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Support for the Shape Concept of Lipid Structure Based on a Headgroup Volume Approach

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ABSTRACT Headgroup volumes of seven dioleoyl lipid species, calculated from covalent radii, are shown to correlate linearly ($r = 0.95$) with the ability of those lipids to alter the midpoint temperature of the lamellar to inverted hexagonal phase transition ($L_\alpha \rightarrow H_{II}$) of a 95 mole fraction percent phosphatidylethanolamine matrix. The results illustrate the utility of the shape concept and basic considerations of headgroup volume as a predictive tool for the determination of lipid structure.

INTRODUCTION

The molecular shape of lipids is generally acknowledged to be an important factor in determining the structure of lipid aggregates (Israelachvili et al., 1976, 1980). Shape-dependent curvature stresses may alter function in biological membranes (Gruner and Shyamsunder, 1991). In its simplest form, the shape theory relies on a simple index, the *critical packing parameter*, which is based on the ratio of acyl and headgroup areas (Israelachvili et al., 1976, 1980).

$$v_h l_c^{-1}/a_o \tag{1}$$

The numerator represents the average hydrocarbon area (hydrocarbon volume, v_h, divided by the critical, or extended, fluid chain-length, l_c). The denominator, a_o, represents the optimal surface area per molecule at the hydrocarbon/water interface, where "optimal" denotes that the area corresponds to the local free energy minimum. Lipids characterized by a critical packing parameter less than 0.5 (e.g., lysophospholipids, detergents) are cone-shaped and tend to form micelles. Packing parameters between 0.5 and 1.0 (e.g., PtdCho) correspond to cylindrical shapes that tend to form bilayers. Packing parameters greater than 1.0 (e.g., unsaturated PtdEtn) reflect an inverted cone shape and tend to form inverted structures. Increases in temperature induce greater acyl chain motion and more facile *trans-gauche* isomerization, which, in turn, leads to a reduction in the critical chain-length, and an increase in the critical packing parameter that may be sufficient to induce an interchange between lipid structures.

Gruner and co-workers (for a review see Gruner (1992)) have commented that the observed surface area of lipids in bilayers may not coincide with the area of minimum free energy of an idealized monolayer, since the forces that promote curvature in a leaflet are frustrated by opposing forces in the other leaflet of the bilayer. These investigators, consequently, have chosen to focus on the elastic curvature expressed in the inverted hexagonal (H_{II}) state as a more reliable measure of packing forces, since deviations due to frustration in the H_{II} state originate from hydrocarbon packing constraints and are believed smaller. Nonetheless, the shape formalism was acknowledged to retain considerable heuristic value (Gruner, 1992).

Qualitative applications of the shape concept to the fluid bilayer to inverted hexagonal ($L_\alpha \rightarrow H_{II}$) equilibrium were pioneered by de Kruijff, Cullis, and colleagues (de Kruijff et al., 1985; Cullis et al., 1985). Factors that increase the effective hydrocarbon area, such as increased temperature, unsaturation, or branching, were shown to promote the formation of the H_{II} phase (also see Seddon et al. (1983), Lewis et al. (1989)). Lipids with a small effective headgroup size also have a tendency to form the H_{II} phase. Quantitative applications, however, remain elusive (LaFleur et al., 1990).

Our interest in quantitative applications of the shape concept to the $L_\alpha \rightarrow H_{II}$ equilibrium was sparked by our observation that the potency of the anionic phospholipid PtdEth to promote the H_{II} state was unexpectedly large (Lee et al., 1993). In this study, we investigate whether this observation can be reconciled using the shape formalism.

MATERIALS AND METHODS

Preparation of liposomes

Lipids, obtained from Avanti Polar Lipids (Alabaster, AL), were assayed for purity (> 99%) by thin-layer chromatography. The 1,2-diolein isomer, PtdEth, and PtdOH were also shown pure at the 99% level by ^{1}H NMR. Phospholipid concentrations were determined according to Bartlett (1959). Diolein concentrations were determined by weight. Lipids were mixed in

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Abbreviations used: PtdCho, 1,2-diacyl-*sn*-glycero-3-phosphatidylcholine; PtdGro, 1,2-diacyl-*sn*-glycero-3-phosphatidylglycerol; PtdSer, 1,2-diacyl-*sn*-glycero-3-phosphatidylserine; PtdEtn, 1,2-diacyl-*sn*-glycero-3-phosphatidylethanolamine; PtdOH, 1,2-diacyl-*sn*-glycero-3-phosphatidic acid; PtdEth, 1,2-diacyl-*sn*-glycero-3-phosphatidylethanol; mf%, mole fraction percent; PO, 1-palmitoyl,2-oleoyl; DO, 1,2-dioleoyl; L_α, fluid bilayer structure; H_{II}, inverted hexagonal structure; I, lipid structure that yields an isotropic P-31 NMR resonance; T_H, midpoint temperature of the $L_\alpha \rightarrow H_{II}$ transition; NMR, nuclear magnetic resonance; CSA, chemical shielding anisotropy; TLC, thin-layer chromatography.

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chloroform, dried to a thin film under a stream of dry nitrogen, and evacuated overnight in a 10-mm NMR tube. Liposomes were formed by hydrating 100 μmol of the phospholipid mixture with 1.5 ml buffer (150 mM NaCl, 10 mM Tris, 0.2 mM EDTA, pH 7.2). The suspension was vortexed for 10 min, allowed to stand for 30–60 min, and vortexed for an additional 10 min at room temperature. A glass rod was used to manually agitate the suspension and liberate the film from the tube walls. The sample was flushed with nitrogen and sealed.

Synthesis of DOPtdEth

DOPtdEth, enzymatically synthesized from DOPtdCho using peanut phospholipase D (Sigma) and purified on a Merck Hibar RT preparative high-pressure liquid chromatograph, was the kind gift of G. Moehren.

^{31}P nuclear magnetic resonance

The ^{31}P NMR spectra were acquired on a Bruker AM-360 spectrometer operating at 145.8 MHz (8.5 Tesla) on nonspinning samples with a 10-mm double resonance probe. Bloch decays were obtained with a 90° pulse, scalar multipulse proton decoupling during data acquisition, and 6-s interpulse spacings. Approximately 500 transients were averaged using a 25-kHz spectral window and 4K data points. An exponential filter of 50–100 Hz was applied to the decay prior to transformation. The temperature controller was calibrated with ethylene glycol and methanol. Samples were allowed to equilibrate at each temperature for 20 min prior to acquisition. All temperature titrations were heating scans to minimize hysteresis.

The midpoint temperature, T_H, of the $L_\alpha \rightarrow H_{II}$ equilibrium was determined in the following manner. The overlapping resonance due to L_α and H_{II} (and any isotropic signal, I) were deconvoluted by subtracting a pure H_{II} spectrum, leaving only the L_α (and I). When present, isotropic components were deconvoluted by subtracting a pure isotropic spectrum (halothane-induced isotropic POPtdEtn defined as 0 ppm.). All areas were integrated in triplicate. The T_H reported here is the temperature at which equal amounts of L_α and H_{II} are observed. The midpoint of the bilayer → nonbilayer (H_{II} + I) transition was 0–0.2°C less than T_H, excepting a 0.9°C decrease for the DOPtdCho/POPtdEtn system. The linearity in the response of T_H was validated by titrations of the dilute lipid in the POPtdEtn matrix (5, 10 mole fraction percent (mf%) of DOPtdEtn; 1, 5, 10 mf% DOPtdEth; 1, 5 mf% of diolein).

RESULTS AND DISCUSSION

In order to relate headgroup chemistry to membrane structure within the context of the shape concept, we have chosen to investigate a binary lipid mixture consisting of a 95 mf% POPtdEtn matrix into which dilute levels (5 mf%) of other lipids are introduced. POPtdEtn undergoes a thermotropic transition from a bilayer structure to an inverted hexagonal structure. According to the shape theory, the transition stems from a temperature-dependent increase of the acyl chain term ($\nu_h\ l_c^{-1}$) such that the packing parameter exceeds a critical value at the transition temperature. Our paradigm is to use this transition temperature as a measure of the packing parameter imparted upon the matrix by dilute lipids of varied headgroup composition, each possessing a constant dioleoylglycerol hydrocarbon core (the temperature dependence of ν_h l_c^{-1} remains unchanged). Changes in the transition temperature are monitored by wideline ^{31}P NMR and correlated with the headgroup size. According to the shape theory, the introduction of dilute lipids with a large headgroup should increase the average optimal surface area, a_o, and decrease the packing parameter. To restore the packing parameter to its critical value at the transition midpoint, it is necessary for the transition temperature (i.e., $\nu_h\ l_c^{-1}$) to increase. Conversely, the introduction of dilute lipids with a small headgroup should decrease a_o, increase the packing parameter, and decrease the midpoint temperature.

The determination of the headgroup dependence of optimal surface area is problematic. The experimental data for expressed surface area is largely incomplete. When available, the relationship between the experimental, expressed surface area and the idealized, optimal surface area (a_o) is subject to the uncertainties associated with frustration of expression in both the bilayer and inverted hexagonal structures as noted by Gruner (1992). Furthermore, pure anionic lipids express surface areas that are charge-dependent. Rather than try to account for the degree of frustration and charge repulsion in an incomplete data set, we have chosen a more primitive approach that focuses on the chemical composition of the headgroup. The virtue of the approach resides in its simplicity, which derives from purposeful neglect of the nontrivial complications in interpreting optimal areas from the experimental data.

Headgroup volumes were calculated from the headgroup composition and Pauling's covalent radii (Pauling, 1960). The headgroup is considered to be the part of the lipid molecule attached to the glycerol moiety ($ROCH_2$-(RO)CH-CH_2-X). Thus, the headgroup of diolein is -OH, whereas the phosphocholine headgroup is $-OPO_3CH_2CH_2N(CH_3)_3$. Headgroup volumes, ν_a, are tabulated by summing the constituent covalent volumes.

$$\nu_a = (4\pi/3) \sum (N_i r_i^3) \tag{2}$$

N_i is the prevalence of the ith atom in the headgroup (i.e., five carbons for phosphocholine), and r_i is the covalent radius (77.2 pm for carbon). Volumes calculated in this manner will slightly underestimate the true volumes, but nonetheless provide a reasonable means of headgroup discrimination.

Presented in Fig. 1 are selected ^{31}P NMR spectra which show the perturbations of the POPtdEtn matrix that are induced by the presence of various dilute lipids. In the transition from the L_α to the H_{II} state, the phosphorus nucleus undergoes an additional axis of rapid motion that changes the sign and reduces the breadth of the chemical shielding anisotropy (CSA) by a factor of 2 (de Kruijff et al., 1985). The transition temperature of the pure POPtdEtn matrix (340 K) is similar to previous results (Epand, 1985a). Lipids with small headgroups decrease the transition temperature of the matrix, as shown for diolein in Fig. 1 *A* in agreement with a previous survey (Epand, 1985b). Lipids with large headgroups increase the transition temperature as shown for PtdSer in Fig. 1 *C*.

The small isotropic signal observed at 0 ppm in Fig. 1, represented 1.2% or less of the total intensity at the transition midpoint, with the exception of PtdCho where the isotropic signal represented 20% of the total signal. The signal was independent of changes in solvent viscosity (50 wt% sucrose) which broaden the resonance from sonicated lecithin vesicles, thereby excluding small vesicular structures as the

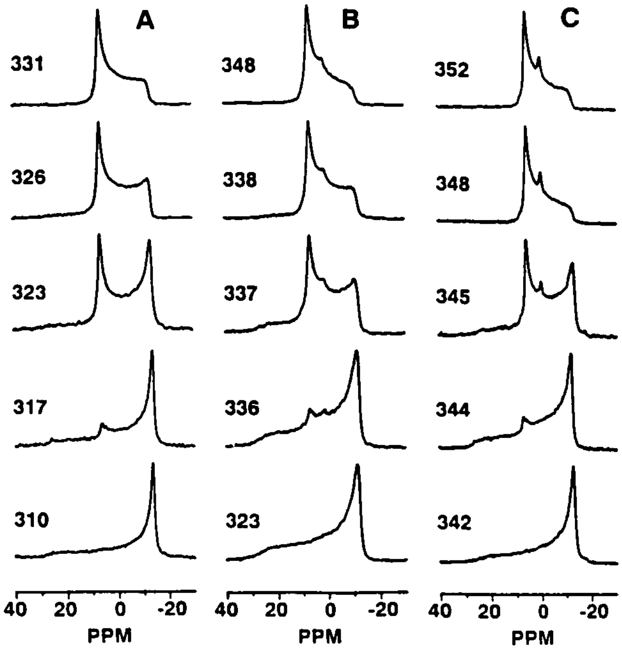

FIGURE 1 ^{31}P NMR spectra of 5 mf% (*A*) diolein, (*B*) DOPtdOH, (*C*) DOPtdSer, in a 95 mf% POPtdEtn matrix are shown at selected temperatures.

origin of the isotropic signal. Cubic phases commonly occur during the $L_\alpha \rightarrow H_{II}$ transition to yield isotropic ^{31}P lineshapes. The cubic structure may represent a thermodynamically stable, but kinetically hindered, state that minimizes the frustration associated with both the bilayer and the inverted hexagonal structures (Shyamsunder et al., 1988; Gruner, 1992). Other nonbilayer structures, such as lipidic particles, are also possible.

The correlation between headgroup volume and change in the $L_\alpha \rightarrow H_{II}$ transition temperature of the POPtdEtn matrix is shown in Fig. 2. The correlation, fit to a linear function on purely empirical grounds, yields an excellent correlation coefficient of 0.95. Inherent in our application of headgroup volumes is the assumption that the headgroup length and surface area, a_o, are proportional to the volume calculated from covalent radii. No corrections are made for charge, conformation, or the extent of headgroup hydration. These factors are all expected to play a role in the effective headgroup volume and area, but these factors are apparently secondary to absolute size considerations for a series of homologous amphiphiles.

Anionic phospholipids are widely believed to stabilize the lamellar state of a membrane matrix due to a charge-induced increase in headgroup size (de Kruijff et al., 1985; Cullis et al., 1985; Gruner, 1992). Surprisingly, dilute levels of the anionic lipids shown in Fig. 2 (*filled symbols*) show no evidence for this effect. Anionic PtdSer and PtdGro correlate well with zwitterionic PtdCho and PtdEtn. The largest discrepancies from the trend are observed for the anionic lipids PtdOH and PtdEth; however, the deviations are in opposite senses and not apparently charge related. Most of the previous observations, however, were based on systems containing nondilute concentrations of the anionic phospholipids, where charge repulsion between the anions may become significant.

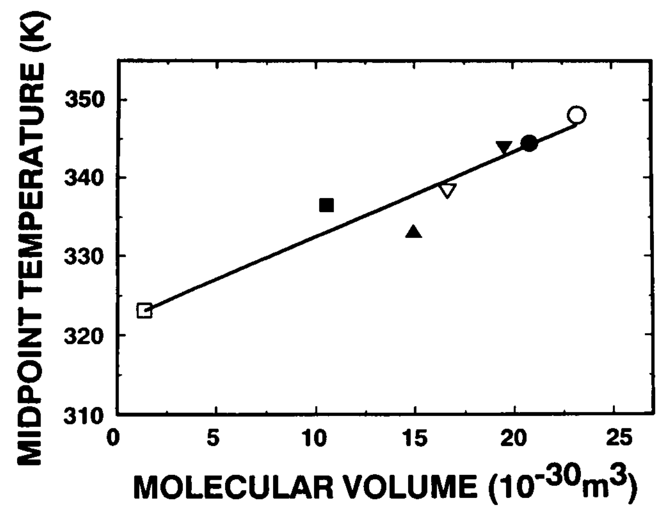

FIGURE 2 Headgroup volume, calculated as described in the text, is correlated with the T_H observed for the binary lipid mixture consisting of a 95 mf% POPtdEtn matrix and 5 mf% DOPtdCho (○), DOPtdSer (●), DOPtdGro (▼), DOPtdEtn (▽), DOPtdEth (▲), DOPtdOH (■), and diolein (□).

The largest deviations are observed for PtdOH and PtdEth. Given the simplicity of the empirical approach, *a posteriori* rationalization of these deviations risks overinterpretation; however, a few comments are appropriate. PtdOH stabilizes the L_α state more than anticipated, while PtdEth stabilizes the H_{II} state more than anticipated. As mentioned, the deviations are in an opposite sense and not likely to be due to the common negative charge. PtdOH is the only lipid with a phosphate moiety that can act as a hydrogen bond donor and acceptor, while PtdEth is the only lipid studied with a hydrophobic headgroup. The main phase transition temperatures of the dipalmitoyl derivatives are dramatically different; PtdOH is the highest of the dipalmitoyl derivatives, while PtdEth is among the lowest (reviewed in Boggs (1987)). Furthermore, the PtdEth alkyl headgroup is believed to be quite labile, oriented parallel to the bilayer surface (or perhaps penetrating the hydrocarbon region for longer alkyl derivatives, see Boggs (1987)) when steric constraints are low, and perpendicular when constraints are high (Browning, 1981).

Based on a comparison of PtdEth and PtdOH using traditional qualitative shape reasoning, we tentatively suggested that the observation that PtdEth is more potent than PtdOH in promoting the formation of highly curved, nonbilayer, H_{II} structures, would indicate that the hydrophobic ethyl headgroup of PtdEth assumes a very unusual conformation that dramatically reduces the effective headgroup size (Lee et al., 1993). The headgroup volume correlation shown in Fig. 2, however, suggests that a qualitative comparison with PtdOH may artefactually accentuate the degree of anomaly that might be attributed to PtdEth.

This study is designed to describe the shape of the isolated amphiphile in a lipid matrix by monitoring changes imparted upon the properties of the matrix. In more complex membranes, higher concentrations of anionic lipids may lead to a charge repulsion contribution to the headgroup volume. In biomembranes, the presence of cholesterol, proteins, variations in lipid composition, nondilute levels of anionic lipids, and the presence of divalent cations may induce nonrandom lateral distribution of lipid components and may lead to heterogeneities in shape and curvature stress absent in the simple model system.

This is the first quantitative application of the shape concept to the headgroup dependence of the $L_\alpha \rightarrow H_{II}$ equilibrium. The remarkably simplistic basis of the correlation, headgroup volumes determined from covalent radii, strongly supports the idea that molecular shape is a dominant factor determining the structural properties of lipid aggregates.

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