retention on polymeric stationary phases. It appears that different scales may be needed, especially for complex solutes that have several hydrogen bond acceptor sites.

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Nearest-Neighbor Recognition in Phospholipid Membranes: A Molecular-Level Approach to the Study of Membrane Suprastructure[1]

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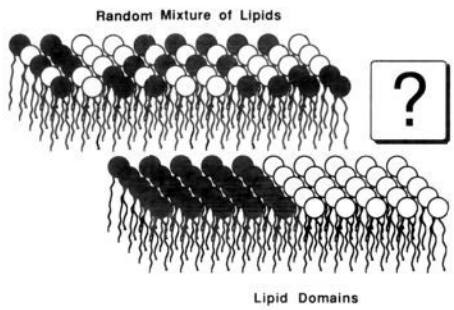
Abstract: A molecular-level approach has been devised for probing the lateral organization of phospholipid bilayers. This method is based on the equilibration of disulfide-based phospholipid dimers via thiolate–disulfide interchange reactions. Analysis of resulting product mixtures defines the tendency of one phospholipid monomer to become a covalently attached nearest neighbor of another. Investigation of equilibrium mixtures derived from *dimeric* analogs of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) has yielded conclusive evidence that the lipid monomers are randomly distributed throughout the membrane in the fluid phase and also in the gel–fluid coexistence region. Additional support for random organization in the gel–fluid region has been obtained by use of differential scanning calorimetry (DSC). When the difference in alkyl chain length between the equilibrating monomers is increased from two to four methylene groups (i.e., dimeric analogs of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) are used instead of DPPC), the membrane suprastructure remains random in the fluid phase. In the gel–fluid coexistence region, however, a significant deviation from randomness is observed. This deviation implies the presence of lipid domains and is consistent with the appearance of phase separation, as indicated by DSC analysis. Examination of the temperature dependence of this nearest-neighbor recognition supports the hypothesis that the packing forces that govern such recognition can be very similar to those that govern domain formation. Previous conclusions that have been drawn from quick-freeze DSC experiments with DMPC/DPPC bilayers are critically examined in light of these findings.

Introduction

One of the most significant challenges that lies at the interface of chemistry and biology is to define the suprastructure of biological membranes. In particular, the specific time-averaged, lateral distribution of the lipids and proteins that make up these biological enclosures remains to be established.[3-5] Do lipids organize themselves into nonrandom clusters, i.e., domains? If domains exist, do they have any functional importance? Are they intimately involved, for example, in basic membrane processes such as fusion, transport, recognition, and catalysis? Do changes in lateral organization accompany the formation of a diseased state, e.g., the malignant transformation of cells? Can such changes alter the presentation of receptors at the cell surface or the activities of membrane-bound enzymes? Are lipid domains in cancer cells unique, and can they serve as specific targets for chemotherapy? These questions are not only of considerable theoretical interest but they also have important practical implications. A firm understanding of the suprastructure of biological membranes has the potential for bringing exploitable targets into clear focus, which could assist the rational design of novel classes of membrane-disrupting drugs.[6]

Despite the considerable amount of effort that has been spent in investigating the lateral organization of lipid membranes, definitive proof of suprastructure in even the simplest of systems has remained elusive. In nearly all studies to date, a combination of thermal, spectroscopic, electron microscopic, and chemical methods has been used to infer the presence or absence of lipid domains.[7-12] While strong evidence has been obtained which supports the existence of domains in certain binary mixtures of phospholipids at temperatures in which the gel and fluid phase coexist (i.e., the gel–fluid coexistence region), the precise organization of lipid bilayers in the physiologically relevant fluid phase remains ill-defined. The major difficulty has been the absence of experimental techniques that can be applied directly to the fluid phase.



In this article we describe a fundamentally new approach to the study of the lateral organization of lipid bilayers. Our

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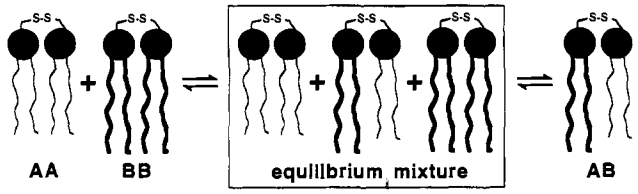
Scheme I



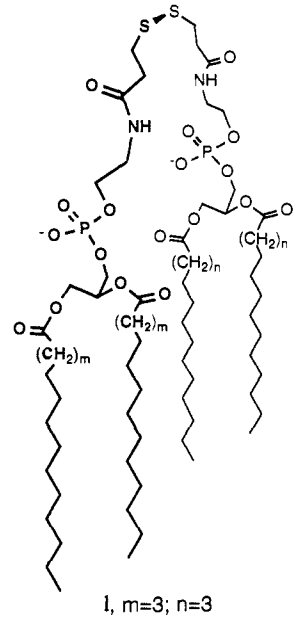
technique is not only directly applicable to the fluid phase but under certain circumstances it also yields conclusive proof of membrane suprastructure.[13] An illustration of the underlying concept is presented in Scheme I. A 1/1 molar mixture of two different symmetrical dimers (homodimers AA and BB) is equilibrated via thiolate–disulfide interchange.[14] In order to ensure that an equilibrium state has been reached, a similar equilibration reaction is carried out starting with the corresponding heterodimer, AB. The extent to which the ratio of AA/AB/BB deviates from a molar ratio of 1/2/1 (a random distribution) reflects the thermodynamic preference for one phospholipid to become a covalently attached, nearest neighbor of another; i.e., it defines the ability of an equilibrating phospholipid monomer to "recognize" a nearest neighbor.

How does nearest-neighbor recognition relate to membrane suprastructure? When dimer distributions are found to be purely statistical (i.e., when no recognition is observed), such a finding, in and of itself, *proves that the lipid components are randomly distributed throughout the membrane at the molecular as well as the supramolecular level.* It establishes that there is no thermodynamic driving force for either nearest-neighbor recognition or domain formation. For those cases in which nearest-neighbor recognition is found, *the existence of domains is inferred.* This inference rests on the assumption that the packing forces that govern nearest-neighbor recognition are the same (or very similar) as those which govern domain formation.

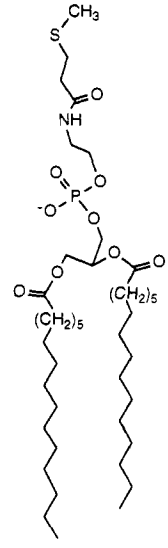
One caveat to this approach is that natural biological membranes are largely comprised of single phospholipid molecules, not dimers; the exception is the glycerol-bridged cardiolipin. Thus, bilayers that consist of chemically equilibrating phospholipid dimers must be regarded as membrane mimetic in character. Nonetheless, we believe that this approach provides a truly unique opportunity for exploring membrane composition–suprastructure relationships in ways that have not, heretofore, been possible.

Results

Design of Lipid Dimers. The phospholipid dimers that we have chosen as targets for this study are shown as structures **I–V**. Our primary reason for investigating these specific molecules was to examine the dependency of nearest-neighbor recognition on the length of the alkyl chains of the phospholipids. On the basis of their fatty acid content, **I**, **II**, and **IV** may be regarded as dimeric analogs of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), respectively; lipids **III** and **V** represent the corresponding heterodimers. Examination of CPK models indicates that these dimers possess a reasonable degree of flexibility due to a significant spacing between the phosphate headgroups. This fact, together with the known similarity in melting behavior of phosphatidylcholines and phosphatidylglycerols,[15] and the fact that the headgroup charges of our dimers and the phosphatidylglycerols are the same suggested to us that **I**, **II**, and **IV** would melt like their corresponding phosphatidylcholines. While we expected that the covalent attachment of two phospholipids, in the manner indicated, would lead to a minimum perturbation of the lipid's melting behavior,



I, m=3; n=3
II, m=7; n=7
III, m=3; n=7
IV, m=5; n=5
V, m=3; n=5



VI

a monomeric analog of **IV** (i.e., **VI**) was chosen in order to test this hypothesis.

Previous high-sensitivity differential scanning calorimetry (hs-DSC) studies have shown that bilayers constructed from DMPC and DPPC are miscible in the gel–fluid coexistence region, producing a single broadened endotherm that appears at a temperature which lies midway between the endotherms of the pure phospholipids.[16,17] In one stunning report, which employed a novel quick-freeze high-sensitivity DSC technique, it was proposed that DMPC/DPPC bilayers are immiscible in the fluid phase.[8] If dimeric analogs were to behave similarly, then one would expect to observe equilibrium mixtures of dimer that are statistical in the gel–fluid coexistence region and nonrandom in the fluid phase.

In marked contrast, bilayers made from DMPC and DSPC have been shown to undergo phase separation at temperatures that lie midway between the gel to liquid–crystalline phase-transition temperatures of the pure lipids. Here, gel-phase domains that are rich in DSPC are presumed to exist in a "sea" of fluid-phase lipid that is rich in DMPC; i.e., two distinct endotherms are observed. If our dimers behaved in a similar way, one would observe significant nearest-neighbor recognition in the gel–fluid coexistence region. Although no experimental data have yet appeared concerning the miscibility of DMPC/DSPC in the fluid phase, we expected that our dimeric analogs would exhibit a degree of recognition that is at least as great as that found for our DMPC/DPPC dimer analogs in the fluid phase.

Phospholipid Synthesis. The synthetic approach that we have used to prepare **I–V** is outlined in Figure 1. In brief, appropriate phosphoethanolamines were first derivatized with the heterobifunctional coupling agent *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) to give a protected form of the thiol-bearing lipid monomers, **1–3**. Subsequent deprotection with dithiothreitol (yielding **4–6**) and coupling with the appropriate protected thiol lipids afforded the requisite set of dimers **I–V**. Lipid **VI** was synthesized from 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine and 3-(methylthio)propionic acid, using *N*,*N*-dicyclohexylcarbodiimide as the condensing agent and 4-(dimethylamino)pyridine as a catalyst.

Thermotropic Phase Behavior of the Lipids. The temperature at which a phospholipid bilayer is half-converted from a gel into

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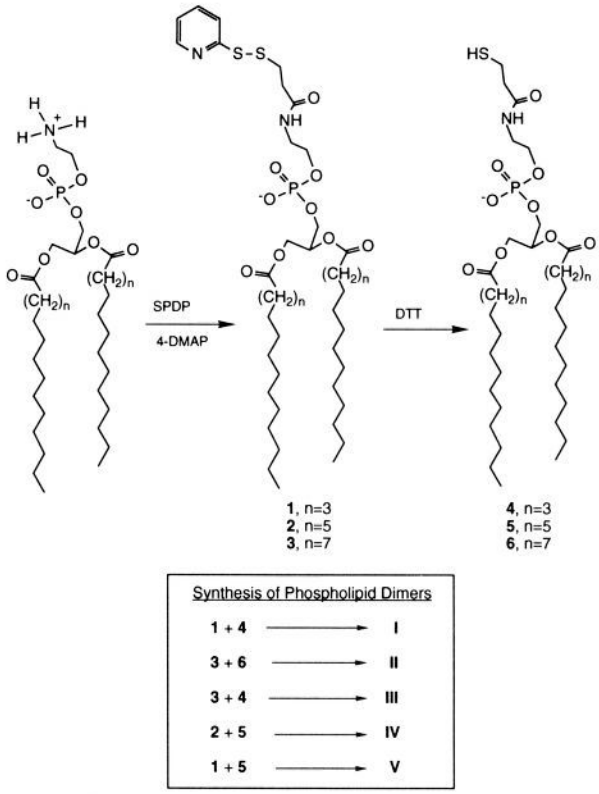

Figure 1. Synthetic approach used for the preparation of phospholipid dimers.

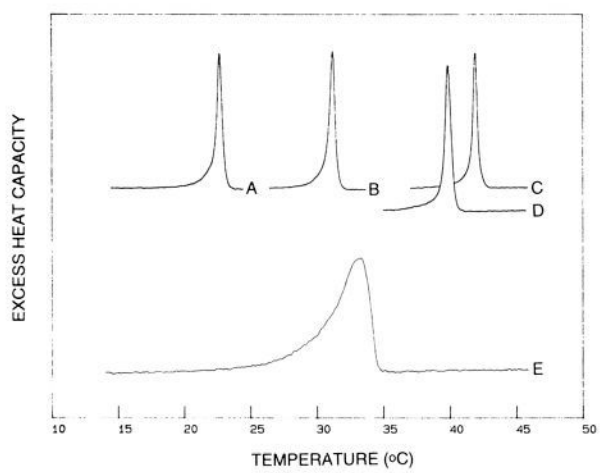

Figure 2. High-sensitivity excess heat capacity profile of (A) **I**, (B) **V**, (C) **IV**, (D) **VI**, and (E) **I/V/IV** (1/2/1 molar ratio).

a fluid phase is, by definition, its characteristic gel to liquid–crystalline phase-transition temperature (T_m). At temperatures below the onset of such melting, the alkyl chains are in an ordered, all-anti configuration. When melting does occur, gauche conformations are introduced into the alkyl chains, and they become disordered and more fluidlike in character.[15,18,19] In Figure 2 we show the DSC thermograms that characterize the melting behavior of bilayer membranes derived from **I**, **IV**, **V**, and **VI**. Figure 3 shows the analogous thermograms for **I**, **II**, and **III**. A summary of the calorimetric data which characterize these phase transitions is presented in Table I.

Vesicle Formation. Large unilamellar vesicles (1000 Å diameter) were used for all of the dimer equilibration studies that are described in this work. By use of standard extrusion methods,

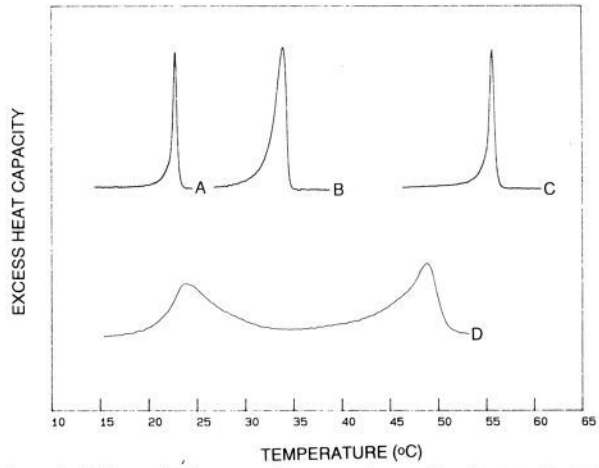

Figure 3. High-sensitivity excess heat capacity profile of (A) **I**, (B) **III**, (C) **II**, and (D) **I/III/II** (1/0.76/1 molar ratio).

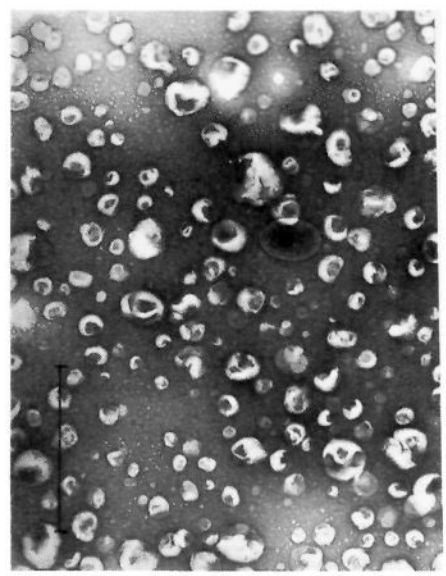

Figure 4. Transmission electron micrograph (2% uranyl acetate) of large unilamellar vesicles derived from **V**. The bar represents 1 μm.

Table I. Gel to Liquid–Crystalline Main Phase-Transition Properties for Phospholipids **I–VI** and Their Corresponding Phosphatidylcholines[a]

phospholipid	T_m (°C)	$\Delta T_{1/2}$ (°C)[b]	ΔH (kcal/mol)	ΔS (cal/K·mol)
DMPC	24.0		6.5	21.9
I	22.7	0.5	14.7	49.7
DPPC	41.5	0.3	8.7	27.7
IV	41.9	0.4	18.7	59.4
VI	39.9	0.5	9.3	29.7
DSPC	54.3		10.4	33.3
II	55.4	0.4	21.7	66.1
III	33.9	1.3	18.7	60.9
V	31.2	0.5	16.7	54.9

[a] Data for phosphatidylcholines were taken from Blume, A. *Biochemistry* **1983**, *22*, 5436. [b] Width at half-maximum excess specific heat.

all of the lipids (and mixtures of lipids) yielded stable and well-behaved vesicular dispersions.[20] A typical transmission electron micrograph of one such dispersion is shown in Figure 4. In all cases, the mean diameter of the vesicles (ca. 1000 Å) was confirmed by dynamic light scattering.

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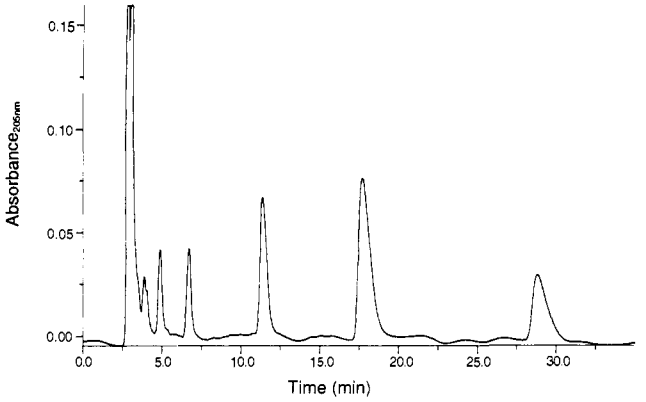

Figure 5. High-pressure liquid chromatogram of an equilibrated dimer mixture formed from **V** after 2 h at 53 °C. Retention times for **I**, **V**, and **IV** are 11.4, 17.7, and 28.8 min, respectively. Thiol lipid monomers **4** and **5** have retention times of 4.9 and 6.7 min, respectively.

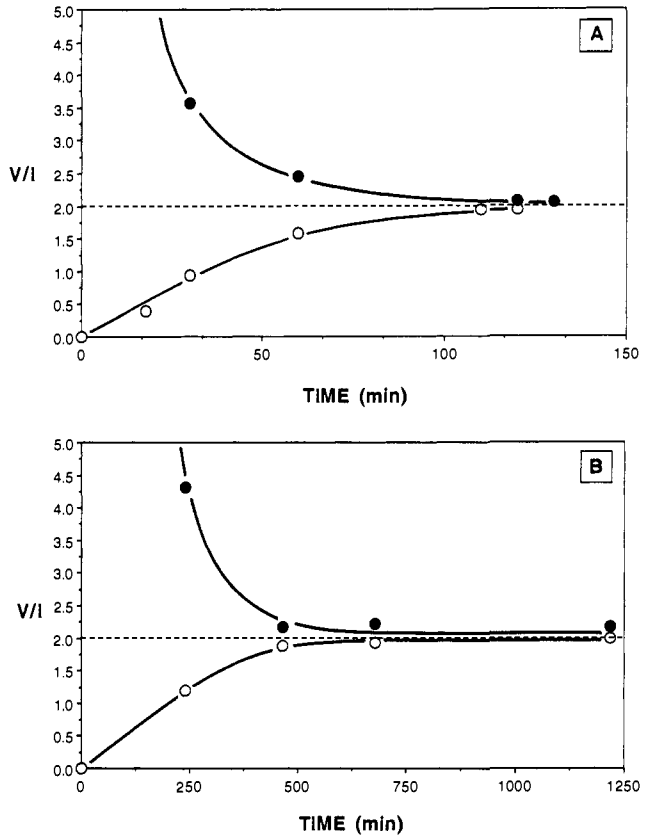

Figure 6. Plots of the molar ratio of **V**/**I** as a function of equilibration time for vesicles prepared from pure **V** (●) and a 1/1 molar mixture of **I**/**IV** (○). The equilibration temperature was maintained at (A) 53 ± 1 and (B) 33 ± 1 °C. In all cases, equal molar ratios of symmetrical dimers were produced (±5%).

Equilibration Experiments. The standard protocol that we have used for carrying out dimer equilibration experiments is detailed in the Experimental Section. In brief, after the pH of an appropriate dispersion is raised from 7.4 to 8.5, it is then treated with 1 equiv of dithiothreitol (DTT) and allowed to react at a given temperature for a specified period of time. The thiolate–disulfide interchange reaction is then quenched by lowering the pH to 5.0. After freeze-drying, the mixture of dimers is analyzed by high-pressure liquid chromatography. The mass balance for recovered lipid was ca. 90% in all cases (HPLC). A typical HPLC chromatogram is presented in Figure 5. Control experiments, carried out in the absence of DTT and also in the presence of DTT (pH 5), resulted in no detectable exchange. Using our standard interchange conditions, ca. 20% of the dimers were converted into equal molar amounts of the two corresponding thiol monomers.

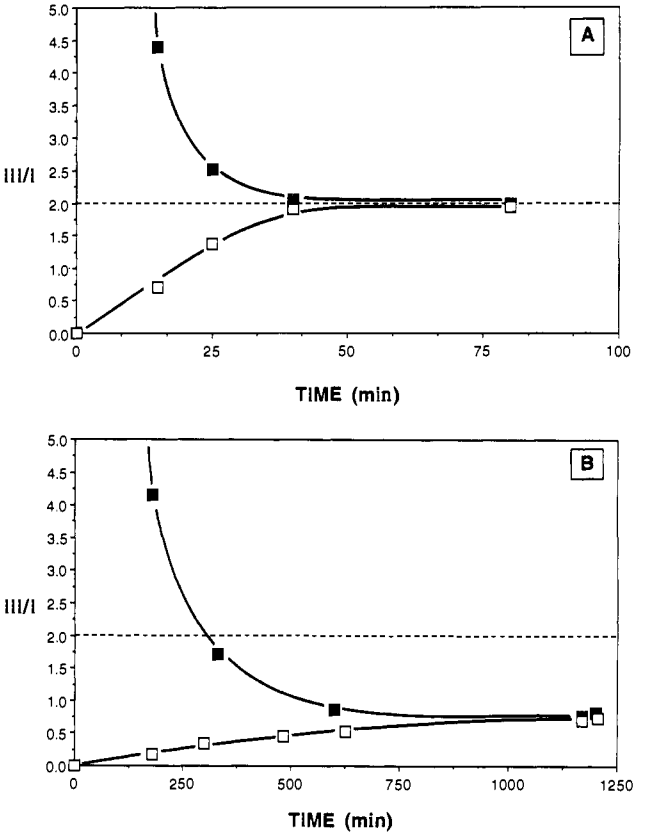

Figure 7. Plots of the molar ratio of **III**/**I** as a function of equilibration time for vesicles prepared from pure **III** (■) and a 1/1 molar mixture of **I**/**II** (□). The equilibration temperature was maintained at (A) 60 ± 1 and (B) 33 ± 1 °C. In all cases, equal molar ratios of symmetrical dimers were produced (±5%).

When similar experiments were carried out using 0.3 equiv of DTT, product mixtures contained ca. 7% of total monomer and *identical* equilibrium mixtures of dimers; the time that was required to reach equilibrium, however, was significantly increased.

Plots of the molar ratio of **V**/**I** (i.e., the ratio of heterodimer/"shorter" homodimer) as a function of time for vesicles prepared from pure **V** and also from a 1/1 molar mixture of **I**/**IV** are shown in Figure 6. At all stages of the equilibration, the molar amounts of the homodimers were identical. At temperatures in which the bilayer is maintained in the fluid phase (53 °C), the equilibrium ratio of **V**/**I** was 1.99 ± 0.04/1 (two standard deviations from the mean, four independent experiments); when the thiolate–disulfide interchange was carried out in the gel–fluid coexistence region (33 °C), the ratio was 2.03 ± 0.05/1. Analogous experiments that were carried out with fluid-phase membranes made from **III**, and 1/1 molar mixtures of **I**/**II**, yielded random distributions of lipid dimers (i.e., 1.98 ± 0.06/1 at 60 ± 1 °C). However, when a similar exchange was carried out in the gel–fluid coexistence region (33 °C), the equilibrium ratio of **III**/**I** was 0.76 ± 0.06/1 (Figure 7).[21] Measurement of equilibrium dimer distributions at two other temperatures within the gel–fluid coexistence region gave ratios of heterodimer/homodimer of 0.57/1 (30 °C) and 1.06/1 (40 °C).

Thermotropic Phase Behavior of Equilibrium Mixtures of Dimers. After equilibrium dimer distributions were determined, similar mixtures of lipids were then examined by DSC under nonexchangeable conditions (absence of DTT, pH 7.4). The thermogram recorded for membranes prepared from a 1/2/1 molar mixture of **I**/**V**/**IV** shows a single broadened endotherm

(21) While our preliminary results suggested a slight deviation from randomness in the fluid phase for the equilibrating **I**/**III**/**II** system, a careful reexamination of the high-pressure liquid chromatograms revealed a systematic error. Ratios reported herein (for fluid and fluid–gel coexistence temperatures) are corrected values.

having a T_m of 33 °C (Figure 2E). Figure 3D shows the thermogram recorded for membranes prepared from a 1/0.76/1 molar mixture of I/III/II, which is the equilibrium dimer distribution determined at 33 °C. Under nonexchangeable conditions, this lipid mixture produced two broadened endotherms at 23 and 48 °C.

Discussion

Thermotropic Phase Behavior of the Phospholipids. Comparison of the gel to liquid-crystalline phase-transition temperatures of DMPC, DPPC, and DSPC with their dimeric analogs reveals a striking similarity (Table I). The T_m value for IV was also found to be very similar to that of its monomeric counterpart, VI. Moreover, the calorimetric enthalpies and entropies that characterize the melting of each of the dimers were similar to those of their phosphatidylcholine analogs, after one takes into account the fact that each mole of dimer contains two phospholipid components; i.e., when dividing the enthalpies and entropies of the dimers by a factor of 2, the resulting values are very similar to those of the monomeric phosphatidylcholines. With the exception of III, the peak widths at half-maximum excess specific heat ($\Delta T_{1/2}$) for all of the phospholipids were narrow and similar to those that are normally observed for pure phospholipids. The unusually broad endotherm for III is noteworthy. The fact that its purity is >99% (HPLC) suggests that the breadth of the endotherm is an intrinsic property of the lipid. It further implies that chain melting is not a very cooperative process and that the effective size of the molecular aggregate, over which the motion of the molecules undergoing the phase transition is transmitted, is relatively small. On the basis of the observed $\Delta T_{1/2}$ and the measured calorimetric enthalpy of the transition (ΔH), we estimate the unit size to be ca. 27 molecules. Such an estimate comes from the relationship, CU = $\Delta H_{VH}/\Delta H$, where CU is the cooperative unit (number of molecules per aggregate) and ΔH_{VH} is the van't Hoff enthalpy, which is defined by $\Delta H_{VH} \approx 6.9(T_m^2/\Delta T_{1/2})$.[15,22] The cooperative unit for all other lipid dimers is approximately 3 times larger than this value. The relatively low degree of cooperativity that characterizes the melting of bilayers of III is a likely consequence of the significant mismatch of the chain length of the two lipid components.

Randomly Organized Lipid Bilayers. Proof of Membrane Suprastructure. The fact that random equilibrium mixtures of dimers are generated from 1/1 molar ratios of I/IV (and from V) in the fluid phase (53 °C), and also in the gel-fluid coexistence region (33 °C), establishes that these lipid components are randomly arranged throughout the bilayer; i.e., it proves that lipid domains do not exist. These results are also fully consistent with the DSC data, which indicate complete mixing of the lipids in the gel-fluid coexistence region; i.e.,, a single broadened endotherm is observed for an equilibrium (1/2/1) mixture of the dimers. Similarly, membranes derived from I/II and from III, which have been chemically equilibrated in the fluid phase (60 °C), also display a random organization.

Nearest-Neighbor Recognition. In marked contrast to the fluid state, equilibrium mixtures produced from I/II (and from III) show a significant deviation from randomness in their gel-fluid coexistence region. This result is in agreement with the DSC data which show two distinct endotherms for such equilibrium mixtures of lipids, indicating that solidlike domains that are rich in II exist in a fluid "sea" that is rich in I. Thus, while a difference of four methylene groups per alkyl chain between two equilibrating monomeric units is insufficient to produce nearest-neighbor recognition in the fluid phase, it is sufficient in the gel-fluid coexistence region.

If we define an equilibrium constant, K, for the interchange between the two phospholipid homodimers (AA and BB) and the heterodimer (AB) via eq 1, then the thermodynamic parameters which govern this equilibrium are defined in eq 2. On the basis of the temperature dependence of the dimer distribution that is observed within the gel-fluid coexistence region, we estimate the enthalpy and entropy that are associated with this recognition to be $\Delta H° = 22.5 \pm 2.3$ kcal/mol and $\Delta S° = 72 \pm 8$ cal/K·mol (Figure 8). It should be noted that this entropy includes a statistical component, R ln (4) or 2.75 cal/K·mol, which derives from the fact that the heterodimer is statistically favored over each homodimer by a ratio of 2/1.

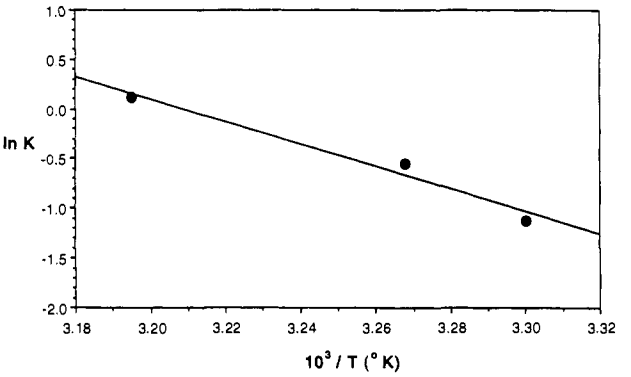

Figure 8. Plot of ln K for the equilibration among I/II and III as a function of $10^3/T$.

$$AA + BB \rightleftharpoons^{K} 2AB$$

$$K = \frac{[AB]_{eq}^2}{[AA]_{eq}[BB]_{eq}} \quad (1)$$

$$\Delta G° = -RT \ln \left(\frac{[AB]_{eq}^2}{[AA]_{eq}[BB]_{eq}} \right) = \Delta H° - T\Delta S° \quad (2)$$

It is especially noteworthy that these thermodynamic data are very similar to those that define the gel to liquid-crystalline phase transition of the higher melting homodimer, II. We do not view this similarity as merely coincidental, but rather as lending strong support for our assumption that the packing forces that govern nearest-neighbor recognition are (at least for this system) very similar to those that govern domain formation. In particular, it is the thermodynamic preference of the higher melting homodimer to self-associate in the gel-fluid coexistence region that serves as the driving force for phase separation, nearest-neighbor recognition, and domain formation.

Lipid Domains in the Physiologically Relevant Fluid Phase? While our predictions that equilibrium mixtures of I/V/IV would favor random mixing and that I/III/II would favor domain formation in the gel-fluid coexistence region have been verified experimentally, the absence of nearest-neighbor recognition for the I/V/IV system within the fluid phase was unexpected. Our results with dimeric analogs of DMPC/DPPC lead to a definitive conclusion regarding membrane suprastructure, but it is one that appears to be inconsistent with conclusions that have been drawn for DMPC/DPPC on the basis of quick-freeze DSC data.

How can one explain this paradox? We believe that the earlier quick-freeze DSC data were misinterpreted, and we present the following brief critique which highlights our misgivings.[8] The essence of that DSC experiment was as follows: A 1/1 mixture of DMPC/DPPC was quickly frozen from +85 (fluid phase) to −173 °C (gel phase) and then subjected to normal melting and analysis by DSC. Samples were scanned from −30 to +60 °C at a rate of 5 °C/min. Under these conditions, two endotherms were observed (28 and 53 °C). In a parallel experiment, a quickly frozen sample, which was "aged" at −196 °C for 3 h prior to DSC analysis, showed melting behavior that was very similar to that of a conventional DSC scan of a 1/1 mixture of DMPC/DPPC, i.e., both exhibited one dominant endotherm at 34 °C. The principal assumptions that were made were as follows: (i) that the lateral organization of the fluid state can be captured by quickly freezing the bilayer to 77 K and (ii) that diffusion of phospholipids within the gel phase is sufficiently slow such that a DSC thermogram (heating cycle only) reflects the lateral organization of the "captured" fluid phase. The appearance of the

(22) Mabrey, S.; Sturtevant, J. M. *Methods Membr. Biol.* **1978**, *9*, 237.

two distinct endotherms, under these DSC conditions, was then attributed to two lipid domains that were captured from the fluid phase. The effect of aging was rationalized in terms of slow lateral diffusion of the lipids in the gel phase, which leads to a new equilibrium state.

While the above interpretations would appear to be reasonable, there is one troubling issue. In particular, lateral diffusion of phospholipids in the gel phase is known to be strongly dependent on temperature.[23,24] On the basis of the temperature dependence of lateral diffusion that has been measured for a fluorescent phospholipid probe molecule in gel-phase DMPC, an activation energy was estimated to be ca. 36 kcal/mol.[23] If a similar activation energy is associated with lateral diffusion in DMPC/DPPC bilayers and if 3 h are a sufficient amount of time for the elimination of DMPC/DPPC domains at liquid nitrogen temperatures, then at +10 °C (where the reported thermograms begin) a similar loss would be expected within ca. 10^{-71} s (estimated by use of the Arrhenius form of the rate expression, i.e., $k_{283}/k_{77} = e^{-E_a/R(283)}/e^{E_a/R(77)}$). Even if one were to make a most conservative estimate for the activation energy of diffusion by assuming a value of 3 kcal/mol (found in fluid lecithin bilayers),[24] then the time required for the loss of domains would be ca. 10^{-2} s. Such time constraints would make it impossible to perform this DSC experiment over the reported 10-min period and have the lipids maintain their lateral organization. While the quick-freeze DSC results that were obtained for the DMPC/DPPC system are very intriguing, we believe that the interpretations that were presented are unlikely and that further investigation is warranted.

Can lipid domains exist in the physiologically relevant fluid phase? We believe that the answer is probably *yes*, but that greater differences in lipid structure and/or composition will be required for their formation. The nearest-neighbor technique that we have introduced herein should help to sort out membrane composition–suprastructure relationships in ways that have not previously been possible.

Conclusions

A chemical approach to the study of lateral organization of lipid bilayers has been devised on the basis of the equilibration and analysis of disulfide-based phospholipid dimers. The appearance of nearest-neighbor recognition (defined as the thermodynamic preference for one phospholipid to become a covalently attached nearest neighbor of another) furnishes inferential evidence for the existence of domains, when it is assumed that the packing forces that govern nearest-neighbor recognition are the same (or very similar) as those which govern domain formation. The absence of such recognition provides conclusive evidence for a random array of equilibrating lipid monomers. Application of this method to equilibrating monomers, differing by two methylene groups per alkyl chain, has established that the suprastructure of the resulting membranes in the fluid phase, and in the gel–fluid coexistence region, consists of a random arrangement of monomeric units throughout the bilayer. For equilibrating monomers that differ by four methylene groups per alkyl chain, nearest-neighbor recognition has been observed in the gel–fluid coexistence region, but not in the fluid phase. The inference that domains are present in such bilayers has been confirmed by DSC analysis. The enthalpy and entropy that characterizes this recognition support the view that the packing forces that govern nearest-neighbor recognition can be very similar to those which govern domain formation.

The results reported herein provide the first definitive evidence for membrane suprastructure in the physiologically relevant fluid phase and in the gel–fluid coexistence region. The loss of lipid domains that has been observed, when one goes from the gel–fluid to the fluid phase, is a likely consequence of reduced intermolecular forces. These findings, together with a critical examination of conclusions that have been drawn from previous quick-freeze DSC studies, cast serious doubt on the possibility that DMPC/DPPC membranes exist as domains in the fluid state.

Experimental Section

General Methods. Unless stated otherwise, all chemicals and reagents were obtained commercially and used without further purification. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Pierce), dithiothreitol (DTT, Aldrich, 99+%), methyl 3-(methylthio)propionate (Aldrich), and *N,N'*-dicyclohexylcarbodiimide (Fluka) were used as obtained. 1,2-Dimyristoyl-*sn*-glyero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids, Inc., and used as obtained. 4-(Dimethylamino)pyridine (DMAP, Aldrich) was recrystallized once from toluene prior to use. The chloroform and methanol that were used for thin-layer chromatography, and for the preparation of vesicles, were HPLC grade (Burdick and Jackson). Absolute ethanol (Midwest Solvents) was used as obtained. The hexane (Burdick and Jackson) and ethanol (denatured, Aldrich) that were used for high-pressure liquid chromatography were HPLC grade and were filtered through 0.45-μm nylon filters (Rainin) prior to use. Choline chloride (Aldrich, 99+%) was dissolved in ethanol (3.7 g/L) and filtered through a 0.45-μm nylon filter prior to use in HPLC. House-deionized water that was used for HPLC, TLC, and vesicle formation was purified by use of a Milli-Q system consisting of one carbon, two ion exchange, and one organex-Q stages. Chromatographic separations were carried out by use of precoated Whatman 500- and 1000-μm silica gel TLC plates, Analtech GF 1000-μm silica gel TLC plates, and Aldrich 70–230 mesh, 60-Å silica gel for column chromatography. Detection on thin-layer chromatographic plates was made by using iodine vapor, a UV lamp, molybdenum blue, or Ellman's reagent. All ^{1}H NMR spectra were recorded using a Bruker 500-MHz instrument and $CDCl_3$ as the solvent. Chemical shifts are reported relative to tetramethylsilane. High-resolution mass spectrometry was performed at the Midwest Center for Mass Spectrometry (Lincoln, NE). Phase-transition temperatures were determined using a differential scanning calorimeter (Microcal MC-2 equipped with DA-2 data acquisition and analysis software, Northampton, MA). Dynamic light scattering measurements were carried out by use of a Nicomp 270 sub-micrometer particle size analyzer. Transmission electron microscopy was performed on a Phillips 300 instrument. Polyvinyl formal (0.25% in CH_2Cl_2, w/w) and uranyl acetate used for TEM sample preparations were purchased from Polysciences and used as obtained. High-pressure liquid chromatographic analyses were carried out using a Waters 600E pump and Model 484 UV detector interfaced to a Maxima 820 chromatography workstation for data collection and manipulation. Chromatography that was performed above room temperature was controlled using a Waters temperature control module and column heater. Vesicle dispersions were prepared in 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 7.4) and extruded through 0.4-, 0.2-, and 0.1-μm polycarbonate filters (Nuclepore) in a Lipex extrusion apparatus (Vancouver, British Columbia, Canada). Vortex mixing was carried out by using a Thermolyne mixer. Freeze-drying of vesicle dispersions was carried out by using a Savant Speed Vac Model SVC100 system. Degassing of solutions and vesicle dispersions was carried out by use of a Firestone Valve (Aldrich). Typically, evacuation under reduced pressure (water aspirator, 1 min) followed by argon purge (1 min) was used to degas samples (eight times in succession). In all cases, the test tube or flask containing the sample was fitted with a no-air rubber septum, and the sample was rapidly stirred (Teflon-coated stir bar) throughout the procedure.

***N*-[3-(2-Pyridyldithio)propionyl]-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (1).** 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine (45.9 mg, 0.0723 mmol) and 4-(dimethylamino)pyridine (10.7 mg, 0.0876 mmol) were dissolved in 3.0 mL of $CHCl_3/CH_3OH/H_2O$ (120/1.5/0.2, v/v/v) at 40 °C. The resulting solution was added to *N*-succinimidyl 3-(2-pyridyldithio)propionate (30.8 mg, 0.0986 mmol) in 0.4 mL of $CHCl_3$. The clear mixture was stirred magnetically, using a Teflon-coated stirring bar, for 1 h at 40 °C under an argon atmosphere. The mixture was then stirred for an additional 44 h at 35 °C. The progress of the reaction was monitored by thin-layer chromatography. Evaporation of the solvent under reduced pressure yielded a crude product that was redissolved in a minimal volume of $CHCl_3$ and subjected to preparative TLC (Whatman silica gel, 60 Å, 20 × 20 cm, 500-μm layer on glass), eluting with $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v). The product band (R_f 0.58–0.42) was removed from the silica gel by extraction with 90 mL of eluent, concentrated under reduced pressure, and freeze-dried (24 h, 0.10 mmHg). A second purification via preparative TLC (Analtech silica gel GF, 20 × 20 cm, 1000-μm layer on glass) afforded a product band (R_f 0.78–0.56) that was removed from the silica gel as described above to yield 52.2 mg (87%) of the protected

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(24) Small, D. M. *The Physical Chemistry of Lipids*; Plenum: New York, 1986; p 531.

thiol lipid (1): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.86 (t, 6 H, CH_3), 1.07–1.40 (br m, 40 H, CH_2), 1.53 (br m, 4 H, $CH_2CH_2C{=}O$), 2.25 (virtual q, 4 H, $CH_2C{=}O$), 2.64 (t, 2 H, $C{=}OCH_2CH_2S$), 3.02 (t, 2 H, $C{=}OCH_2CH_2S$), 3.44 (br m, 2 H, CH_2N), 3.87 (br m, 4 H, CH_2OPOCH_2), 4.12 (d of d, 1 H, CH_2CH), 4.37 (d, 1 H, CH_2CH), 5.19 (m, 1 H, CH), 7.06 (m, 1 H, SCCH), 7.63 (m, 2 H, NCHCHCH), 7.90 (br s, 1 H, NH), 8.40 (m, 1 H, NCH); HRMS for $(C_{41}H_{72}O_9N_2PS_2Na_2)^+$ calcd 877.421 29, found 877.4176.

N,N'-(Dithiodipropionyl)bis[1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine] (I). Into a 25-mL round-bottomed flask was placed an ethanolic solution of dithiothreitol [87.2 mg (0.565 mmol) in 1.5 mL of absolute ethanol] that was previously degassed under an argon atmosphere. To this solution was slowly added (ca. 1 h) 3.0 mL of chloroform containing *N*-[3-(2-pyridyldithio)propionyl]-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (15.7 mg, 0.0188 mmol). The mixture was then heated to 45 °C for 3 h under an argon atmosphere. After thin-layer chromatography indicated a single, phosphorus- and thiol-positive spot (R_f 0.49, $CHCl_3/CH_3OH/H_2O$, 65/24/4, v/v/v), the mixture was concentrated under reduced pressure. The crude material was chromatographed on a silica gel column (3 g, 1.0 cm × 6.5 cm), eluting with $CHCl_3$ followed by $CHCl_3/CH_3OH$ mixtures from 40/1 to 5/1 (v/v). The eluted sample of *N*-(3-mercaptopropionyl)-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (4) was then concentrated under reduced pressure, redissolved in 2 mL of $CHCl_3$, and added to *N*-[3-(2-pyridyldithio)propionyl]-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (17.2 mg, 0.0207 mmol) in 3.3 mL of $CHCl_3$. The reaction mixture was stirred for 16 h under an argon atmosphere. Subsequent analysis by TLC indicated the complete disappearance of thiol lipid (R_f 0.49, $CHCl_3/CH_3OH/H_2O$, 65/25/4, v/v/v). The mixture was then concentrated under reduced pressure, and the resulting lipid dimer was purified by preparative TLC (Whatman silica gel, 60 Å, 20 × 20 cm, 500-μm layer on glass), eluting with $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v). The product band (R_f 0.39–0.29) was removed from the silica gel by extraction with 90 mL of eluent, concentrated under reduced pressure, and freeze-dried (24 h, 0.10 mmHg) to yield 24.8 mg (91%) of I: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.87 (t, 12 H, CH_3), 1.08–1.40 (br m, 80 H, CH_2), 1.56 (br m, 8 H, $CH_2CH_2C{=}O$), 2.28 (virtual q, 8 H, $CH_2C{=}O$), 2.62 (br t, 4 H, $C{=}OCH_2CH_2S$), 2.98 (br t, 4 H, $C{=}OCH_2CH_2S$), 3.43 (s, 4 H, CH_2N), 3.86 (br m, 8 H, CH_2OPOCH_2), 4.13 (d of d, 2 H, CH_2CH), 4.40 (d, 2 H, CH_2CH), 5.18 (m, 2 H, CH), 7.73 (br s, 2 H, NH); HRMS for $(C_{72}H_{136}O_{18}N_2P_2S_2Na_3)^+$ calcd 1511.8398, found 1511.8362.

***N*-[3-(2-Pyridyldithio)propionyl]-1,2-distearoyl-*sn*-glycerol-3-phosphoethanolamine (3).** Procedures used for the preparation of 3 were similar to those used to synthesize 1. Starting from 86.1 mg (0.115 mmol) of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine was obtained 97.7 mg (90%) of protected thiol lipid 3: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.86 (t, 6 H, CH_3), 1.07–1.40 (br m, 56 H, CH_2), 1.53 (br m, 4 H, $CH_2CH_2C{=}O$), 2.25 (virtual q, 4 H, $CH_2C{=}O$), 2.65 (t, 2 H, $C{=}OCH_2CH_2S$), 3.04 (t, 2 H, $C{=}OCH_2CH_2S$), 3.46 (s, 2 H, CH_2N), 3.93 (br s, 4 H, CH_2OPOCH_2), 4.12 (d of d, 1 H, CH_2CH), 4.35 (d, 1 H, CH_2CH), 5.21 (s, 1 H, CH), 7.06 (m, 1 H, SCCH), 7.63 (m, 2 H, NCHCHCH), 7.82 (br s, 1 H, NH), 8.40 (m, 1 H, NCH); HRMS for $(C_{49}H_{88}O_9N_2PS_2Na_2)^+$ calcd 989.5464, found 989.5460.

N,N'-(Dithiodipropionyl)bis[1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine] (II). Procedures used for the preparation of II were similar to those described for the synthesis of I. The thiol lipid 6 was first prepared by DTT reduction of 19.5 mg (0.0207 mmol) of *N*-[3-(2-pyridyldithio)propionyl]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine and then reacted with *N*-[3-(2-pyridyldithio)propionyl]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (21.4 mg, 0.0227 mmol) to afford 30.8 mg (90%) of II: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.87 (t, 12 H, CH_3), 1.08–1.45 (br m, 112 H, CH_2), 1.56 (br m, 8 H, $CH_2CH_2C{=}O$), 2.28 (virtual q, 8 H, $CH_2C{=}O$), 2.62 (br t, 4 H, $C{=}OCH_2CH_2S$), 2.99 (br t, 4 H, $C{=}OCH_2CH_2S$), 3.47 (s, 4 H, CH_2N), 3.87 (br m, 8 H, CH_2OPOCH_2), 4.13 (d of d, 2 H, CH_2CH), 4.39 (d, 2 H, CH_2CH), 5.18 (m, 2 H, CH), 7.78 (br s, 2 H, NH); HRMS for $(C_{88}H_{168}O_{18}N_2P_2S_2Na_3)^+$ calcd 1736.0902, found 1736.0886.

1,2-Dimyristoyl-1',2'-distearoyl-*N*,*N*'-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (III). Reduction of *N*-[3-(2-pyridyldithio)propionyl]-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (1) (10.4 mg, 0.0125 mmol) by DTT, using procedures similar to those described above, followed by reaction with *N*-[3-(2-pyridyldithio)-propionyl]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (3) (13.7 mg, 0.0145 mmol) for 15 h in 2.1 mL of $CHCl_3$ afforded the desired lipid dimer III (16.6 mg, 85%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.87 (t, 12 H, CH_3), 1.07–1.43 (br m, 96 H, CH_2), 1.56 (br m, 8 H, $CH_2CH_2C{=}O$), 2.28 (virtual q, 8 H, $CH_2C{=}O$), 2.62 (br t, 4 H, $C{=}OCH_2CH_2S$), 2.99 (br t, 4 H, $C{=}OCH_2CH_2S$), 3.43 (s, 4 H, CH_2N), 3.87 (br m, 8 H, CH_2OPOCH_2), 4.13 (d of d, 2 H, CH_2CH), 4.39 (d, 2 H, CH_2CH), 5.19 (m, 2 H, CH), 7.75 (br s, 2 H, NH); HRMS for $(C_{80}H_{152}O_{18}N_2P_2S_2Na_3)^+$ calcd 1623.9650, found 1623.9686.

***N*-[3-(2-Pyridyldithio)propionyl]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (2).** Using procedures similar to those used for the preparation of *N*-[3-(2-pyridyldithio)propionyl]-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, 61.1 mg (0.0884 mmol) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine was converted to 57.1 mg (73%) of the protected thiol lipid 2: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.86 (t, 6 H, CH_3), 1.05–1.42 (br m, 48 H, CH_2), 1.53 (br m, 4 H, $CH_2CH_2C{=}O$), 2.25 (virtual q, 4 H, $CH_2C{=}O$), 2.66 (br t, 2 H, $C{=}OCH_2CH_2S$), 3.06 (br t, 2 H, $C{=}OCH_2CH_2S$), 3.50 (s, 2 H, CH_2N), 3.95 (br s, 4 H, CH_2OPOCH_2), 4.13 (m, 1 H, CH_2CH), 4.36 (d, 1 H, CH_2CH), 5.22 (s, 1 H, CH), 7.05 (m, 1 H, SCCH), 7.62 (m, 2 H, NCHCHCH), 7.81 (br s, 1 H, NH), 8.41 (m, 1 H, NCH); HRMS for $(C_{45}H_{80}O_9N_2PS_2Na_2)^+$ calcd 933.4838, found 933.4828.

N,N'-(Dithiodipropionyl)bis[1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine] (IV). A 15.3-mg (0.0172 mmol) sample of *N*-[3-(2-pyridyldithio)propionyl]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (2) was reduced with DTT, using procedures similar to those described above, to give the thiol lipid *N*-(3-mercaptopropionyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (5), which was then reacted with *N*-[3-(2-pyridyldithio)propionyl]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (16.9 mg, 0.0190 mmol) under argon for 17 h. Purification of the lipid dimer IV, by preparative TLC, yielded 22.8 mg (85%) of product: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.87 (t, 12 H, CH_3), 1.08–1.42 (br m, 96 H, CH_2), 1.56 (br m, 8 H, $CH_2CH_2C{=}O$), 2.28 (virtual q, 8 H, $CH_2C{=}O$), 2.62 (br t, 4 H, $C{=}OCH_2CH_2S$), 2.98 (br t, 4 H, $C{=}OCH_2CH_2S$), 3.43 (s, 4 H, CH_2N), 3.86 (br m, 8 H, CH_2OPOCH_2), 4.13 (d of d, 2 H, CH_2CH), 4.39 (d, 2 H, CH_2CH), 5.18 (s 2 H, CH), 7.74 (br s, 2 H, NH); HRMS for $(C_{80}H_{152}O_{18}N_2P_2S_2Na_3)^+$ calcd 1623.9650, found 1623.9644.

1,2-Dimyristoyl-1',2'-dipalmitoyl-*N*,*N*'-(dithiodipropionyl)bis[*sn*-glycero-3-phosphoethanolamine] (V). A sample 14.9 mg (0.0168 mmol) of *N*-[3-(2-pyridyldithio)propionyl]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (2) was reduced to its thiol analog, *N*-(3-mercaptopropionyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (5), and reacted with *N*-[3-(2-pyridyldithio)propionyl]-1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (1) (16.7 mg, 0.0201 mmol) to give 21.8 mg (87%) of V: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.86 (t, 12 H, CH_3), 1.08–1.45 (br m, 88 H, CH_2), 1.56 (br m, 8 H, $CH_2CH_2C{=}O$), 2.28 (virtual q, 8 H, $CH_2C{=}O$), 2.61 (br t, 4 H, $C{=}OCH_2CH_2S$), 2.98 (br t, 4 H, $C{=}OCH_2CH_2S$), 3.43 (s, 4 H, CH_2N), 3.86 (br m, 8 H, CH_2OPOCH_2), 4.13 (m, 2 H, CH_2CH), 4.38 (d, 2 H, CH_2CH), 5.18 (s, 2 H, CH), 7.73 (br s, 2 H, NH); HRMS for $(C_{76}H_{144}O_{18}N_2P_2S_2Na_3)^+$ calcd 1567.9024, found 1567.8990.

***N*-[3-(Methylthio)propionyl]-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (VI).** 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (51.2 mg, 0.0741 mmol) and 4-(dimethylamino)pyridine (12.2 mg, 0.0889 mmol) were dissolved in 3.0 mL of $CHCl_3/CH_3OH/H_2O$ (120/1.5/0.2, v/v/v) at 45 °C. To this solution was added a mixture of 3-(methylthio)propionic acid (55.9 mg, 0.465 mmol) (prepared by saponification of the corresponding methyl ester) and *N*,*N*'-dicyclohexylcarbodiimide (566 mg, 2.74 mmol) in 1.5 mL of $CHCl_3$. The mixture was stirred magnetically, using a Teflon-coated stirring bar, for 17 h at 45 °C under an argon atmosphere. The progress of the reaction was monitored by TLC. Evaporation of the solvent under reduced pressure yielded a crude product that was redissolved in a minimal volume of $CHCl_3$ and subjected to preparative TLC (Whatman silica gel, 60 Å, 20 × 20 cm, 1000-μm layer on glass) eluting with $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v). The product band (R_f 0.33–0.44) was removed from the silica gel by extraction with 40 mL of the eluent, concentrated under reduced pressure, and freeze-dried at 100 mTorr for 24 h to yield 10.0 mg (17%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.87 (t, 6 H, CH_3), 1.10–1.40 (br m, 48 H, CH_2), 1.57 (br m, 4 H, $CH_2CH_2C{=}O$), 2.10 (s, 3 H, SCH_3), 2.29 (virtual q, 4 H, $CH_2C{=}O$), 2.51 (t, 2 H, $C{=}OCH_2CH_2S$), 2.76 (t, 2 H, $C{=}OCH_2CH_2$), 3.49 (s, 2 H, CH_2N), 3.98 (br m, 4 H, CH_2OPOCH_2), 4.14 (d of d, 1 H, CH_2CH), 4.36 (d, 1 H, CH_2CH), 5.22 (s, 1 H, CH), 7.11 (br s, 1 H, NH); HRMS for $(C_{41}H_{79}O_9NPSNa_2)^+$ calcd 838.5009, found 838.5009.

Preparation of Liposomes by Extrusion. Typically, 0.5 mL of chloroform containing 1 mg of lipid was injected into a small test tube (Pyrex, 13 × 100 mm), and the solvent was removed under a stream of nitrogen. The tube containing the thin film of lipid was fitted with a no-air rubber septum and repeatedly degassed by evacuating the test tube under reduced pressure (50 mmHg, 1 min) and replenishing the test tube with nitrogen (four times in succession). After this procedure, the test tube was placed under reduced pressure for 12 h at 0.4 mmHg (23 °C). After addition of 2 mL of borate buffer (10 mM borate, 140 mM NaCl, 2 mM NaN_3, pH 7.4), the test tube was immersed in a water bath for 30 min at 60 °C. The lipid was dispersed in the buffer solution with the aid of

intermittent vortex mixing (23 °C, 2 min), resulting in a translucent dispersion. In order to prepare a more homogeneous liposomal dispersion, the solution was frozen in liquid nitrogen, thawed in the heated water bath (60 °C), and finally vortex-mixed for 5 min at room temperature. This procedure was repeated four times. The resulting lipid dispersion was sequentially extruded through two stacked polycarbonate filters of 0.4, 0.2, and 0.1 μm (four extrusions each). The Lipex Biomembrane chamber that was used was maintained at a temperature at least 5 deg above the gel to liquid–crystalline phase-transition temperature of the lipid (or the higher melting lipid of a mixture) via an external water bath.

Sample Preparation for Transmission Electron Microscopy. Copper grids (200 mesh, 3.05 mm diameter) were coated with a thin film of formvar to provide mechanical support for the vesicle dispersion. This was accomplished by first filling a large crystallizing dish (19 × 10 cm) with Milli-Q water and then sweeping the surface with a Kimwipe to remove any traces of dust. To this water surface was applied one drop of the formvar solution (0.25% polyvinyl formal in CH_2Cl_2), resulting in a thin transparent film. Copper grids (10–20) were gently placed onto the surface film, which was then transferred to a clean microscope slide (2.5 × 7.5 cm). The transfer was performed by lowering the slide over the grids and rapidly sweeping the slide through the water. The formvar-coated grids were then dried overnight in a desiccator. A thin film of carbon (10–20 μm) was then deposited onto the formvar surface of the copper grids using a Denton vacuum DV502 evaporator. Immediately after carbon evaporation, the grids were exposed to an ion stream from a glow discharge apparatus. This treatment rendered the carbon surface hydrophilic, which reduced clustering of the vesicle dispersion. The vesicle dispersion was applied to the copper grid immediately after the glow discharge procedure. Typically, a carbon-coated copper grid was removed from the surface of the microscope slide and secured with forceps (carbon side up). One drop of the vesicle dispersion (0.6 mg/mL) was placed on the grid. After 30 s, the excess liquid was absorbed by touching the edge of the copper grid with filter paper. In order to stain the vesicles, one drop of an aqueous solution of uranyl acetate (2%, w/w) was applied to the grid, and the excess was removed after 30 s with filter paper. The grid was placed in a dust-free grid holder for 3–4 h prior to examination by transmission electron microscopy. Micrographs were recorded at magnifications between 20 000× and 80 000× with accelerating voltages of 80 and 100 kV.

Thiolate–Disulfide Interchange Reaction. In a standard experiment, large unilamellar vesicles (1000 Å diameter) of **III** were prepared via extrusion. A separate dispersion was prepared from a 1/1 molar ratio of **I** and **II**. In the latter case, appropriate aliquots of a stock solution of each lipid (2 mg/mL in chloroform) were first combined in the test tube, followed by solvent evaporation. Each of these dispersions was prepared, typically, from 0.70 μmol of lipid in 2.2 mL of borate buffer (pH 7.4) and was treated identically throughout the entire experiment. Immediately after extrusion, a 0.3-mL aliquot (0.095 μmol of lipid) was removed from each test tube and freeze-dried for use as a control. The remaining portion of each of the two dispersions was then repeatedly degassed via argon using a Firestone valve (8 repetitions), and the pH was then raised to 8.5 by adding 20 μL of a 0.15 M NaOH solution. These dispersions were then divided into 0.3-mL samples and placed into separate test tubes (Pyrex, 13 × 100 mm) that were previously purged with argon. The test tubes, which were equipped with Teflon-coated magnetic stirring bars, were then placed in an oil bath maintained at the desired temperature, typically 33 ± 1, 53 ± 1, or 60 ± 1 °C. After 30 min of thermal equilibration, 20 μL of an aqueous solution of 4.8 mM dithiothreitol (1 equiv relative to moles of lipid) was injected into each tube followed by brief vortex mixing. The thiolate–disulfide interchange reaction was allowed to proceed for times ranging between 10 and 1800 min. Reactions were quenched at a given temperature by lowering the pH to 5.0 via direct injection of 55 μL of a 0.01 M HCl solution. Each aliquot was then vortex-mixed followed by immediate freeze-drying via the use of a Savant Speed Vac.

Analysis of Dimer Distributions by High-Pressure Liquid Chromatography. Phospholipids were analyzed by high-pressure liquid chromatography using a 4.6 × 250 mm Altex Ultrasphere C18 reversed-phase column (5-μm particle size, Beckman Instruments) with 20 mM choline chloride in ethanol/water/hexane (78/13/9, v/v/v). The flow rate was 0.8 mL/min and detection was made by UV at 205 nm.

Freeze-dried vesicle samples were first prepared for HPLC analysis by dissolving the lipid portion in 2 mL of chloroform and vortex mixing for 1 min, followed by centrifugation for 5 min (clinical centrifuge). The chloroform solution was carefully removed from the undissolved buffer components and filtered through glass wool. Chloroform was subsequently removed via freeze-drying (Savant Speed Vac), and the lipid was then redissolved in 50 μL of eluent and injected onto the HPLC column. The retention times for lipids **I**–**V** were 11, 71, 26, 28, and 17 min, respectively. Chromatography was performed at room temperature for the **I**/**V**/**IV** system and at 31.2 °C for the **I**/**III**/**II** system. Based on the quantity of lipid that was used to prepare the initial multilamellar dispersions, the mass balance of lipids that was recovered and analyzed by HPLC was typically ca. 90%.

Differential Scanning Calorimetry (DSC). Multilamellar vesicles were prepared by dispersing a thin lipid film (1 mg) in 1.6 mL of 10 mM borate buffer (140 mM NaCl and 2 mM NaN_3, pH 7.4). Their melting behavior was measured using that same buffer solution as a reference. Heating scans were recorded between 10 and 65 °C at a scan rate of 30 deg/h. Three DSC runs were performed for each sample; no difference was observed among the scans. A borate buffer baseline was also collected and subtracted from each thermogram. The calorimetric data were analyzed to yield phospholipid excess heat capacities as a function of temperature, and the transition enthalpies were calculated by employing software supplied by Microcal. Thermograms were normalized from mcal/min to mcal/deg by dividing by the scan rate. The heat capacity in the units of kcal/deg·mol was determined by dividing by the number of moles of lipid present in the vesicle dispersion. Analysis of DPPC, using this protocol, gave thermodynamic data which were in agreement with those that were previously reported (see Table I).