases of the ammonium ion lipids, **3**–**5**, where the pK_a's of the G′ head groups are ~5. When subjected to an exo/endo pH gradient of 8/4, endovesicular deprotonation (to pH ~6), of G′ in (saturated) **4**-F/**4**-NF liposomes occurred with $\tau_{1/2} \sim 21$ min, whereas in (unsaturated) **5**-F/**5**-NF liposomes $\tau_{1/2}$ was only ~14 s.[11]

a greaselike, soft solid that lacked a clear melting point. The product gave one spot on TLC (R_f 0.57, elution with the above solvent, detection by fluorescence quenching and by I_2 adsorption): ^{1}H NMR ($CDCl_3$) δ 0.88 (crude t, 6 H, 2 terminal CH_3's), 1.3 (broad s, 40 H, 2 $(CH_2)_4$'s and 2 $(CH_2)_6$'s), 1.52 (broad m, 4 H, 2 CH_2's β to carbonyls), 1.99 (broad m, 8 H, 4 allylic CH_2's), 2.22 (2 overlapping t's, 4 H, 2 CH_2's α to carbonyls), 3.28 (s, 6 H, $N^+(CH_3)_2$), ca. 4.0 and 4.4 (overlapping m's, 8 H, choline CH_2's and glyceryl CH_2's), 5.19 (m, 1 H, glyceryl CH), 5.33 (broad m, 4 H, vinyl), 5.42 (s, 2 H, benzyl CH_2), ca. 7.6 and 8.2 (m, 8 H, aromatic). Anal. Calcd for $C_{57}H_{91}N_2O_{12}P\cdot H_2O$: C, 65.49; H, 8.97; N, 2.68. Found: C, 65.26; H, 9.11; N, 2.39.

Preparation of Liposomes. Typically, 0.40 mL of a 5 mM functionalized phosphatidylcholine (6-F or 7-F) stock solution in chloroform was mixed with 2.80 mL of a 5 mM nonfunctionalized phosphatidylcholine solution (6-NF or 7-NF) in chloroform, and the chloroform was removed through evaporation under a gentle stream of nitrogen gas. This was followed by further drying under vacuum. To the film of the phosphatidylcholines thus obtained was added 20 mL of 10 mM BisTris buffer (containing 10 mM potassium chloride, with the pH adjusted to 6.0 with hydrochloric acid), and the mixture was sonicated at 55–60 W for 10 min under a nitrogen atmosphere. The temperature was controlled by a water bath at 55 °C for the 6-F/6-NF system and at room temperature for the 7-F/7-NF system. There resulted an almost clear solution that was filtered through a Millex membrane filter (pore size, 0.8 μm) and was then allowed to cool to room temperature if necessary. The liposome solution thus obtained was diluted with an equal volume of the BisTris buffer and employed in further experiments.

Kinetic Studies. Reactions were initiated in a UV cuvette by mixing the appropriate liposome preparation (6-F/6-NF or 7-F/7-NF) at pH 6 in BisTris buffer–KCl (see above) with 1 N aqueous NaOH solution at 25 °C to bring the pH to 12. Saponification of the benzoate began immediately and was followed at 400 nm while the *p*-nitrophenylate product absorption was monitored. The initial rapid exovesicular benzoate hydrolysis was followed by a second, slower, endovesicular hydrolysis (see Figure 1). At the end of a kinetic run, the pH was lowered to 10 by the addition of a drop of HCl solution. The solution was then warmed to 65 °C for 30 min to ensure total benzoate hydrolysis. The absorbance at this point was taken as the infinity titer (point X in Figure 1). The pseudo-first-order rate constants for the slow and fast hydrolyses were calculated from absorbance vs time data by computer analysis. Kinetic data are summarized in Table I.

Permeation Experiments. Liposomes functionalized with the *p*-nitrophenylate moiety were obtained after complete hydrolysis of 6-F/6-NF or 7-F/7-NF as described above. The pH of the liposome sample was then reduced to 6 by the addition of aqueous HCl, and the solution was warmed to 65 °C to equilibrate the inner and outer liposomal pH. The *p*-nitrophenol functionalized sample was then cooled to 25 °C, and the external pH was raised to 12 by the addition of 1 N NaOH. An instantaneous (exovesicular) deprotonation led to a "jump" in the 400-nm absorbance. This was followed by a time-dependent increase in absorption corresponding to endovesicular deprotonation.

Flip-Flop Studies. Typically, 40 mL of the 6-F/6-NF or 7-F/7-NF liposome solution was mixed with ca. 0.5 mL of 1 N aqueous NaOH solution to bring the pH to 12. After 30 min, the hydrolysis was stopped by the addition of ca. 0.5 mL of 1 N aqueous HCl, which brought the pH of the solution back to 6. Then 2 mL of the acidified liposome solution was placed in a UV cuvette and incubated in a water bath at a controlled temperature for a certain period of time. After the solution had been cooled to 25 °C, its pH was brought back to 12 by the addition of ca. 0.25 μL of the 1 N NaOH solution. This caused the immediate dissociation of protons from *p*-nitrophenol groups on the outer surface of the liposomal bilayer membrane. The process was monitored at 400 nm. When there had been any flip-flop of the cleavage-intact functionalized phosphatidylcholine molecules from the inner surface to the outer surface of the liposomes during the incubation, it was detected as a new rapid hydrolysis. After the completion of the new fast hydrolysis, if any, the sample was hydrolyzed at 65 °C, as described above, to obtain the total absorbance.

The extent of flip-flop was determined using eq 1, as described under Results. Pertinent data are summarized in Figures 2 and 3 and in Table I. Further details of our flip-flop protocol are discussed in ref 8.

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Aromatic Nitration with Electrophilic *N*-Nitropyridinium Cations. Transitory Charge-Transfer Complexes as Key Intermediates

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Abstract: Electrophilic aromatic nitration of various arenes (ArH) is shown to be critically dependent on labile charge-transfer complexes derived from *N*-nitropyridinium cations. The electrophiles $XPyNO_2^+$, with X = CN, CO_2CH_3, Cl, H, CH_3, and OCH_3, form a highly graded series of electron acceptors that produce divers [ArH,$XPyNO_2^+$] complexes, with charge-transfer excitation energies ($h\nu_{CT}$) spanning a range of almost 50 kcal mol^{-1}. The latter underlie an equally broad spectrum of aromatic substrate selectivities from the different nitrating agents ($XPyNO_2^+$), but they all yield an isomeric product distribution from toluene that is singularly insensitive to the X substituent. The strong correlation of the nitration rates with the HOMO–LUMO gap in the [ArH,$XPyNO_2^+$] complex is presented (Scheme III) in the context of a stepwise process in which the charge-transfer activation process is cleanly decoupled from the product-determining step—as earlier defined by Olah's requirement of several discrete intermediates. This charge-transfer formulation thus provides a readily visualized as well as a unifying mechanistic basis for the striking comparison of $XPyNO_2^+$ with other nitrating agents, including the coordinatively unsaturated nitronium cation ($NO_2^+BF_4^-$), despite their highly differentiated reactivities.

Introduction

Nitration of various aromatic compounds is commonly carried out with nitric acid, either alone or in conjunction with Brønsted and Lewis acids.[1] Strong acids are particularly effective as catalysts for the steady-state production of the nitronium ion (NO_2^+) as the active electrophile.[2] A wide variety of other nitronium "carriers" YNO_2 have also been employed directly, with their nitrating activity generally paralleling the base (nucleofugal)

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(2) Ingold, C. K. *Structure and Mechanism in Organic Chemistry*, 2nd ed.; Cornell University Press: Ithaca, NY, 1953; Chapter 6. See also: Taylor, R. *Electrophilic Aromatic Substitution*; Wiley: New York, 1990. De La Mare, P. B. D.; Ridd, J. H. *Aromatic Substitution, Nitration and Halogenation*; Butterworths: London, 1959.

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