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Spontaneous Formation of Stable Unilamellar Vesicles†

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ABSTRACT: Stable unilamellar vesicles form spontaneously upon mixing aqueous suspensions of long-chain lecithins (fatty acid chain lengths 14 carbons or longer) with small amounts (20 mol %) of micellar synthetic short-chain lecithins (fatty acid chain lengths 6–8 carbons). These vesicles are potentially ideal for any experiment (i.e., membrane protein reconstitution, drug delivery, etc.) that requires an easily formed, nonleaky unilamellar structure.

Phospholipid bilayers are widely used in biochemistry as model membranes. Vesicles have been used for functional reconstitution of membrane-bound proteins [for example, see Christianson & Carlsen (1983)], as substrates for a variety of lipolytic enzymes (Kensil & Dennis, 1979; DeBose & Roberts, 1983), as permeability barriers in transport studies (Papahadjopoulos & Watkins, 1967; Papahadjopoulos & Kimelberg, 1973; Racker & Stoeckenius, 1974), and for encapsulation of various agents for assays, drug delivery, etc. (Ryman & Tyrrell, 1980). When naturally occurring and synthetic long-chain phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidic acid, etc.) are dispersed in aqueous solution, they form large multilamellar structures. Several methods have been developed to form unilamellar vesicles from these multibilayers: (i) sonication, (ii) reverse evaporation from organic solvent (Szoka & Paphadjopoulos, 1978), (iii) detergent dialysis or dilution (Brunner et al., 1976), and (iv) pressure/mechanical filtration. All these methods require either special equipment [sonicator, French press (Hamilton et al., 1980)] or extrusion through Nucleopore filters (Farmer & Gaber, 1984) or depend on the addition and partial removal of nonphospholipid material (organic solvent, detergents) and hence are multistep processes. In many cases the vesicles produced are not stable and tend to aggregate, fuse, and release contents (particularly if made with phospholipids containing saturated fatty acids). We have discovered a novel and unique way to form unilamellar vesicles spontaneously without (i) special equipment or (ii) nonphospholipid additives (Gabriel & Roberts, 1984). The method we have developed takes advantage of the difference in fatty acid chain length of lecithin species and packing of the hydrophobic chains to minimize contact with water.

Short-chain lecithins (6–8 carbon length fatty acids) form micelles (nonbilayer, nonencapsulating aggregates) when dispersed in aqueous solutions (Tausk et al., 1974a,b). When (i) small amounts (20 mol % total lipid) of aqueous micellar short-chain lecithins are incubated with aqueous solutions of preexisting multibilayers or when (ii) films of short-chain lecithin/long-chain lecithin (1:4) deposited by evaporation from organic solvent are hydrated with an aqueous solution, unilamellar vesicles form spontaneously. A similar experiment with a variety of single-chain detergents produces a few mixed micelles and mostly multilamellar structures. Presumably because of phospholipid head-group/head-group interactions and chain-length mismatching, short-chain lecithins lead to an unexpected result: insertion into bilayers and formation of unilamellar vesicles. These spontaneously forming, unilamellar vesicles may be formed with dihexanoylphosphatidylcholine (PC), diheptanoyl-PC, or dioctanoyl-PC as the short-chain component (15–20 mol %) and any chain length (longer than 13 carbons), saturated or unsaturated, long-chain phosphatidylcholine as the basic bilayer matrix. They have been characterized by ^{1}H NMR spectroscopy. The stability of these mixed-chain vesicles has been examined by fluorescence over the temperature range 4–55 °C and is maintained for at least 5 days without vesicle aggregation and up to 2 months with some vesicle aggregation but no release of entrapped material.

Experimental Procedures

Materials. Dihexanoyl-PC,[1] diheptanoyl-PC, dioctanoyl-PC, and distearoyl-PC were obtained from Avanti-Polar

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[1] Abbreviations: diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; T_1, spin–lattice relaxation time.

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Lipids, Inc. Dipalmitoyl-PC, dimyristoyl-PC, and HEPES were obtained from Calbiochem Behring Corp. $N(CD_3)_3$-dipalmitoyl-PC was the gift of Dr. William Curatolo, Francis Bitter National Magnet Laboratory, M.I.T. Lecithins, checked for purity by thin-layer chromatography (Burns & Roberts, 1980), were used without further purification. 6-Carboxyfluorescein (Kodak) and praesodymium chloride, $PrCl_3 \cdot 6H_2O$ (Alfa Products), were used for encapsulation and stability studies.

Vesicle Preparation. Two methods were used for vesicle preparation. (i) Vesicles were usually prepared by cosolubilizing both long- and short-chain lecithins (4:1) in $CHCl_3$. Organic solvent was evaporated under N_2, and all remaining traces were removed by evacuation at low pressure for at least 12 h. Samples were then hydrated in the appropriate aqueous solvent containing 0.15 M NaCl (physiological salt concentration), bath sonicated for 1 min, and equilibrated at room temperature for 6–8 h. The pH was adjusted if necessary to be within the range 6.5–7.5. Vesicles containing dioctanoyl-PC as the short-chain lecithin were hydrated with 0.2 M KSCN. This ionic species helps to suppress the micelle phase separation that occurs for high concentrations of dioctanoyl-PC (Tausk et al., 1974a,b). (ii) Vesicles were also prepared by adding an aliquot of a diheptanoyl-PC aqueous stock solution (~50 mM, where micelles are the dominant species) to an aqueous dispersion of dipalmitoyl-PC (20 mM) such that the final diheptanoyl-PC concentration was 5 mM.

NMR Spectroscopy. The 500-MHz ^1H NMR spectra were obtained on a home-built spectrometer at the Francis Bitter National Magnet Laboratory, M.I.T. Twenty transients with a 90° flip angle (15 μs) and 5-s repetition time were collected and transformed with a 1.0-Hz exponential weighting function. Spin–lattice relaxation times were measured by inversion–recovery (Vold et al., 1968). Lanthanide shift experiments were done by adding microliter amounts of 5 or 20 mM Pr^{3+} stock solution to 0.3-mL samples at pH 6.5–7.

Fluorescence Spectroscopy. A Perkin-Elmer LS-3 fluorescence spectrometer was used to measure fluorescence of 6-carboxyfluorescein at room temperature: λ_{max} = 490 nm, λ_{em} = 520 nm. Mixed lipid films were hydrated in the presence of 100 mM 6-carboxyfluorescein. Most of the free dye was separated from the entrapped dye by passage through a (0.9 × 21.5 cm) Sephadex G-25 fine column. The solution collected in the void volume was used for fluorescein stability studies.

Results

^1H NMR Spectral Features of Lecithin Mixtures. When dipalmitoyl-PC and diheptanoyl-PC are cosolubilized in organic solvent in a 4:1 ratio and solvent is removed and the film rehydrated with an aqueous solution to yield a 20 mM/5 mM mixture, an opalescent solution is produced. At lower temperatures it is often slightly cloudy, but it can be clarified with a little heat. By comparison, 20 mM dipalmitoyl-PC hydrated with an aqueous solution is milky white. The slightly cloudy to opalescent solution of the long-chain/short-chain mixture gives rise to high-resolution NMR spectra. At all temperatures line widths are narrower than for multibilayer dispersions but broader than for short-chain lecithin micelles (Hershberg et al., 1976). In general, all resonances broaden as the temperature is increased toward the dipalmitoyl-PC phase transition temperature of 41 °C and then narrow. As an example, the bulk methylene resonance (~1.35 ppm) of diheptanoyl-PC/dipalmitoyl-PC vesicles broadens from 57 Hz at room temperature to 106 Hz at 40 °C and then narrows above that temperature (95 Hz at 45 °C). The most interesting feature

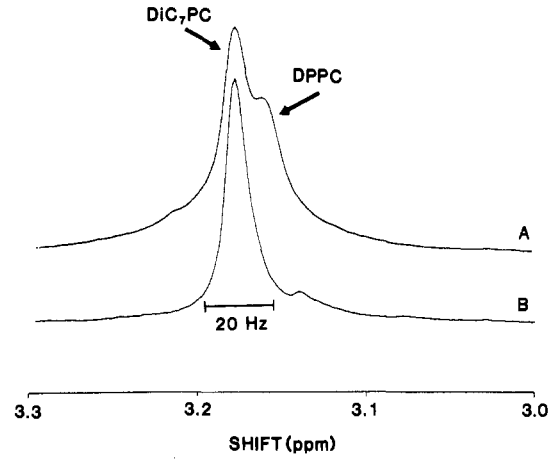

FIGURE 1: 500-MHz ^1H NMR spectra of the *N*-methylcholine region for (A) diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM) and (B) diheptanoyl-PC/$N(CD_3)_3$-dipalmitoyl-PC (5 mM/20 mM) at 25 °C.

of these spectra is the clear splitting observed for the $N(CH_3)_3$ group below 41 °C which is most prominent at room temperature (Figure 1A). With an increase in temperature the two resonances shift downfield differentially such that they coalesce around 40 °C (near the dipalmitoyl-PC phase transition temperature). Above that temperature the line width of the $N(CH_3)_3$ peak narrows, and occasionally two discrete resonances can again be discerned.

There are several possible explanations for the observed splitting in the $N(CH_3)_3$ region: (i) one resonance arises from diheptanoyl-PC molecules while the other arises from the long-chain lecithin (and no information is easily extracted about particle identity); (ii) two different types of particles (for example, vesicles, micelles, and monomers) are formed with lecithin molecules in slow exchange between the different environments; (iii) vesicles are present, and the splitting represents lecithin molecules in the outer monolayer in slow exchange with molecules on the inner monolayer; (iv) only the short-chain component is visible (the long-chain lecithin is in a gel-type conformation and immobile on this NMR time scale), and the short-chain lecithin is in slow exchange between two environments of different mobility. In order to determine which of these alternatives is correct, we prepared similar lecithin mixtures using $N(CD_3)_3$-dipalmitoyl-PC and diheptanoyl-PC (Figure 1B). Only one narrow resonance is visible in the choline methyl region; this can only arise from the short-chain lecithin. Therefore, the two resonances observed when both components are the proteo species are due to the chemically and possibly environmentally distinct diheptanoyl-PC and dipalmitoyl-PC. The narrower resonance belongs to the short-chain species. As the temperature increases the single diheptanoyl-PC resonance shifts downfield (~20 Hz/5 °C) but not as rapidly as the component from dipalmitoyl-PC which eventually obscures and then overtakes it.

Similar spectra are produced if micellar diheptanoyl-PC (5 mM) is added to multibilayer dispersons of dipalmitoyl-PC (20 mM). Within 4 min of mixing the two aqueous solutions, high-resolution spectral features are apparent (Figure 2). Initially resonances are broad but narrow on the time scale of hours. After 7 h at room temperature the mixture has reached equilibrium since no further spectral changes occur.

Of our four explanations for the split choline resonance we have ruled out (iii) and (iv) and limited (ii) to the case where diheptanoyl-PC micelles/monomers are one particle and dipalmitoyl-PC bilayers must be the other. ^1H NMR spin–lattice relaxation times, T_1, obtained for pure diheptanoyl-PC micelles and a mixed lecithin mixture provide evidence that

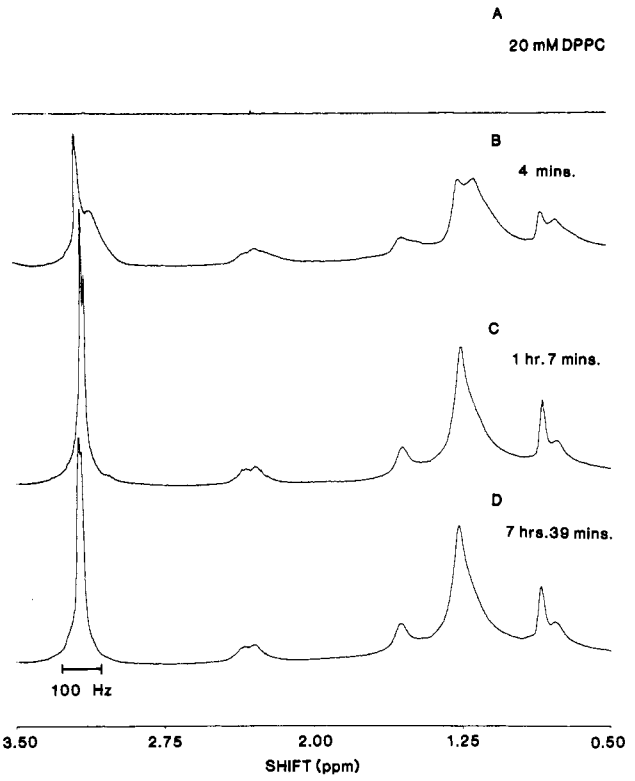

FIGURE 2: Normalized 500-MHz ^{1}H NMR spectra of preexisting multibilayers (20 mM dipalmitoyl-PC) incubated with short-chain lecithin (5 mM diheptanoyl-PC) at 25 °C as a function of time: dipalmitoyl-PC multibilayers (A) alone, (B) 4 min after addition of micellar diheptanoyl-PC, and (C) 67 and (D) 459 min after addition of short-chain species.

Table I: ^{1}H NMR Spin–Lattice (T_1) Relaxation Times for Diheptanoyl-PC (5 mM) as Pure Micelles and Mixtures with Dipalmitoyl-PC (20 mM)[a]

	T_1 (s)	
resonance	diheptanoyl-PC micelles	diheptanoyl-PC/dipalmitoyl-PC mixtures
$N(CH_3)_3$	0.61	0.62/0.63
$(CH_2)_n$	0.93	0.75/0.73
CH_3	1.26	0.87/1.07

[a] Data obtained by inversion–recovery pulse sequence; samples at 25 °C.

the short-chain lecithin is not present as a micelle (or monomer). For bulk methylene and terminal methyl groups [as well as $N(CH_3)_3$] in the mixed particles two components are observed in the ^{1}H NMR spectrum at room temperature. While $N(CH_3)_3$ T_1 values are not very structure dependent, T_1 values for the chain protons differ dramatically (Table I). The short-chain lecithin in the mixed particles has shorter T_1 values than in pure lecithin micelles (T_1 values for monomers would be even longer). This is best illustrated with the diheptanoyl-PC terminal methyl group. Its T_1 is 1.26 s in micelles and 0.87 s when mixed with dipalmitoyl-PC at 25 °C. In the mixed particle the diheptanoyl-PC methyl T_1 is also significantly less than the corresponding dipalmitoyl-PC methyl T_1 (1.07 s). This may reflect some type of motional restraints on heptanoyl chains by neighboring palmitate chains.

Detection of Bilayer Vesicles. The types of particles present in solution could be multibilayers, unilamellar vesicles, or mixed micelles. Multibilayers as the dominant particle are unlikely, given the clarity of the solution. Lanthanide shift reagents can be used to distinguish between vesicle and micellar or monomer species. It has been well documented that Pr^{3+} added to a population of vesicles complexes with the

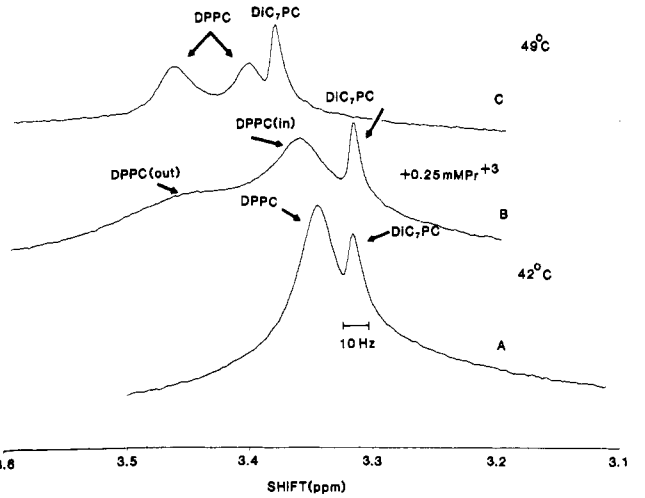

FIGURE 3: 500-MHz ^{1}H NMR spectra of the *N*-methyl region illustrating the effect of added lanthanide on diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM): (A) 42 °C, no lanthanide; (B) 0.25 mM Pr^{3+} added; (C) temperature increased to 49 °C.

phospholipid head group and shifts resonances from exterior molecules downfield without affecting resonances from interior lecithin molecules (because the aqueous compartment that these molecules surround is Pr^{3+} free). If all phospholipid head groups are exposed to the exterior Pr^{3+} solution (which would be the case for micelles or monomers), then all resonances will be shifted with possibly a different shift increment for short-chain vs. long-chain species. ^{1}H NMR (500-MHz) lanthanide shift experiments to detect vesicles were undertaken at temperatures above and below the T_c of the long-chain lecithin. In Figure 3 is shown the $N(CH_3)_3$ region (A) without lanthanide at 42 °C (two peaks visible), (B) with Pr^{3+} at 42 °C, where three components are visible, and (C) with Pr^{3+} at 49 °C, where the three components have narrower line widths and are more easily seen. The resonance from diheptanoyl-PC is invariant to the addition of small amounts of Pr^{3+}, while the broader dipalmitoyl-PC resonance splits into two peaks. The most downfield shifted of these is due to the exterior monolayer of dipalmitoyl-PC, while the upfield, broader peak is due to interior layer dipalmitoyl-PC. With further addition of Pr^{3+} to concentrations that cause a shift in pure micelles of diheptanoyl-PC, the $N(CH_3)_3$ resonance of the short-chain component in the vesicles also shifts downfield, but not as much as for micellar samples (with that level of Pr^{3+}, the dipalmitoyl-PC peaks are broadened dramatically). Two discrete short-chain peaks are not observed under these conditions. This suggests that the short-chain species is either all on the outer surface and interacts more weakly with Pr^{3+} than dipalmitoyl-PC or is rapidly exchanging across the bilayer. Similar results are seen for other diheptanoyl-PC/long-chain lecithin (dimyristoyl-PC, distearoyl-PC, egg lecithin) mixtures both above and below the phase transition temperature of the long-chain species. Preliminary quasi-elastic light scattering experiments detect particle distributions that are bimodal or more complex for temperatures below the T_c and around 610 Å (variance = 0.2) for diheptanoyl-PC/dipalmitoyl-PC vesicles at 45 °C.

Vesicle Stability. Stability (integrity) studies have been conducted by encapsulating the fluorescent dye 6-carboxyfluorescein inside the vesicles. The ratio of fluorescence in each vesicle sample compared to the Triton X-100 mixed micelle standard is monitored in Figure 4 for diheptanoyl-PC/dipalmitoyl-PC vesicles made and stored at 4, 25, and 45 °C. A 3-fold increase in fluorescence above background was ob-

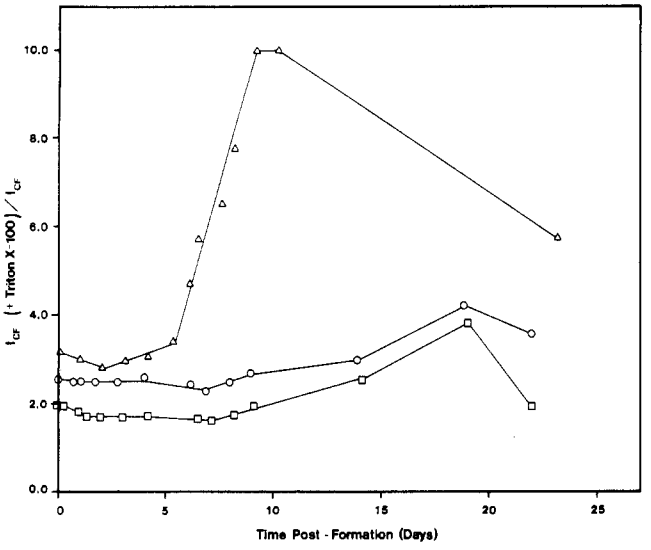

FIGURE 4: Stability study of diheptanoyl-PC/dipalmitoyl-PC (5 mM/20 mM) vesicles prepared in 100 mM 6-carboxyfluorescein and stored at room temperature (△), 4 °C (○), and 45 °C (□).

served for the samples to which Triton X-100 was added. [At 4 and 25 °C this may not be enough Triton to micellize dipalmitoyl-PC completely, but it should cause at least partial micellization (Ribeiro & Dennis, 1974).] The vesicle sample fluorescence remained unchanged for at least 5 days; an increase in the fluorescence ratio is detected as the vesicles aggregate. This is most pronounced for the sample formed and stored at 25 °C. On the same time scale empty vesicles do not aggregate so that the clumping must reflect electrostatic interactions of dye loaded in the vesicles.

NMR experiments also suggest excellent vesicle stability. Samples stored with Pr^{3+} for up to 2 weeks show no large change in the relative amount of outer/inner resonances. Initial preparations of vesicles are also stable to dilution at least 5-fold, as judged by no change in the appearance of the ^1H NMR spectra under these conditions. [Any release of monomer short-chain lecithin would give rise to very narrow peaks, as has been seen for bile salt/lecithin vesicle formation (R. E. Stark, M. F. Roberts, and M. Carey, unpublished results).]

Discussion

We have developed a technique for spontaneously forming unilamellar vesicles from mixtures of short-chain and long-chain lecithins. The method is easy, rapid, and requires no elaborate equipment. There is no need to use detergents or organic solvents that might denature material to be encapsulated or that might remain with the formed vesicles in small but toxic amounts.

Experiments following vesicle formation and equilibration by ^1H NMR suggest a tentative mechanism for formation of these mixed chain length vesicles (Figure 5). Resonances of the long-chain multibilayers are very broad and not detectable. After the addition of the micellar short-chain lecithin, two resonances are observed for each proton species. The integrated intensity increases along with spectral shifts until equilibrium is reached. The short-chain lecithin may insert itself in the outer layer of a multibilayer aggregate, initiating a mismatch in fatty acyl chain lengths that can lead to a locally convexed area as more short-chain lecithin molecules are inserted into the bilayer. Smaller regions can then bud off to form vesicles with the short-chain species residing in the outer monolayer of the vesicle. Alternatively, the short-chain species

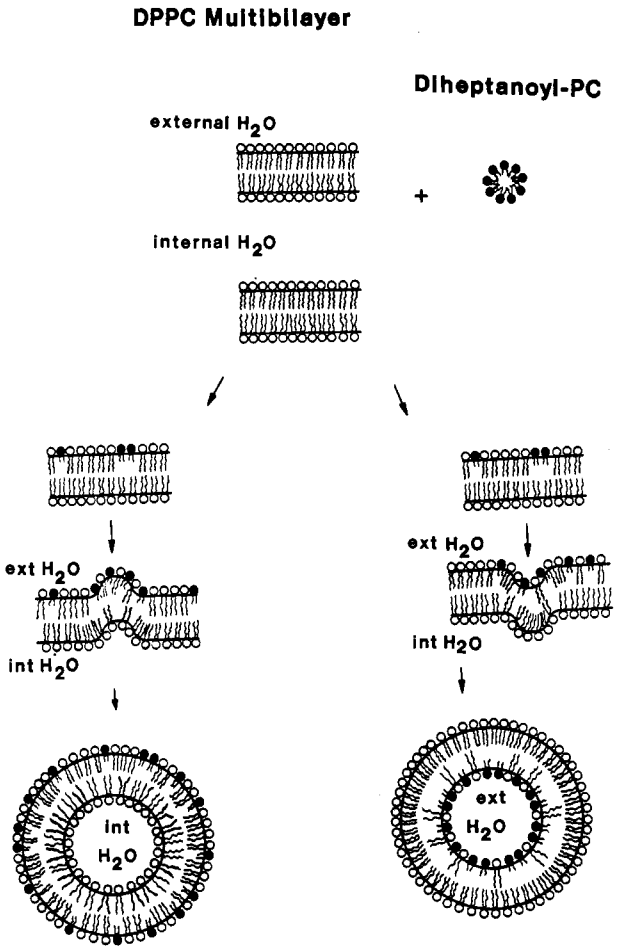

FIGURE 5: Proposed mechanism for spontaneous formation of unilamellar vesicles from multibilayer and micellar aggregates.

could cause a concaved area as it inserts into the bilayer with the short-chain species now residing in the inner monolayer of the vesicle. Since transbilayer flip-flop rates of long-chain lecithins are slow (Kornberg & McConnell, 1971), the short-chain species might be expected to remain on one side, although they may show an enhanced transbilayer diffusion rate and equilibrate rapidly. The ^1H NMR experiments with relatively high concentrations of Pr^{3+} may be slightly more consistent with the first alternative, since the short-chain *N*-methyl group does shift downfield eventually. Whichever distribution occurs it must also be the thermodynamically stable arrangement, since cosolubilized short-chain/long-chain lecithins form vesicles that are spectroscopically identical with respect to the vesicles formed from mixing multibilayers and micellar aggregates.

Given the ease of formation and stability of short-chain/long-chain lecithin unilamellar vesicles, they should be useful in a wide variety of fields. If size can be modulated by appropriate selection and ratio of short- and long-chain components, these vesicles may be very useful for delivery of drugs or reagents for imaging experiments (for example, encapsulating paramagnetic ions or stable nuclear labels for NMR imaging or encapsulating technitium complexes for real-time radio-label imaging). Perhaps an intriguing and less applied use is to adapt the vesicle formation mechanism as a general method of forming plasma membrane vesicles. If short-chain lecithins insert into bilayers and cause regions of local curvature, which bud off to form vesicles, then perhaps adding small amounts of diheptanoyl-PC to cells will initiate plasma membrane vesicle formation. By analogy to the simple lipid case, an asymmetric orientation of proteins, lipids, etc. should

result. Further investigations into the size distribution of different long-chain/short-chain pairs and the effect of cholesterol and/or other head-group phospholipids will allow better definition of these possibilities.

Acknowledgments

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Registry No. Diheptanoyl-PC, 35387-75-8; dipalmitoyl-PC, 2644-64-6.

References

Brunner, J., Skrabol, P., & Hauser, H. (1976) *Biochim. Biophys. Acta 455*, 322–331.

Burns, R. A., Jr., & Roberts, M. F. (1980) *Biochemistry 19*, 3100–3106.

Christiansen, K., & Carlsen, J. (1983) *Biochim. Biophys. Acta 735*, 225–233.

DeBose, C. D., & Roberts, M. F. (1983) *J. Biol. Chem. 258*, 6327–6334.

Farmer, M. C., & Gaber, B. P. (1984) *Biophys. J. 45*, 201a.

Gabriel, N. E., & Roberts, M. F. (1984) *Biophys. J. 45*, 41a.

Hamilton, R., Jr., Goerke, L., Guo, L., Williams, M., & Havel, R. (1980) *J. Lipid Res. 21*, 981–992.

Hershberg, R. D., Reed, H. G., Slotboom, A. J., & de Haas, G. H. (1976) *Biochim. Biophys. Acta 424*, 73–81.

Kensil, C. A., & Dennis, E. A. (1979) *J. Biol. Chem. 254*, 5843–5848.

Kornberg, R., & McConnell, H. M. (1971) *Biochemistry 10*, 1111–1120.

Papahadjopoulos, D., & Watkins, J. C. (1967) *Biochim. Biophys. Acta 135*, 639–650.

Papahadjopoulos, D., & Kimelberg, H. K. (1973) *Prog. Surf. Sci. 4*, 139–221.

Racker, E., & Stoeckenius, W. (1974) *J. Biol. Chem. 249*, 660–663.

Ribeiro, A. A., & Dennis, E. A. (1974) *Biochim. Biophys. Acta 332*, 26–29.

Ryman, B. E., & Tyrrell, D. A. (1980) in *Essays in Biochemistry* (Campbell, P. N., & Marshall, R. D., Eds.) pp 49–98, Academic Press, London.

Szoka, F., Jr., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.

Tausk, R. J. M., Karmiggelt, J., Oudshoorn, C., & Overbeek, J. (1974a) *Biophys. Chem. 1*, 175–183.

Tausk, R. J. M., Oudshoorn, C., & Overbeek, J. (1974b) *Biophys. Chem. 2*, 53–63.

Vold, R. L., Waugh, J. S., & Klein, M. P. (1968) *J. Chem. Phys. 48*, 3831–3839.