Hydrophobic Alkyl Headgroups Strongly Promote Membrane Curvature and Violate the Headgroup Volume Correlation Due to "Headgroup" Insertion†

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ABSTRACT: The ability of lipid aggregates to form planar bilayers, rather than highly curved micellar or inverted structures, is dependent on the relative geometries of the headgroup and hydrocarbon regions. The headgroup volume approach to lipid structure provided a quantitative link between a lipid's headgroup size and its ability to promote curved, inverted hexagonal (H_{II}) structures in a phosphatidylethanolamine (PtdEtn) matrix [Lee et al. (1993) *Biophys. J. 65*, 1429−1432]. Phosphatidylalkanols (PtdAlks) are shown here to promote curvature with a potency that far exceeds and a chain length dependence contrary to the expectations of the headgroup volume approach, suggestive of an atypical alkyl "headgroup" conformation. A homologous series of 3-substituted triacylglycerols (TAGs), for which 3-acyl "headgroup" insertion is established, exhibits a chain length dependence similar to the PtdAlks, evidence that the deviation is of common origin. The potency of the TAGs to promote curvature is unprecedented, and the onset of saturation, which parallels the dramatic promotion of curvature, occurs at mole fractions as low as 0.0025. The potency of the PtdAlks or TAGs to promote curvature exceeds that of all mammalian phospholipids examined. Thermodynamic analysis implicates the enthalpic curvature stress imparted upon the membrane matrix as the dominant energetic factor. The imparted stress ranges from -930 J mol^{-1} for phosphatidylcholine to $+7.5$ kJ mol^{-1} for 3-palmitoyl TAG. The results affirm the geometric considerations of membrane structure and indicate that alkyl headgroups tend to insert into the bilayer and increase the enthalpic curvature stress within the membrane.

The constraints imposed by the packing of lipids into membranes create energetic stresses that are increasingly seen as important in modulating membrane structure and function [e.g., Cullis et al. (1985), deKruijff et al. (1985), Gruner (1992), and Hui and Sen (1989)] and fusion [e.g., Cheetham et al. (1989)]. In simple organisms, environmental and metabolic challenges are accompanied by a process of membrane remodeling that tends to restore the original packing stresses [e.g., Goldfine et al. (1987), Wieslander et al. (1994), and Rietveld et al. (1994)]. The energetics underlying lipid assembly and packing stresses are incompletely understood and are the subject of considerable inquiry [e.g., Gruner (1985), Kumar (1991), Gawrisch et al. (1992), Lee et al. (1993b), Hui (1993), and Epand and Epand (1994)].

Phosphatidylethanol (PtdEt),[1] a transphosphatidylation product of phospholipase D (PLD), is nearly ubiquitous in tissues exposed to ethanol (Alling et al., 1984; Moehren et al., 1994). This exotic anionic phospholipid exhibits a small hydrophobic ethyl headgroup (-P-O-CH_2-CH_3) and exhibits properties distinct from other mammalian phospholipids. We have found that PtdEt possesses an unusual potency among naturally occurring mammalian phospholipids for its ability to impart a curvature stress upon the bilayer structure (Lee et al., 1993a,b). The dominant PLD substrate, phosphatidylcholine (PtdCho) (Okamura & Yamashita, 1994), strongly stabilizes the bilayer structure (Lee et al., 1993b); consequently, the transphosphatidylation process accentuates the stress created by PtdEt by removing PtdCho. PtdEt undergoes rapid transbilayer movement in PtdCho vesicles, with transfer rates that greatly exceed, by 1−3 orders of magnitude, the transfer rates reported for other naturally occurring phospholipids near physiological pH (Victorov et al., 1994). The fluidity of membranes and the activity of membrane-bound enzymes are sensitive to the presence of PtdEt (Omodeo-Sale, 1991). Eibl (1977) proposed that long-chain alkyl headgroups could penetrate the bilayer, in assessing the main transition temperatures of PtdAlks. Browning (1981) concluded that short-chain alkyl headgroups were extremely labile. Victorov et al. (1994) found that, unlike more polar phospholipids, the 1-ethyl carbon chemical shift of PdtEt is split into inner and outer leaflet signals due to differential packing constraints in sonicated PtdCho vesicles.

Evidence for the presence of triacylglycerols (TAGs) as an integral component of many biological membranes, particularly plasma membranes of stimulated and malignant cells, continues to mount (Mountford & Wright, 1988; Hamilton, 1989; Mackinnon et al., 1992; Lerique et al., 1994). Lipoprotein metabolism is dependent on the presen-

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[1] Abbreviations: PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; PtdEg, phosphatidylethyleneglycol; PtdAlk, phosphatidylalkanol; PtdMe, phosphatidylmethanol; PtdEt, phosphatidylethanol; PtdPr, phosphatidylpropanol; PtdBu, phosphatidylbutanol; PtdPe, phosphatidylpentanol; PtdHx, phosphatidylhexanol; PtdOc, phosphatidyloctanol; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; TAG, triacylglycerol; Lau, lauroyl; Lin, linoleoyl; Myr, myristoyl; Ole, oleoyl; Pam, palmitoyl; PLD, phospholipase D; L_α, fluid bilayer structure; H_{II}, inverted hexagonal structure; I, lipid structure that yields an isotropic ^{31}P NMR resonance; T_H, midpoint temperature of the $L_\alpha \rightarrow H_{II}$ transition; mf%, mole fraction percent; NMR, nuclear magnetic resonance; CSA, chemical shielding anisotropy; TLC, thin-layer chromatography.

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tation of TAGs at the phospholipid surface [e.g., Rojas et al. (1990), and Morton and Steinbrunner (1990)]. In human duodenal contents, small unilamellar vesicles are found to contain TAGs (Hernell et al., 1990). Acylation rates of TAG in human erythrocytes are modified by both acute and chronic ethanol consumption (Lerique et al., 1994).

In describing the behavior of phosphatidylethanol in membranes, this paper touches on three issues of membrane structure and packing. (1) What are the consequences of introducing a hydrophobic alkyl moiety at the polar interface? (2) What are the consequences of triacylglycerol accumulation in membranes? (3) How are these consequences expressed in terms of the packing geometry and thermodynamics?

MATERIALS AND METHODS

Synthesis of Phosphatidylalkanol. A 100 mg amount of OleOlePtdCho (Avanti Polar Lipids) in 5 mL of $CHCl_3$, peanut phospholipase D (20 mg or 5200 units, EC 3.1.4.4; Sigma), 8.0 mL of buffer (0.2 M sodium acetate and 0.1 M calcium chloride at pH 5.6), 5 mL of the appropriate alcohol, and 20 mL of diethyl ether (water washed to remove traces of unlabeled ethanol) were stirred in a sealed flask at ambient temperatures overnight. The reaction is stopped with 5 mL of 0.6 M EDTA and extracted with $CHCl_3$:CH_3OH 2:1. The aqueous layer is decanted, and the organic layer is rewashed with 20 mL of CH_3OH, 2.5 mL of 0.6 M EDTA solution, and 20 mL of water. The organic phase is collected, dried under a rotary evaporator, and dissolved in $CHCl_3$. The lipids (PtdAlk, PtdOH, and PtdCho) were purified on a 250 $\times$ 25 mm Merck Hibar preparative high-pressure liquid chromatography column packed with 7 μM LiChrosorb-Si-60 silica particles (EM Science, Cherry Hill, NJ) using the gradient procedure described previously (Ellingson & Zimmerman, 1987). The purity of the phosphatidylalkanols exceeded 99% using the thin-layer chromatographic (TLC) system described previously (Victorov et al., 1994). Yield decreased with increasing alcohol chain length.

Preparation of Liposomes. 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylethanolamine, PamOlePtdEtn (Avanti Polar Lipids, Alabaster, AL), was assayed for purity (>99%) by high-performance TLC using silica gel 60 plates (Merck) eluted with $CHCl_3$:CH_3OH:H_2O 65:25:4. Monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) were obtained from Matreya (Pleasant Gap, PA; MGDG, 90% 18:0, 10% 16:0, and DGDG, 90% 18:0, 10% 16:0, according to the supplier). The purity was assessed (>99%) by TLC as for PtdEtn. 1,2-Dioleoyl-3-octanoyl-*rac*-glycerol and 1,2-dioleoyl-3-dodecanoyl-*rac*-glycerol (Deva Biotech, Hatboro, PA) and 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (Sigma) were pure (>99%) by TLC on silica gel G plates with a petroleum ether:ethyl ether:acetic acid (90:10:1) elution solvent. Cholesterol (Sigma) was recrystallized twice from ethanol. Lipid concentrations were determined according to Bartlett (1959) or by weight. Liposomes were prepared as described (Lee et al., 1993b).

^{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 Hz was applied to the decay prior to transformation. Quantitation artifacts due to receiver deadtime were not detected and thus were less than the uncertainty in base line definition ($\pm$2%). The temperature controller was calibrated with ethylene glycol and methanol. Samples were equilibrated 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 PamOlePtdEtn). 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 linearity in the response of T_H was validated for the PtdAlks and TAGs by titrations of the dilute lipid, excepting PtdOc, which due to low yield was examined at a single concentration (1 mole fraction percent, mf%), with results that may be less accurate. The PamOlePtdEtn matrix exhibited small lot-dependent differences in transition temperature (340−343 K); consequently, all data have been normalized to 343 K.

Headgroup Volume Approach to Lipid Structure. The integrity of membranes as bilayers is maintained by a balance of intermolecular forces in the acyl and headgroup regions (Israelachvili et al., 1976, 1980). If the balance is upset, the curvature stress imparted upon the membrane may alter membrane function and structure (Cullis et al., 1985; deKruijff et al., 1985; Gruner, 1992; Hui & Sen, 1989). Analytical approaches to the balance of forces in the hydrocarbon and headgroup regions often focus on the molecular geometry expressed by the individual membrane components (the shape theories of lipid polymorphism) (Israelachvili et al., 1980; deKruijff et al., 1985). Cylindrical lipids (e.g., PtdCho) tend to form bilayers. Lipids that possess inverted cone shapes tend to form inverted structures (e.g., unsaturated PtdEtn). Cone-shaped lipids (e.g., lysophospholipids) tend to form micelles.

A highly schematic representation of the role of lipid shape in membrane structure is depicted in Figure 1. Cross sections through the lipid structures are shown with the headgroup region shaded. The cylindrically shaped lipids in Figure 1A pack efficiently to sequester the acyl chain region from water. That is, the mean areas of the headgroup and acyl chain regions, perpendicular to the cross sections, are equal. Introduction of a lipid with a smaller headgroup (an inverted cone-shaped lipid) into the matrix of cylindrically shaped lipids introduces packing defects at the interface that would tend to expose the acyl chain region to water. In order to minimize these interfacial packing defects the membrane exhibits a tendency toward curvature, as depicted in Figure 1B. Packing defects in the acyl chain region and constraints imposed by the maintenance of the vesicular bilayer structure, however, prevent the curvature from being expressed. Thus, the tendency toward curvature is frustrated. The frustrated force is termed a curvature stress, which is imparted upon the bilayer assembly by the presence of the inverted cone-shaped lipid. In a membrane composed of inverted cone-shaped lipids (Figure 1C), the interfacial packing defects in

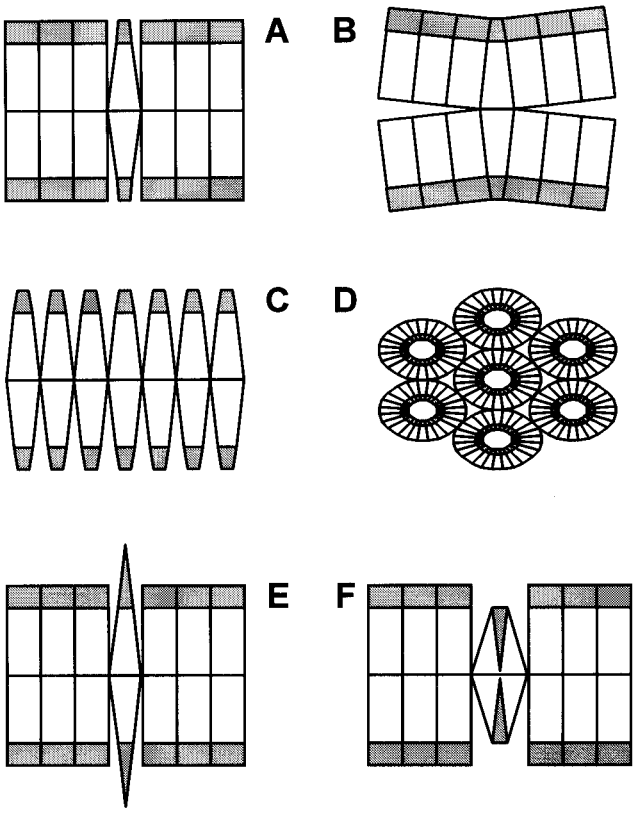

FIGURE 1: Highly schematic representation of the role of shape in modulating the packing and structure of membranes. Cross-sectional areas of lipid structures are shown with shaded and open regions representing the headgroup and chain regions, respectively. (A) A planar bilayer structure, composed mainly of cylindrically shaped lipids (e.g., PtdCho) with an inverted cone-shaped lipid in each leaflet (e.g., PtdEtn), illustrates how mismatches in shape cause packing defects at the membrane−water interface. (B) Illustration of the concept of frustrated curvature stress. Elimination of the interfacial packing defects shown in A favors curvature. Constraints imposed by the vesicular bilayer structure, however, prohibit this curved geometry; thus, the tendency toward curvature in the bilayer is frustrated. (C) A planar bilayer structure composed of inverted cone-shaped lipids exhibits numerous interfacial packing defects that can be accomodated in (D), the highly curved nonbilayer H_{II} structure in which curvature is largely expressed. (E) Headgroups that extend into the aqueous medium exhibit the same degree of curvature stress as would a smaller, more compact, headgroup (cf. A). (F) "Headgroups" that extend into the bilayer hydrocarbon region enhance the curvature stress by increasing interfacial packing defects and expanding the acyl chain region.

the bilayer overwhelm the cohesive forces that maintain the membrane structure at some critical composite geometry and an array of highly curved inverted cylinders (H_{II}) is formed, depicted by the cross-sectional fragment shown in Figure 1D. In the H_{II} structure the curvature is largely expressed, with modest constraints imposed by mismatches in chain packing at the interstices of the cylinders.

In order to inject a quantitative basis to relate the molecular geometry of the individual lipids with the macroscopic geometric tendencies of lipid assemblies, a simple paradigm that we termed the "headgroup volume approach to lipid structure" was developed (Lee et al., 1993b). The basis of the paradigm is to introduce dilute levels of a given lipid into a matrix consisting of PamOlePtdEtn. The matrix undergoes a thermotropic transition from a planar bilayer structure (L_α) to a highly-curved inverted hexagonal structure (H_{II}). Increases in temperature induce more facile *trans*−*gauche* chain isomerization. According to the shape theory, this tends to increase the mean hydrocarbon area and alter the resultant expressed shape such that the cumulative geometry passes through a critical region that distinguishes the modified cylinder and inverted cone shapes, and thereby induces an interchange between lipid structures, such as that depicted in Figure 1C,D. The paradigm uses this transition temperature as a measure of the geometric character imparted upon the matrix by dilute lipids of varied headgroup composition, each possessing a constant dioleoylglycerol hydrocarbon core, and references changes in transition temperature to the headgroup volume obtained from Pauling's (1960) covalent radii. Implicit within the model is that the headgroup volume is proportional to the headgroup area. The introduction of dilute levels of lipids with a small headgroup (inverted cone) should impart its curvature-preferring shape upon the matrix such that the overall critical shape that distinguishes the structures is reached with less hydrocarbon expansion, i.e., at lower temperature. The converse applies to large headgroups.

The ability of the added lipid to modify the matrix transition temperature is termed that lipid's "potency" and is expressed in units of deg per mole fraction percent (mf%), or, alternatively, J mol^{-1}, using the thermodynamic considerations described below.

As described previously (Lee et al., 1993b), the headgroup is considered to be the part of the lipid molecule attached to the glycerol moiety ($ROCH_2$-(RO)CH-CH_2-X). Headgroup volumes, v_a, are tabulated by summing the constituent covalent volumes.

$$v_a = (4\pi/3)\sum(N_i r_i^3) \quad (1)$$

N_i is the prevalence of the ith atom in the headgroup, and r_i is the Pauling (1960) covalent radius. The model neglects all factors affecting headgroup volume external to headgroup atomic composition (e.g., conformation, charge, hydration). Effects due to charge repulsion are minimized in the dilute matrix. Deviations due to conformational effects are anticipated for headgroups which extend well into the aqueous medium, depicted in Figure 1E, in which case the simple proportionality between headgroup volume and mean headgroup area will overestimate the expressed headgroup area. Alternatively, conformational effects which arise from "headgroup" insertion into the acyl chain region, as depicted in Figure 1F, will cause both overestimation of the expressed headgroup area and underestimation of the mean hydrocarbon area.

Ideal Colligative Thermodynamics. Colligative thermodynamic treatments, based on the configurational entropy imparted by solutes to membranes as embodied by ideal solid-solution theory, have been applied to solute action in equilibria among the gel-state → ripple-state → liquid-crystal bilayer state of PtdCho membranes (Janes et al., 1992; Ma et al., 1992; Wang et al., 1993; Janes, 1995). The minor lipid components (solutes) studied here do not exhibit appreciable aqueous solubility, and ideal behavior is described by integration of the Lewis and Randall (1961) differential equation for solid solutions as modified by Mastrangelo and Dornte (1955).

$$T_o - T_m = \frac{RT_oT_mX_2^{tot}[1-K]}{\Delta H_t[K + 0.5(1-K)]} \quad (2)$$

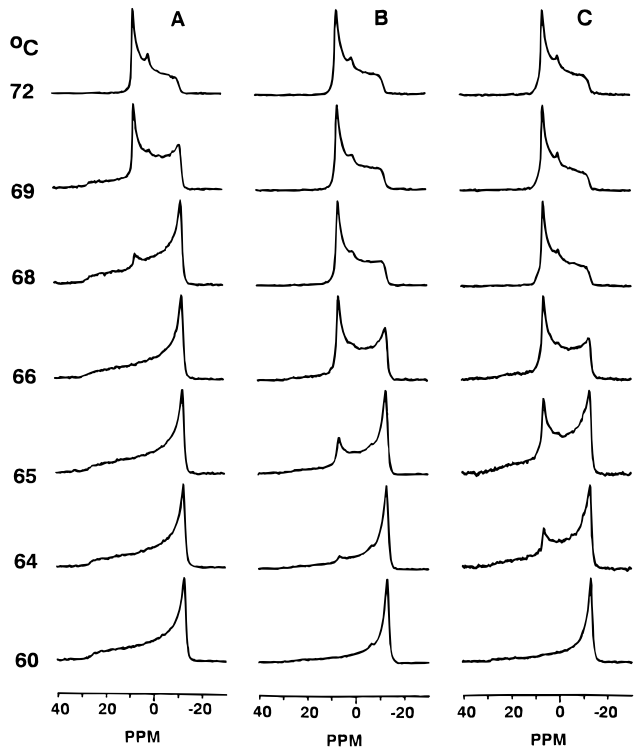

FIGURE 2: ^{31}P NMR spectra of (A) 5 mf% OleOlePtdEtn, (B) 2.5 mf% OleOlePtdPr, and (C) 0.4 mf% OleOleLauTAG incorporated into a PamOlePtdEtn matrix are shown at selected temperatures.

The midpoint temperature is T. The subscripts o and m denote the pure matrix and its mixture, respectively. ΔH_t is the change in transition enthalpy for the PamOlePtdEtn matrix, 1670 ± 125 J mol^{-1} (Epand, 1985). X_2^{tot} is the total mole fraction of solute. K is the partition coefficient of the solute between the states (i.e., $X_2^{L_\alpha}/X_2^{H_{II}}$). The maximum depression obtained uses the freezing point depression approximation ($K = 0$), and corresponds to 11.7 deg per mole fraction percent (mf%).

Thermodynamics of Enthalpic Stress. In cases where the solute exhibits no phase preference, configurational entropy will lower the free energy of each state to the same degree and leave the free energy difference and equilibrium balance unaltered. The enthalpic stress imparted by the solute upon the system can then be calculated directly from changes in the transition temperature, given the usual assumption that changes in heat capacities are small. At the reaction midpoint,

$$\Delta G_o = 0 = \Delta H_t - T_o \Delta S_t \quad (3)$$

$$\Delta G_m = 0 = \Delta H_t - \Delta H_s - T_m \Delta S_t \quad (4)$$

$$\Delta H_s = (T_o - T_m)\Delta S_t \quad (5)$$

The conventional thermal entropy is ΔS_t. The enthalpic contribution imparted by solute is denoted as ΔH_s. The thermal entropy change of the $L_\alpha \rightarrow H_{II}$ reaction is 4.88 J mol^{-1} deg^{-1} (Epand, 1985), thus enthalpic stresses imparted upon the bilayer would lower the reaction midpoint by 1 deg per 4.88 J mol^{-1}. In order to isolate headgroup effects, a small correction for the difference in the ability of the solute OleOle chains and the matrix PamOle chains to promote curved structures is obtained by subtracting 0.3 °C, the mf% potency of OleOlePtdEtn, from T_o.

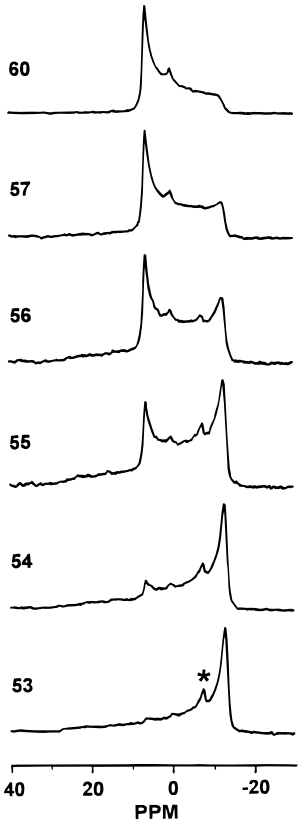

FIGURE 3: ^{31}P NMR spectra of 10 mf% OleOlePtdEt incorporated into a PamOlePtdEtn matrix are shown at selected temperatures. An asterisk marks the perpendicular tensor element of PtdEt.

RESULTS

Representative ^{31}P NMR spectra shown in Figure 2 demonstrate the approach. The PamOlePtdEtn matrix undergoes a thermotropic transition ($L_\alpha \rightarrow H_{II}$) from a lamellar structure of low curvature to an inverted hexagonal structure of high curvature. In this process, the ^{31}P nucleus acquires an additional axis of rapid motion that changes the sign and reduces the breadth of the chemical shielding anisotropy by a factor of 2 (deKruiff et al., 1985). Addition of dilute levels of lipids to the matrix alters the composite membrane shape and structural preference of the matrix, which is expressed as a concentration-dependent shift in the midpoint temperature, as described above. Each lipid can be characterized for its ability to promote the curved H_{II}-state with a "potency" expressed in degrees per mole fraction percent (mf%).

The phase preference of the PtdAlks can be monitored by the intensity of their characteristic signal as shown in Figure 3 (their L_α shielding anisotropy is narrower than that of PtdEtn, and the perpendicular tensor element is distinct as a small peak downfield from that of PdtEtn; Browning, 1981). Monitoring the relative rates of disappearance of this signature PtdEt peak with respect to the matrix bilayer pattern in Figure 3 (also see Figure 2B) demonstrates that whatever phase preference PtdEt has for the H_{II}-state is quite small.

Headgroup Volume Approach. The headgroup volume approach to lipid structure, described above, is shown in Figure 4. Our original correlation ($r = 0.95$) included the following dioleoyl lipids: diolein, PtdEt, PtdOH, PtdEtn, PtdGro, PtdSer, and PtdCho. Dioleoylphosphatidylethyleneglycol (OleOlePtdEg) is now added to the original correlation ($r = 0.95$). The dotted horizontal line represents the potency of cholesterol [see also Epand and Bottega

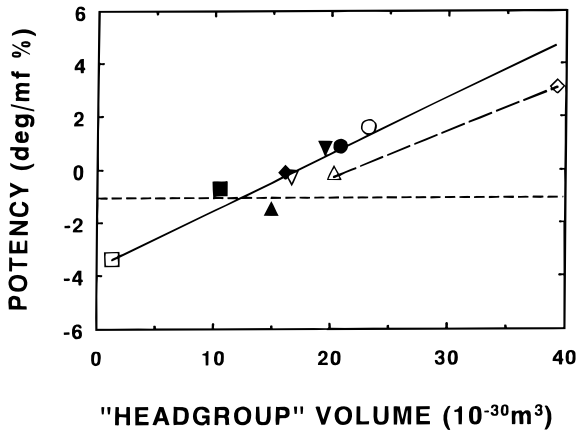

FIGURE 4: Headgroup volume approach to lipid structure. The ability of a variety of lipids to alter the $L_\alpha \rightarrow H_{II}$ midpoint temperature (potency) is plotted against the headgroup volume obtained from Pauling's covalent radii (see text). To the data reported previously (Lee et al., 1993b) are added OleOlePtdEg (♦), monogalactosyl diacylglycerol (△), digalactosyl diacylglycerol (◊), and cholesterol (horizontal dashed line). The remaining points include the following dioleoyl species: PtdCho (○), PtdSer (●), PtdGro (▼), PtdEtn (▽), PtdEt (▲), PtdOH (■), and diolein (□).

(1987)]. Although not homologous, cholesterol is of special interest due to its prevalence as a potent major matrix lipid.

Monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) have been postulated as possible violations of the headgroup volume approach (Hui, 1993). Hydrogenated species are shown in Figure 4, connected by the dashed line. The inclusion of these non-homologous species is perhaps a bit premature, but the slope is nonetheless important with reference to the data shown later for PtdAlks and TAGs. The close apparent agreement of MGDG and DGDG may be, in part, an artifactural consequence of the lack of homology. The main point is the positive correlation between headgroup size and stabilization of the bilayer structure, as is observed for the other polar headgroups.

Shape and Phosphatidylalkanols. PtdEt was previously noted as the most potent mammalian phospholipid in destabilizing the lamellar state (Lee et al., 1993a,b). In order to analyze this behavior with reference to the unusual alkyl moiety, we have expanded the headgroup volume approach in Figure 5 to include the series of homologous PtdAlks (PtdOH, PtdMe, PtdEt, PtdPr, PtdBu, PtdPe, PtdHx, and PtdOc), represented by the filled triangles and fitted to a linear function on purely empirical grounds ($r = 0.99$). The PtdAlks deviate dramatically from the predictions of the headgroup volume approach. That is, the larger alkyl headgroups impart a greater degree of curvature stress to the membrane.

Headgroup Volume Approach to Triacylglycerols. In light of this discrepancy, we questioned whether the concept of the PtdAlk "headgroup" might be considered a misnomer. Perhaps the alkyl moiety was inserted into the bilayer interior. In order to test this hypothesis by analogy, we have examined the chain length dependence of the 3-substituted 1,2-dioleoylglycerol series. Insertion of the TAG chains into the bilayer is established (Spooner & Small, 1987; Hamilton, 1989). Thus, the 3-acyl substituent cannot be considered a "headgroup", but it can be treated as such (eq 1) for purposes of comparison with the PtdAlks. The data for this series (diolein and its 3-substituents 8:0, 12:0, and 16:0) are

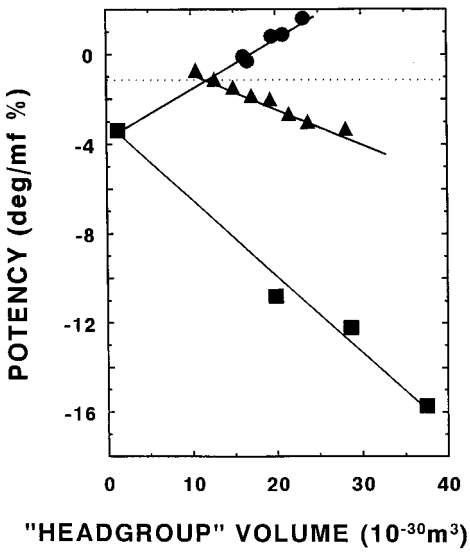

FIGURE 5: Headgroup volume approach to lipid structure is expanded to include results for the phosphatidylalkanols, PtdOH to PtdHx and PtdOc (▲), the 3-substituted dioleoyl (diolein, 3-octanoyl, 3-lauroyl, 3-palmitoyl) glycerols (■), and the phospholipids (●) identified in Figure 4.

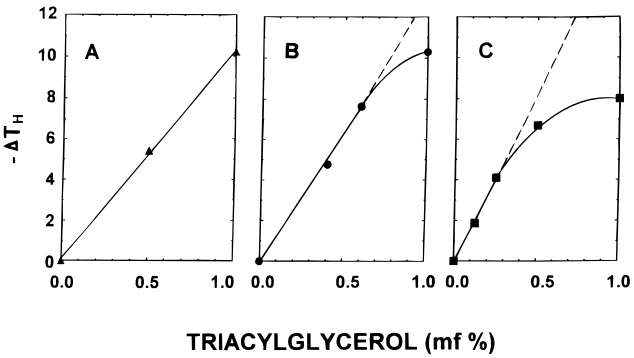

FIGURE 6: Ability of the 3-acyl-substituted dioleoyl triacylglycerols to alter the $L_\alpha \rightarrow H_{II}$ midpoint temperature saturates at low levels depending on the 3-substituent chain length: (A) 8:0; (B) 12:0; (C) 16:0.

represented by the solid squares in Figure 5 and fitted to an empirical linear function ($r = 0.99$). A dramatic deviation from the predictions of the headgroup volume approach is observed with a trend similar to that exhibited by the PtdAlks but with a powerful potency to promote curved structures that to our knowledge is considerably in excess of any other lipid.

Noteworthy is that the depression induced by the 3-palmitoyl TAG indicates an energetic contribution considerably greater than could be expected based solely on the maximum configurational entropy change that the solute can impart upon the system (see below and eq 2 above).

Saturation of Triacylglycerols. Almost as striking as is the powerful potency of the triacylglycerol series to promote membrane curvature is the extremely low levels at which this potency saturates. Shown in Figure 6 are titration curves for the 3-substituted TAGs. Whereas diolein exhibits linearity at the 5 mf% level (not shown) and the 8:0 adduct is linear to the 1 mf% level (Figure 6A), the 12:0 adduct exhibits signs of saturation above the 0.6 mf% level (Figure 6B) and the 16:0 adduct exhibits signs of saturation above the exceedingly low level of 0.25 mf% (Figure 6C).

DISCUSSION

Our interest in alkyl headgroups and curvature was sparked by our observation that PtdEt was able to promote nonbilayer structures with a potency greater than any other naturally occurring phospholipid. It appeared that either this exotic phospholipid violated shape theories of lipid structure or adopted an unusual conformation (Lee et al., 1993a).

In order to more fully investigate the consequences of introducing a hydrophobic alkyl moiety at the membrane interface, the inquiry was extended to the greater PtdAlks. The PtdAlks deviate markedly from the headgroup volume correlation as shown in Figure 5. The more polar PtdEg and PtdGro (Figure 4) yield expected results, in contrast to their alkyl adducts, PtdEt and PtdPr. This confirmed the anomaly of the PtdAlks. The question of whether this anomaly reflected an insufficiency of the shape theories or reflected an unusual headgroup conformation was posed.

Acylglycerol Series. Could the behavior of the PtdAlks be attributed to the hydrophobic effect driving the alkyl headgroup into the bilayer such that it can no longer be considered a "headgroup"? The hypothesis of headgroup insertion was tested by analogy using a series of saturated 3-acyl adducts of 1,2-dioleoylglycerols and diolein. The acylglycerol series (Figure 5) exhibits the same marked deviation from the headgroup volume approach as does the phosphatidylalkanol series, but the 3-substituent of TAGs is not a headgroup at all, rather it acts in the membrane interior to expand the hydrocarbon area (compare Figure 1E and F). Increases in the chain length progressively increase the hydrocarbon area and impart a progressively inverted cone-shaped geometry to the membrane. The analogy between the TAG series and the PtdAlk series provides compelling evidence for a common mechanism of headgroup insertion. The chemical difference between the PtdAlk and TAG series, the anionic phosphate linkage, may provide an energetic tether in the headgroup region that resists insertion and may thereby account for the slightly lesser potency per methylene (slope) exhibited by the phosphatidylalkanols. An insertion mechanism also may provide an explanation for the membrane fluidization induced by PtdEt (Omodeo-Sale, 1991).

Potency of Triacylglycerols. For an integral lipid adopting the characteristic bilayer orientation, the TAGs possess an unusual geometry. They completely lack a headgroup, but owing to the presence of three acyl chains their hydrocarbon volume is substantial. As a consequence, they impart a considerable curvature stress upon the bilayer in a chain length dependent manner. This powerful potency is in accord with a previous survey (Epand et al., 1988). The dramatic ability of TAGs to destabilize planar structures and promote structures of inverted curvature has potentially important implications for lipoprotein structure and metabolism and adds a new dimension to recent suggestions that triacylglycerols are native components of many biological membranes (Mountford & Wright, 1988; Hamilton, 1989; Hernell et al., 1990; Mackinnon et al., 1992; Lerique et al., 1994).

Saturation of Triacylglycerols. The ability of the TAGs to promote curved structures saturates in a chain length dependent manner that parallels the dramatic promotion of curvature (Figures 5 and 6). The chain length dependence of saturation is quite strong. The 3-palmitoyl adduct begins to saturate at half the level (0.25 mf%) of the 3-lauroyl adduct (0.6 mf%). Tristearin saturation at 0.5 mf% was reported in PtdEtn (Epand et al., 1988). At the 0.25 mf% TAG level, only 1.5% of nearest neighbors and 3.0% of next-nearest neighbors are TAGs (hexagonal lattice, random occupancy), which is perhaps a reflection of the unfavorable energetics associated with such a strong promoter of curvature.

By contrast, studies in matrices with less curvature stress, composed of bilayer-preferring and cylindrically shaped PtdCho (see Figure 4), indicate that saturation occurred at much greater levels, 3 mf% for triolein and tripalmitin (Hamilton, 1989). The reported chain length dependence of TAG saturation, 14 mf% for trioctanoin in PtdCho, is consistent with that described here in PtdEtn (Hamilton, 1989). When curvature stress is increased within the PtdCho matrix using the strong promoter of curvature, cholesterol (see Figures 4 and 5), TAG solubility is sharply decreased. Equimolar PtdCho/cholesterol matrices exhibited triolein saturation at 0.15 mf% (Spooner & Small, 1987). These observations are consistent with the concept that the unfavorable energetics associated with cumulative interfacial packing defects and high curvature stress in bilayers may play a role in limiting the incorporation of TAGs.

Galactosyldiacylglycerol. Two lipids invoked as possible violations of the Headgroup Volume correlation are MGDG and DGDG (Hui, 1993). In both cases, the headgroup volume calculated from covalent radii overestimates the headgroup volume obtained from curvature promotion. The source of the discrepancy may reside in the assumption inherent in the headgroup volume approach that presumes proportionality between headgroup volume calculated from chemical composition and mean expressed headgroup area, which is a function of conformation. Such a discrepancy would be anticipated if the galactosyl headgroups are extended into the aqueous milieu, while the other headgroups are expressed in a more compact manner along the surface. An extended headgroup orientation, which is distinct from the perpendicular habit of other phospholipid headgroups (Seelig & Seelig, 1980), has been reported for the glucose moiety of cerebroside (Skarjune & Oldfield, 1982). A simple pictorial representation of how an extended headgroup conformation would lead the headgroup volume to overestimate the mean headgroup area is obtained from a comparison of Figure 1A and E.

Although MGDG and DGDG exhibit a modest deviation from the absolute expectations of the headgroup volume approach, the trends observed do fulfill the expectations. The larger digalactoside is more cylindrical and favors bilayer formation, while the smaller monogalactoside is more inverted cone-shaped and favors inverted curve structures. This is in accord with the MGDG/DGDG index of curvature developed by Lindblom and co-workers (1986; Wieslander et al., 1986) to describe packing homeostasis in Acholeplasma membranes, but contrary to the PtdAlk trend.

Comments on Thermodynamics and Shape. The headgroup volume approach was described as a way to express the frustrated curvature stress (or relief) imparted upon a bilayer matrix by low levels of an added agent (solute). By restricting the focus largely to homologous species we are able to isolate headgroup effects.

Implicit within this formulation is that the solute imparts potential energy or, in thermodynamic terms, solute action is dominated by an enthalpic term that increases the free energy of the bilayer structure and thereby destabilizes it

with respect to the hexagonal state. This enthalpic term is implicitly postulated to dominate the configurational entropy imparted by the solute and any enthalpic contributions imparted to the hexagonal state, wherein the curvature is expressed and the stress is largely relaxed. This reasoning has similarities to that advanced by Gruner (1985, 1989), Epand and Epand (1994), Hui and Sen (1989), and Swedish workers (Rilfors et al., 1993), but see also Marsh (1986).

In agreement with this formulation, ideal solid-solution theory based on the configurational entropy imparted by the solute upon the membrane (eq 2) is unable to account for the potency observed for 1,2-dioleoyl-3-palmitoylglycerol, even given the most extreme assumptions. The maximum depression obtainable, 11.7 deg per mf%, is only relevant to the situation in which there is an absolute preference of the solute for the hexagonal phase; that is, the solute is excluded or phase separated in the bilayer structure. The real situation is far from this extreme limit. *No* phase preference could be detected ($K = 1$) in a variety of PtdCho/PtdEtn systems (Tilcock et al., 1982; Boni & Hui, 1983), suggesting that the actual entropic contribution to the free energy difference is quite small and possibly zero. The phase preference of the phosphatidylalkanols can be monitored by the distinct asymmetry associated with these species, which is shown in Figure 3 (also see Figure 2B). The rate of disappearance of this signature PtdEt peak with respect to that of the matrix bilayer pattern clearly demonstrates that whatever phase preference this agent has for the matrix H_{II}-state is small.

Since the change in transition entropy is small, 4.88 J deg^{-1} mol^{-1}, the transition temperature is quite sensitive to energetic perturbations that are difficult to detect calorimetrically. With the assumption that the enthalpic stress imparted by the solute upon the bilayer is the dominant energetic term, that enthalpic stress can be determined by changes in the transition temperature as described by the discussion after eq 5. For diolein the mf% depression, −3.4 °C, is corrected by the depression (−0.3 °C) caused by OleOlePtdEtn, to isolate headgroup effects, to yield −3.1 °C, which is multiplied by 4.88 J deg^{-1}, to give a stress of 15.1 J per mf% [for PtdCho: (1.6 °C + 0.3 °C)4.88 J deg^{-1} = 9.3 J per mf%]. The most potent agent studied, 1,2-dioleoyl-3-palmitoylglycerol, provides 75 J per mf%. Biochemical processes that transform lipids can accentuate the stress change. The formation of PtdEt from PtdCho by PLD in the presence of ethanol causes a change in stress of 14.9 J per mf%.

This thermodynamic formulation should not apply to agents such as alkanes, which are thought to act by a different mechanism to stabilize the H_{II}-state by filling the interstices between the inverted cylinders (Gruner, 1985), nor should it apply to agents, such as cholesterol (Epand & Bottega, 1987), that alter the expressed "shape" and energetics of the neighboring matrix lipids.

Epand and Epand (1994) have measured curvature stress relief by titration calorimetry of lysophosphatidylcholine into an OleOlePtdEtn-*N*-methyl matrix in the presence and absence of 1 and 2 mf% diolein. Diolein increased the calorimetric heat by 24.5 J per mf%. As noted above, the enthalpic stress imparted by diolein is 15.1 J per mf%. Given the differences in the approaches and matrices this is considered to be good agreement.

Semiempirical calculations of curvature stress in LinLinPtdEtn bilayers were performed by Hui and Sen (1989). Addition of PamOlePtdCho to this matrix at the 20 mf% level was calculated to relieve this stress by 68 J per mf% PtdCho. The relief of stress determined here is somewhat smaller, 9.3 J per mf%. It should be noted, however, that the acyl compositions used by Hui and Sen (1989) should provide both stronger initial matrix stress and stronger solute stress relief.

Implications for Biological Membranes. Major mammalian membrane matrix lipids exhibit a balance of forces among the headgroup and acyl regions that fall in a narrow range demarcated by cholesterol's ability to promote curvature (Figure 4). Lipids that fall below the cholesterol demarcation are either minor components or transient metabolic intermediates.

The PtdAlks and the TAGs exhibit potencies to promote curvature that exceed the demarcation. The presence of these agents in the membrane will alter the balance of intermolecular forces in the headgroup and acyl region. The resultant curvature stress may be sufficient to alter membrane structure and function.

The striking potency and the striking dependence of potency on the matrix composition offers a role for the TAGs in membrane structure, fusion, and assembly.

As the most potent mammalian phospholipid promoter of stress yet described, PtdEt is likely to create local curvature stress that may impact structure and function. PtdEt formation at the expense of PtdCho will exacerbate the effect. Its hydrophobic alkyl headgroup, its negative charge, its propensity for transbilayer diffusion, and its tendency for ethyl headgroup insertion toward the bilayer interior are all features that offer the potential for behavior that could compromise membrane function.

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