Stabilization of H_{II} Phases by Low Levels of Diglycerides and Alkanes: An NMR, Calorimetric, and X-ray Diffraction Study

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ABSTRACT: Traces of hydrophobic molecules like diglycerides and alkanes substantially reduce the lamellar/inverted hexagonal (L_α/H_{II}) phase transition temperature (T_H) of phospholipid systems. Two different mechanisms by which impurities can stabilize nonbilayer phases have been proposed (Gruner, 1985). Each requires a different location for impurity molecules within the H_{II}-phase unit cell. Here, ^2H NMR, ^{31}P NMR, X-ray diffraction, and DSC were used to probe the environment of perdeuterated alkanes and chain-perdeuterated 1,2-dipalmitoylglycerol (DPG-d_{62}) in L_α- and H_{II}-phase lattices. The host phospholipids were dioleoylphosphatidylcholine (DOPC) and monomethylated dioleoylphosphatidylethanolamine (DOPE-Me), and the samples contained several mole percent of perdeuterated additives. The data collected here generally support the two proposed mechanisms of H_{II} stabilization. Our data are consistent with localization of perdeuterated alkane molecules to the periphery of H_{II} tubes and to the hydrophobic interstices between H_{II} tubes. Low levels of alkanes probably decrease an unfavorable phospholipid acyl chain stretching contribution to the H_{II}-phase chemical potential. The alkanes that reduce T_H the most at low alkane concentrations are those that are most thoroughly excluded from the more ordered regions of L_α- and H_{II}-phase lattices. In contrast, DPG-d_{62} molecules appear to be oriented in H_{II} phases with glycerol backbones at the lipid/water interfaces and acyl chains roughly parallel to those of DOPE-Me. X-ray diffraction data show that DPG decreases the lattice constant of the H_{II} phase at constant temperature. Thus, diglycerides appear to reduce T_H by reducing the spontaneous radius of curvature of the lipid/water interfaces, R_0. These results illustrate two different ways in which traces of nonpolar lipid metabolites (e.g., diglycerides and dolichol) can modulate the phase behavior (T_H) of biomembrane lipids. Such modulation may be involved in the control of dynamic processes in biomembranes.

The lamellar/inverted hexagonal (L_α/H_{II}) phase transition temperatures (T_H) of phospholipid systems are very sensitive to the presence of hydrophobic impurities. Siegel et al. (1989) recently showed that 2 mol % of a diglyceride can lower the T_H of some systems by 20 °C, and Tate and Gruner (1987) have shown that 5 lipid volume % of a normal alkane is even more effective. Higher levels of alkanes can induce H_{II}-phase formation in dioleoylphosphatidylcholine (DOPC),[1] which does not normally form this phase at all in excess water (Sjölund et al., 1987, 1989).

This effect of hydrophobic molecules should be investigated for two reasons: first, this may elucidate the principles determining the relative stability of L_α and H_{II} phases [e.g., see Kirk et al. (1984) and Gruner (1985)]; and second, this effect may be one which cells use to control biomembrane function. Cells regulate the lipid composition of their membranes, maintaining them in a fixed region of the lipid/water-phase diagram [e.g., see Lindblom et al. (1986)]. The membrane lipid phase behavior is sensitive to low levels of nonpolar lipids. Perturbations of biomembrane lipid phase behavior via transient production of nonpolar lipid metabolites (e.g., diglyceride) might regulate some aspects of cell membrane function [e.g., see Siegel et al. (1989)]. For example, the susceptibility of phospholipid membranes to membrane fusion is sensitive to the proximity of the system to lamellar/inverted-phase transitions (Ellens et al., 1986, 1989).

In this study, ^2H and ^{31}P NMR powder patterns were utilized to infer the location of perdeuterated alkanes and diglycerides in H_{II}- and L_α-phase lattices. ^{31}P NMR, DSC, and (in some cases) X-ray diffraction data were used to identify the phases present in alkane- and diglyceride-doped samples of N-monomethylated dioleoylphosphatidylethanolamine (DOPE-Me) at different temperatures. ^2H NMR spectra obtained from the same samples yielded the magnitude of the quadrupolar splittings of the alkane or diglyceride resonances, and hence the range of C^2H_2 orientational order parameters experienced by these impurities. These values were compared to those obtained by other workers from deuterated phospholipids and alkane-doped lamellar phases. The relative magnitudes of these parameters in the L_α and H_{II} phases at the same temperature, together with X-ray diffraction data on the effect of alkane or diglyceride additives on the H_{II}-phase lattice constant, permit us to infer the locations of the additives in the phase lattices.

Our findings indicate that alkanes and diglycerides stabilize H_{II} phases via two different mechanisms and that these mechanisms are easily rationalized in terms of the formalism developed by Gruner and co-workers (Kirk et al., 1984; Kirk & Gruner, 1985; Gruner, 1985; Tate & Gruner, 1987; Gruner et al., 1986, 1988).

Materials and Methods

Materials. N-Monomethylated dioleoylphosphatidylethanolamine (DOPE-Me), dioleoylphosphatidylcholine

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[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPE-Me, N-monomethylated dioleoylphosphatidylethanolamine; DPG, 1,2-dipalmitoylglycerol; DPG-d_{62}, 1,2-di[$^2H_{31}$]palmitoylglycerol; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; LWHH, line width at half-height; NMR, nuclear magnetic resonance; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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(DOPC), chain-perdeuterated 1,2-dipalmitoylglycerol (DPG-d_{62}), and chain-perdeuterated dipalmitoylphosphatidylcholine (DPPC-d_{62}) were all obtained from Avanti Polar Lipids (Birmingham, AL). Dodecane, tetradecane, and squalene (2,6,10,15,19,23-hexamethyl-1-tetracosane) were obtained from Sigma Chemical Co. (St. Louis, MO), and hexadecane was from Fisher Scientific Co. (Fair Lawn, NJ). Perdeuterated dodecane, hexadecane, and squalane (dodecane-d_{26}, hexadecane-d_{34}, and squalane-d_{62}) were obtained from MSD isotopes (St. Louis, MO). Spectrophotometric-grade 2-methylbutane (isopentane) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was further purified via simple distillation.

Sample Preparation. Different methods were used for samples containing alkanes and samples containing diglyceride. For diglyceride-containing samples, a weighed amount of lyophilized DOPE-Me or DOPC was dissolved in chloroform, and an appropriate volume of a diglyceride stock solution in chloroform was added. Chloroform was removed by rotary evaporation for 30 min under vacuum. The dried samples were hydrated with several milliliters of buffer solution (150 mM NaCl, 20 mM TES, and 0.1 mM EDTA, pH 7.4) for 2–3 h at 4 °C and were then subjected to several freeze/thaw cycles (dry ice/40 °C water bath). The lipids were then pelleted and either resuspended in a new aliquot of buffer (for DSC) or pelleted into X-ray diffraction capillaries at low acceleration with a small aliquot of buffer.

For alkane-containing samples, the preparation was similar, except that the thin film of DOPE-Me resulting from removal of chloroform was redissolved in isopentane, to which the alkane was added as an isopentane stock solution. The isopentane was then removed via rotary evaporation under reduced pressure (water aspirator) for 5–10 min. The samples were then hydrated as above. Lipids were cosolubilized in isopentane (boiling point 28 °C) so that this highly volatile solvent could be removed rapidly with minimal loss of semivolatile alkanes like dodecane. Gas chromatography demonstrated that ca. 95% of the added dodecane was retained in the final lipid preparation with this procedure, and essentially 100% of added hexadecane (data not shown). Dodecane, tetradecane, and hexadecane were added so as to make up a certain percentage of the volume of the total lipid in a sample (using the assumption that the density of the phospholipid is 1.00 g/cm^3). These concentrations are noted below as "lipid volume %" and are calculated according to the equation:

$$\text{lipid volume \%} = \left(\frac{W_A/\delta}{W_L + W_A/\delta} \right) \times 100$$

where W_L is the weight of phospholipid in the sample, W_A is the weight of added alkane, and δ is the density of the alkane at room temperature obtained either via pycnometry (for perdeuterated alkanes; data not shown) or from the CRC Handbook of Chemistry and Physics.

Samples for NMR studies were stored as dried-down lipid films at −70 °C under argon until use and were hydrated just before use by the same protocol given above. However, in cases where the T_H of the sample was below room temperature, the freeze/thaw cycles were omitted, and the lipids were merely hydrated for 24 h at 4 °C. Prolonged storage of the lipids as dried lipid films did not change the T_H of the hydrated systems as determined via DSC (data not shown).

NMR Methods. ^{2}H and ^{31}P NMR spectra were obtained on the same sample without removing the sample from the spectrometer, data collection for the second nucleus beginning within 1–2 min after data collection for the other. Sample temperatures were also changed without removing samples from the spectrometer.

^{31}P NMR spectra were obtained with a JEOL FX270 Fourier-transform spectrometer in a broad-band-tunable probe in 10-mm tubes. A total of 2000 scans were accumulated at 1 s^{-1}. A fully phase-cycled (32 pulse) Hahn echo sequence was used with a 40-μs echo. The echo sequence eliminates base-line artifacts, removing the need for first-order phase corrections (Rance & Byrd, 1983). Data were collected prior to the refocusing of the echo and the FID transformed from the top of the echo. Because of the short echo time that must be employed to capture all the resonance intensity (due to very short T_2*), the finite length of the transmitter pulses (10 μs, 20 μs) must be taken into account in determining the refocusing point of the echo. The ^{1}H decoupler was gated on during acquisition and off the remainder of the time to prevent sample heating. The effectiveness of the decoupling was improved by using single-frequency decoupling, rather than noise-modulated decoupling, with the frequency set at the resonance frequency of the phospholipid headgroup protons. Nine kilohertz of decoupling power was used. Exponential line broadening of 50–200 Hz was used. Recalculations showed that this induced no artifacts.

^{2}H NMR spectra were obtained at 41.36 MHz using a solid echo (pulse width = 10 μs). Fifty-kilohertz spectra in the frequency domain were obtained. Data points (2K) were collected in the time domain. Depending on the width of the powder patterns obtained, 0–200-Hz exponential line broadening was used in calculating the spectra (0 in cases where the spectra were hundreds of hertz side). A total of 10 000–30 000 scans were collected for each spectrum with a repetition rate of 10 s^{-1}.

DSC. Thermograms were obtained with a Microcal MC-2 calorimeter scanning at 13 or 27 °C/h. Some DSC samples were run with buffers containing only 100 mM NaCl instead of 150 mM NaCl: this had no effect on the results.

X-ray Diffraction. Patterns were obtained by using a Rigaku RU-100H rotating-anode source and a flat-film camera.

Results

Differential Scanning Calorimetry. DSC was used to determine the T_H of DOPE-Me systems with various levels of different additives. Typical thermograms of DOPE-Me in the absence and presence of dodecane are displayed in Figure 1. The enthalpies (ca. 250 cal/mol) and peak temperatures of the endotherms are consistent with L_α/H_{II}-phase transitions in pure DOPE-Me systems (Gagné et al., 1985; Ellens et al., 1986, 1989; Siegel et al., 1989). The identities of the phases existing above and below the temperatures of the endotherms in alkane- and DPG-d_{62}-doped systems were confirmed by separate X-ray diffraction and NMR experiments (below).

Addition of dodecane lowers the onset temperatures and broadens the endotherms. At alkane concentrations above 2 lipid volume %, the endotherms sometimes contain two peaks (Figure 1D). The temperatures corresponding to the largest peak in the endotherms of several alkane- and diglyceride-doped DOPE-Me systems are recorded in Table I. These data show that T_H decreases roughly linearly with increasing alkane and diglyceride content in the range 0–3 lipid volume % (alkanes) and 0–3 mol % (diglyceride). This linear decrease of T_H with increasing alkane or diglyceride content indicates that these additives are not forming separate neat phases within this concentration range.

Note that different alkanes reduce T_H to significantly different extents when they represent the same volume fraction within the L_α-phase lattice (i.e., different effectiveness in re-

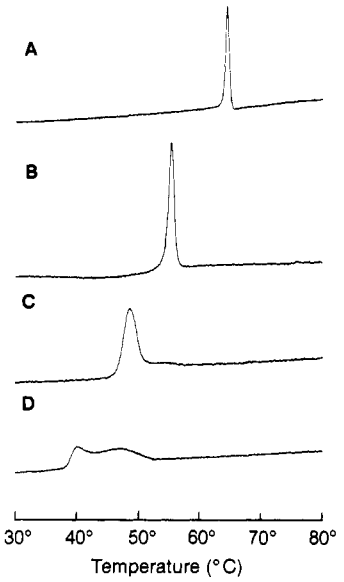

FIGURE 1: Thermograms of DOPE-Me samples containing (A) 0, (B) 1.0, (C) 2.0, and (D) 3.0 lipid volume % dodecane. The thermogram in (A) was obtained at half the sensitivity used to obtain the other endotherms. The enthalpies of these phase transitions are ca. 0.2 kcal/mol.

Table I: T_H of Alkane/DOPE-Me and DPG-d_{62}/DOPE-Me Systems As Determined via Differential Scanning Calorimetry

additive	amount	temp (°C) of peak in endotherm (±2 °C)
none		66–67
dodecane	1.0 volume %	57
	2.0 volume %	48
	3.0 volume %	40[a]
tetradecane	0.50 volume %	60
	1.0 volume %	56
	2.0 volume %	43
	3.0 volume %	33[a]
hexadecane	0.50 volume %	61
	1.0 volume %	55
	2.0 volume %	43
	3.0 volume %	32[b]
squalane	1.0 volume %	47[c]
DPG-d_{62}	2.0 mol %	52
	3.0 mol %	41

[a] The endotherms are quite broad, with a second peak visible at 47 °C. [b] Very broad and poorly reproducible. [c] Broad, with a second peak visible at 54 °C.

ducing T_H on a volume-for-volume basis). Tetradecane and hexadecane are significantly more effective than dodecane, and squalane, a saturated C_{30} analogue of isoprenoid lipids, is almost twice as effective as tetradecane and hexadecane.

X-ray Diffraction Experiments. The results of X-ray diffraction experiments on representative systems are displayed in Table II. The samples were determined to be in the H_{II} phase if a minimum of three diffraction rings were observed, indicating spacings in the ratios $1:1/\sqrt{3}:1/\sqrt{4}:1/\sqrt{7}$. The H_{II} lattice constants (the center–center spacing of H_{II} tubes) were determined via a least-squares analysis of these spacings and are accurate to within ±0.1 nm. These results demonstrate that the hexadecane-, squalane-, and DPG-doped systems were indeed in the H_{II} phase at temperatures above the putative L_α/H_{II}-phase transition tempertures identified via DSC (Table I). The identities of the phases present in NMR experiments were further confirmed by the shape of the ^{31}P NMR resonances (below).

The H_{II} lattice constants of the hexadecane-containing DOPE-Me samples are about the same as or a little larger than the lattice constant of pure DOPE-Me. The lattice constant of pure DOPE-Me is 7.4 nm at 65 °C, and the extrapolated value at 55 °C is about 7.6 nm (Gruner et al., 1988). This is 0.1–0.3 nm smaller than the values in the presence of hexadecane or squalane at the same temperature (Table II).

Table II: Lattice Constants of DOPE-Me H_{II} Phases

additive	temp (°C)	lattice constant[a] (nm) (±0.1 nm)
2.0 lipid volume % hexadecane	55	7.8
4.0 lipid volume % hexadecane	55	7.7
2.0 lipid volume % squalane	55	7.9
2.0 mol % DPG	61	7.5
5 wt % dodecane[b]	61	7.7
2.0 mol % diolein[c]	58	7.15
5 wt % dodecane[b]	58	7.75
2.0 mol % 1-oleoyl-2-arachidonoylglycerol[c]	50	7.6
5 wt % dodecane[b]	50	8.0

[a] The center-to-center distance of rods in the H_{II} phase. [b] Data from Gruner et al. (1988). [c] Data from Siegel et al. (1988).

In contrast, addition of diglyceride to DOPE-Me always reduces the H_{II}-phase lattice constant relative to the value obtained in the presence of low levels of alkane at the same temperature (by 0.2–0.6 nm; Table II). The basis of comparison is alkane-containing DOPE-Me because this system is thought to have the equilibrium value of the spontaneous radius of curvature (Kirk & Gruner, 1985; see Discussion).

Nuclear Magnetic Resonance Spectroscopy. ^{31}P NMR and ^2H NMR spectra were obtained from DOPE-Me samples containing different amounts of perdeuterated alkane and DPG-d_{62} as a function of temperature. The ^{31}P NMR powder patterns were used to verify the identity of the phases present in a sample under each set of conditions. The ^{31}P NMR powder patterns of phospholipids in the L_α, H_{II}, and inverted cubic (or isotropic) phase are quite different (Cullis & de Kruijff, 1978). Theoretically, a ^{31}P NMR resonance of the symmetry usually associated with the H_{II} phase does not guarantee the presence of an H_{II} phase in the sample. However, reliable phase assignments can generally be made on the basis of ^{31}P NMR data [e.g., see Cullis and de Kruijff (1978), Tilcock et al. (1986), and Yeagle (1987)]. Separate X-ray diffraction experiments confirmed the phase assignments in some alkane and DPG systems [see below; also see Siegel et al. (1989)]. ^{31}P NMR spectra were obtained for each sample either immediately before or immediately after ^2H NMR spectra were obtained from the same sample in the same instrument without removing the sample. This was done so that the different components of the ^2H NMR resonances could be reliably assigned on the basis of the phases present as determined by ^{31}P NMR.

^2H NMR and ^{31}P NMR spectra obtained from a sample of DOPE-Me containing 5.0 lipid volume % (12 mol %) of hexadecane-d_{34} at three different temperatures are displayed in Figure 2A–C. The ^{31}P NMR spectra (top) consist of two overlapping spectral components: one arising from DOPE-Me in the L_α phase and one from DOPE-Me in the H_{II} phase. These spectra show that L_α and H_{II} phases coexisted in the sample at 13 °C (Figure 2A), that the proportion of the lipid in the H_{II} phase increased with increasing temperature (20 °C, Figure 2B), and that the transition to the H_{II} phase was nearly complete at 25 °C (Figure 2C).

The ^2H NMR spectra of the perdeuterated alkane in the sample (bottom) also consist of two overlapping spectral components. The first is a single peak with a line width at half-height (LWHH) of about 1.5 kHz. The second compo-

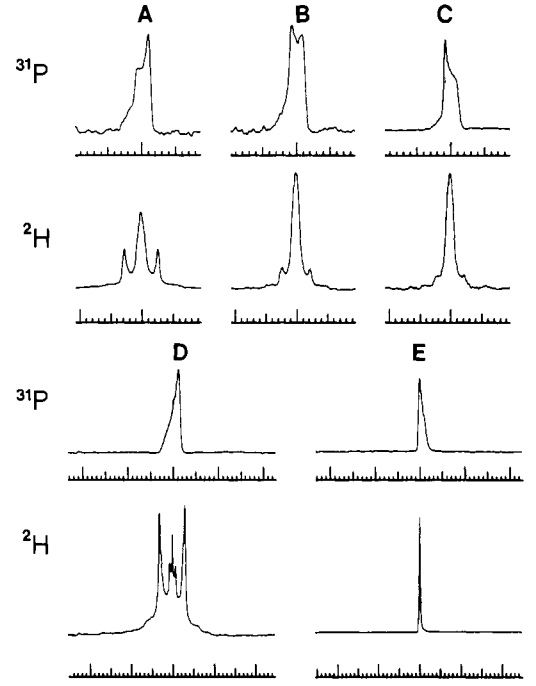

FIGURE 2: ^{31}P NMR (top) and ^{2}H NMR (bottom) spectra of DOPE-Me samples containing perdeuterated alkanes. The ^{31}P and ^{2}H spectra in each pair were obtained consecutively from the same sample in the same spectrometer under the same conditions. (A–C) is a series of spectra obtained from a sample of DOPE-Me containing 5.0 lipid volume % hexadecane-d_{34} at three consecutive temperatures: 13 °C (A), 20 °C (B), and 25 °C (C). (D) is a pair of spectra obtained from a sample containing 2.0 lipid volume % dodecane-d_{26} at 20 °C. The spectra in (E) were obtained from a sample of DOPE-Me containing 2.0 lipid volume % hexadecane-d_{34} at 62 °C. The smallest divisions on the horizontal scales are 10 ppm (^{31}P NMR) and 1 kHz (^{2}H NMR).

nent has a quadrupole splitting of ca. 5 kHz; there may also be a central maximum in this second component underlying the peak due to the first component. The relative intensities of these spectral components change in parallel with the relative intensities of the two ^{31}P NMR powder pattern components. Clearly, the intensity of the narrow ^{2}H NMR component increases as the proportion of H_{II}-phase phospholipid in the sample (as indicated by ^{31}P NMR) increases with increasing temperature. Similarly, the intensity of the component with the 5-kHz quadrupole splitting decreases as the fraction of phospholipid in the L_α phase decreases. Moreover, the narrow component resembles ^{2}H NMR spectra obtained from alkane-containing samples of lipid that were completely in the H_{II} phase (Figure 2E), and the broader component resembles spectra obtained from purely L_α-phase samples (Figure 2D). Therefore, the broad component represents perdeuterated alkane in the L_α phase and the narrow peak component perdeuterated alkane in the H_{II} phase. The LWHH of the H_{II}-phase ^{2}H NMR spectrum in Figure 2E is narrower than in Figure 2C because the spectrum in Figure 2E was obtained at a much higher temperature (62 °C), and the order parameters of CD_2 groups (which are proportional to the observed quadrupole splittings) in such phases decrease with increasing temperature.

The ^{2}H NMR resonances of perdeuterated alkanes in the L_α phase closely resemble ^{2}H NMR resonances of hexane-d_{14} in L_α-phase dioleoylphosphatidylcholine (DOPC) at similar temperatures (Jacobs & White, 1984b) and spectra of long-chain perdeuterated alkanes in other L_α-phase phosphatidylcholine systems (Jacobs & White, 1984a; Pope et al., 1984; Pope & Dubro, 1986; Sjölund et al., 1987). The hexane-d_{14} quadrupole splittings observed by Jacobs and White in DOPC at 20 °C were ca. 5 and 1.3 kHz. Sjölund et al. (1987) observed splittings of 1.4, 4.6, and 5.4 kHz in dodecane-d_{26}/DOPC samples at 35 °C. The larger quadrupole splitting we observed in Figure 2A,B is also ca. 5 kHz at 20 °C. In Figure 2A,B, the inner doublet that appears in the data of Jacobs and White and Sjölund et al. is obscured by the powder pattern associated with the H_{II} phase, which has a LWHH slightly larger than the 1.3-kHz splitting. However, this second doublet is visible in the spectrum of a predominantly L_α-phase sample of dodecane-d_{26} in DOPE-Me at 20 °C, displayed in Figure 2D. Apparently, the intrinsic spin–spin relaxation is shorter in the spectra in Figure 2A–C than in Figure 2D, resulting in broader line widths which obscure the quadrupole splitting pattern. At temperatures close to the chain-melting temperature of the host phospholipid, spectra of samples containing dodecane-d_{26} and hexadecane-d_{34} should contain several pairs of peaks with different quadrupole splittings (Pope et al., 1984; Pope & Dubro, 1986; Sjölund et al., 1987). At temperatures far above the chain-melting temperature, as is the case here, the quadrupole splittings of the outer peaks in the resonance are very nearly equal (e.g., as in the case of dodecane-d_{26}; Pope et al., 1984; Pope & Dubro, 1986). These components appear as a single doublet in Figure 2A–C. In Figure 2D, the outermost peaks in the L_α-phase spectra show evidence of these additional quadrupole splittings.

The ^{2}H NMR powder patterns from perdeuterated alkanes in L_α and H_{II} phases at the same temperature are quite different. This difference is emphasized in Figure 3, which displays ^{2}H and ^{31}P NMR spectra of hexadecane-d_{34}-doped and dodecane-d_{26}-doped DOPC (L_α phase; Figure 3A and Figure 3C, respectively) and hexadecane-d_{34}-doped and dodecane-d_{26}-doped DOPE-Me (H_{II} phase; Figure 3B and Figure 3D), all obtained at 62 °C.

In order to determine the differences in the environments of perdeuterated alkanes in L_α and H_{II} phases, it is necessary to compare data obtained from L_α and H_{II} phases of host phospholipids that are as similar as possible. The magnitudes of phospholipid CH_2 order parameters (and hence the environment of perdeuterated alkanes in these phases) are sensitive to lipid composition and temperature [e.g., see Jacobs and White (1984b), Pope et al. (1984), and Pope and Dubro (1986)]. Ideally, one would obtain powder patterns of perdeuterated alkanes in DOPE-Me at temperatures slightly below and slightly above T_H. However, incubation of DOPE-Me samples at temperatures just below T_H induces rapid formation of isotropic-phase ^{31}P and ^{2}H NMR resonances (described in more detail below). ^{2}H NMR quadrupole splittings of perdeuterated alkane molecules in the L_α phase could not be obtained from such samples.

Therefore, we used alkane/DOPC powder patterns at 62 °C (Figure 3A,C) as models of the patterns expected from alkanes at infinite dilution in an L_α phase of DOPE-Me at 62 °C. DOPC and DOPE-Me have the same acyl chain substituents and have very nearly the same chain-melting temperature (−15 to −20 °C and −10 to −15 °C, respectively; Gruner et al., 1988). 62 °C is very far from the chain-melting temperatures of these lipids, and the order parameters of phospholipid acyl chains (and ^{2}H NMR quadrupole splittings) vary modestly with temperature in this regime (Yeagle & Frye, 1987). For example, in [11,11-$^{2}H_2$]DOPC, the ^{2}H NMR quadrupole splitting changes little between 40 and 70 °C (Chupin et al., 1987). Moreover, in L_α-phase samples of mixtures of [11,11-$^{2}H_2$]phosphatidylethanolamine (DOPE) and [11,11-$^{2}H_2$]DOPC, ^{2}H NMR spectra of these lipids are equivalent at 40 °C. DOPE-Me is intermediate in structure

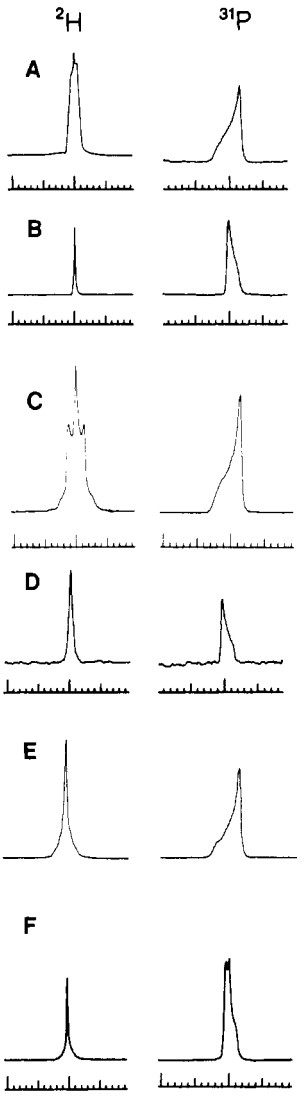

FIGURE 3: ^{2}H (left) and ^{31}P NMR (right) spectra of DOPC and DOPE-Me samples containing low levels of perdeuterated alkanes. The spectra in each pair were obtained consecutively on the same sample of lipid at the same temperature (62 °C in all cases). (A and B) Hexadecane-d_{34} DOPC (3.0 lipid volume %) and DOPE-Me (2.0 lipid volume %), respectively. (C and D) Dodecane-d_{26} in DOPC (3.0 lipid volume %) and DOPE-Me (2.0 lipid volume %), respectively. (E and F) 1.0 lipid volume % squalane-d_{62} in DOPC and 0.67% squalane-d_{62} in DOPE-Me. The smallest divisions on the horizontal scales are 10 ppm (^{31}P NMR) and 1 kHz (^{2}H NMR).

between DOPE and DOPC (i.e., one methyl group on the primary amine vs none and three for DOPE and DOPC, respectively), and its properties should be even more similar to DOPC than those of DOPE. These similarities indicate that the CH_2 order parameter at each position on the acyl chains should be similar in L_α phases of DOPC and DOPE-Me at 62 °C. Therefore, these lipids provide nearly equivalent L_α-phase environments for perdeuterated alkanes at that temperature.

The ^{2}H NMR powder pattern of hexadecane-d_{34} in L_α-phase DOPC is narrower than for dodecane-d_{26} at the same temperature (Figure 3A,C). This may at first seem to contradict Pope and Dubro (1986), who found that the powder pattern of hexadecane-d_{34} was broader than that of dodecane-d_{26} at temperatures just above the chain-melting temperature of the host phospholipid. However, Pope and Dubro also found that the width of the powder patterns decreased much more rapidly with increasing temperature for samples containing dodecane-d_{26} than for samples with octane-d_{18}. This implies that the LWHH decreases faster with increasing temperature for longer perdeuterated alkane chain lengths. This is consistent with our data (Figure 3A,C), obtained at a temperature more than 70 °C above the chain-melting temperature of DOPC.

The ^{2}H NMR powder patterns of perdeuterated alkanes in the DOPE-Me samples do not contain discernible doublets (Figure 3B,D,F). We therefore use the line width at half-height (LWHH) as a rough measure of the largest quadrupole splitting (and hence the largest CD_2 order parameter) compatible with a given powder pattern. The ^{2}H NMR spectrum of hexadecane-d_{34} in H_{II}-phase DOPE-Me (ca. 0.3 kHz, Figure 3B) has a ca. 5-fold smaller LWHH than in L_α-phase DOPC (1.7 kHz, Figure 3A). The spectrum of dodecane-d_{26} in H_{II}-phase DOPE-Me is ca. 6-fold narrower than in L_α-phase DOPC (0.5 kHz in the H_{II} phase vs 2.9 kHz in the L_α phase; Figure 3C,D). These reductions are much greater than the 2-fold reduction ascribable to the change from lamellar to cylindrical geometry during the L_α- to H_{II}-phase transition (Wennerström et al., 1974; Seelig, 1977). Sjölund et al. (1987) also observed very large reductions in ^{2}H NMR powder pattern width in perdeuterated alkane-containing H_{II} phases.

^{2}H and ^{31}P NMR spectra of 1.0 lipid volume % of squalane-d_{62} in DOPC at 62 °C are shown in Figure 3E. The LWHH of the ^{2}H NMR pattern of squalane in L_α-phase DOPC is ca. 1.4 kHz, narrower than the corresponding L_α-phase spectra of dodecane-d_{26} and hexadecane-d_{34}. Spectra of 0.67 lipid volume % squalane-d_{62} in DOPE-Me are displayed in Figure 3F. The LWHH of the ^{2}H NMR powder pattern is only ca. 0.3 kHz in the H_{II} phase, much smaller than that of dodecane-d_{26} and about equal to that of hexadecane-d_{34}.

The appearance of narrow (0.3–0.5 kHz) ^{2}H NMR resonances in spectra of H_{II}-phase samples is unlikely to be due to phase separation of neat perdeuterated alkane. Alkane-containing samples yielded spectra similar to Figure 3A until the samples were heated above T_H (data not shown), indicating that the samples were well mixed with alkane and fully equilibrated to start with. The linear decrease in the transition temperature with increasing alkane content (Table I) also indicates that the alkane was completely incorporated into at least one and probably both of the L_α and H_{II} phases in such samples. Finally, the LWHH of ^{2}H NMR spectra of neat hexadecane-d_{34} at 27 °C is less than 35 Hz under the same accumulation conditions as in the other experiments, much narrower than the LWHH of our H_{II}-phase powder patterns.

The shape of the H_{II}-phase ^{2}H NMR powder pattern of squalane (Figure 3F) is somewhat different than for the H_{II}-phase patterns of the other alkanes. The disparity between the width of the base of the powder pattern and the LWHH is much larger for squalane (Figure 3F) than for the other alkanes (Figure 3B,D). The width of the squalane powder pattern at the base (3 kHz) is also larger than for the other alkanes. Part of the greater disparity between base width and LWHH in the ^{2}H NMR pattern for squalane may be due to the presence of the same sort of structures that produce a very narrow isotropic component in the corresponding ^{31}P NMR powder pattern. In these samples, such a very narrow ^{31}P NMR component is always associated with a narrow isotropic component of similar magnitude in the ^{2}H NMR pattern (see below). However, the isotropic component of the ^{31}P NMR resonance is only a few percent of the total intensity in the powder pattern (Figure 3F), so this influence on the shape of the ^{2}H NMR pattern should be small. The disparity in base width and LWHH of the ^{2}H NMR pattern is consistent with either of two possibilities. This shape may reflect the time-averaged distribution of order parameters in squalane mole-

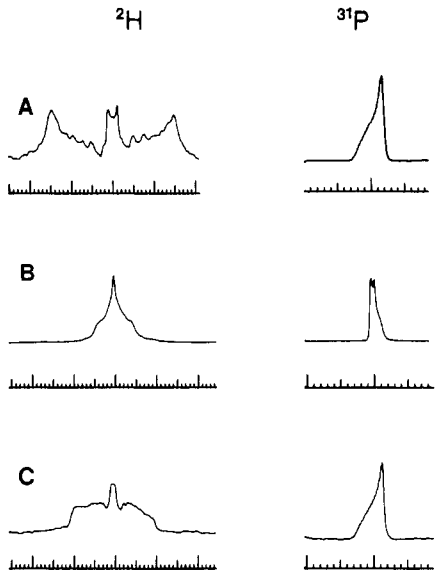

FIGURE 4: ^{2}H (left) and ^{31}P NMR (right) spectra of sample containing DPG-d_{62}. Spectra in each pair of ^{2}H and ^{31}P NMR spectra were obtained consecutively from the same sample at the same temperature. (A) 2.0 mol % DPG-d_{62} in DOPE-Me at 20 °C. (B) 2.0 mol % DPG-d_{62} in DOPE-Me at 62 °C. (C) 5.0 mol % DPG-d_{62} in DOPC at 62 °C. The smallest divisions on the horizontal scales are 10 ppm (^{31}P NMR) and 1 kHz (^{2}H NMR).

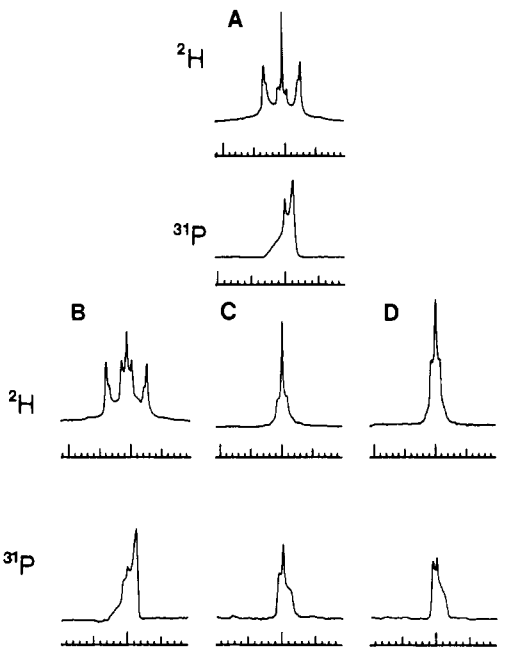

FIGURE 5: Isotropic ^{2}H NMR resonances associated with the appearance of isotropic ^{31}P NMR resonances. (A) ^{2}H (bottom) and ^{31}P NMR (top) spectra of the same sample of DOPE-Me containing 2.0 lipid volume % dodecane-d_{26} at 21 °C. (B–D) Spectra obtained successively from the same sample of DOPE-Me containing 5.0 lipid volume % dodecane-d_{26} at 10 °C (B), 20 °C (C), and 25 °C (D). The isotropic ^{31}P NMR components are probably due to the formation of inverted cubic-phase or cubic-phase precursors. Note that there is a corresponding isotropic ^{2}H NMR component that grows in parallel with the isotropic ^{31}P NMR resonance. The smallest divisions on the horizontal scales are 10 ppm (^{31}P NMR) and 1 kHz (^{2}H NMR).

cules existing in one class of sites, in which different C^2H_2 and C^2H_3 groups of the molecule experience environments of very different time-averaged order. Alternatively, the squalane molecules may be exchanging between multiple sites, in at least one of which the molecules are comparatively well-ordered. This exchange would have to occur at rates slower than ca. 3 kHz. We cannot distinguish between these two explanations on the basis of our data.

The ^{2}H NMR powder patterns of chain-perdeuterated 1,2-dipalmitoylglycerol (DPG-d_{62}) are also quite different in the L_α and H_{II} phases. This is illustrated in Figure 4A,B, which display ^{2}H NMR (left) and ^{31}P NMR (right) spectra obtained from a sample of DOPE-Me containing 2.0 mol % DPG-d_{62} at 20 °C (A) and 62 °C (B). For comparison, ^{31}P and ^{2}H NMR spectra of 5.0 mol % DPG-d_{62} in DOPC at 62 °C are displayed in Figure 4C.

At 20 °C (Figure 4A), the ^{31}P NMR powder pattern indicates that the phospholipid is in the L_α phase, consistent with DSC (Table I) and X-ray diffraction (Table II) data. The largest quadrupole splitting in the ^{2}H NMR powder pattern of this sample is ca. 30 kHz. The shape and width of this powder pattern are similar to patterns of perdeuterated phospholipids in the L_α phase at similar reduced temperatures [e.g., see Paddy et al. (1985)]. This implies that the DPG-d_{62} acyl chains are oriented in roughly the same fashion as the phospholipid chains in the L_α phase and that the glycerol backbone of DPG-d_{62} remains anchored at the lipid/water interface within that phase. The basic line shape of the DPG-d_{62} ^{2}H NMR resonance in the L_α phase (DOPC, Figure 4C) is unaltered at 62 °C, and the largest quadrupole splitting at 62 °C is ca. 19 kHz.

The ^{31}P NMR powder pattern of the DPG-d_{62}-doped DOPE-Me sample obtained at 62 °C showed that the phospholipid was predominantly in the H_{II} phase (Figure 4B), in agreement with DSC (Table I) and X-ray diffraction (Table II) data. A small fraction of the intensity appeared as a very narrow isotropic resonance. This small isotropic component probably represents phospholipid in inverted cubic phase or inverted cubic-phase precursors. The ^{2}H NMR resonance of the DPG-d_{62} is very similar to that obtained by Sternin et al. (1988), using perdeuterated tetradecanol in POPE. The most strking difference between the L_α- and H_{II}-phase powder patterns in Figure 4 is that the largest quadrupole splitting in the H_{II}-phase spectrum (Figure 4B) is ca. 8 kHz, only 40% of the largest splitting (19 kHz) in Figure 4C. This assumes a simple reduction in motional order as revealed by the ^{2}H NMR quadrupole splittings, and not a change in rate or type of motion. If the DPG enters a motional regime characterized by an intermediate time scale (10–100 kHz), the powder patterns are directly modulated by the rate of motion, and interpretation becomes more complex. At this time, however, no evidence is available to support the latter possibility.

Sternin et al. (1988) observed a similar 60% reduction in the largest quadrupole splitting when they compared the ^{2}H NMR powder patterns of perdeuterated tetradecanol in L_α and H_{II} phases of POPE at the same temperature. The fact that we observed a reduction in LWHH of the same magnitude when we compared powder patterns from DPG-d_{62} in DOPC and in DOPE-Me indicates that DOPC is a good model for the L_α phase of DOPE-Me at the same temperature.

^{31}P NMR spectra of some L_α-phase samples of alkane-doped DOPE-Me contained isotropic components as well as components typical of L_α phases, particularly if the sample had been incubated for extended periods at temperatures within ca. 20 °C of T_H. In all these cases, the corresponding ^{2}H NMR spectrum obtained nearly simultaneously from the same sample always contained an additional isotropic component, as well. Figure 5A displays the ^{31}P and ^{2}H NMR spectra of a sample of DOPE-Me doped with 5.0 lipid volume % dodecane-d_{26} at 21 °C. A substantial fraction of the total intensity in the ^{31}P NMR spectrum is in an isotropic component (bottom). Note that the central, isotropic component of the

^{2}H NMR spectrum (top) is also larger relative to the other components of the spectrum than in Figure 2D, in which no isotropic ^{31}P NMR resonance was observed. Isotropic ^{31}P NMR resonances are closely associated with the formation of inverted cubic phase or precursors to this phase (Eriksson et al., 1985; Sjölund et al., 1987, 1989; Lindblom & Rilfors, 1989). Such resonances are often observed in spectra of multilamellar samples of DOPE-Me systems under similar conditions and have been reported to appear in the hysteretic and sample history dependent manner observed here [e.g., see Gagné et al. (1985), Gruner et al. (1988), and Ellens et al. (1989)].

The isotropic ^{2}H NMR resonances associated with isotropic ^{31}P NMR resonances persisted when the samples were heated above T_H. The LWHHs of these ^{2}H NMR resonances are approximately the same as those of the ^{2}H NMR components due to perdeuterated alkane in H_{II} phases. This can complicate interpretation of ^{2}H NMR data from H_{II}-phase samples. Figure 5B–D shows spectra of a DOPE-Me sample containing 5.0 lipid volume % dodecane-d_{26} that was heated through T_H. At 10 °C (Figure 5B), where the sample is in the L_α phase, low-intensity isotropic components are visible in ^{31}P and ^{2}H signals. At 20 °C (Figure 5C), the ^{31}P spectrum contains a substantial isotropic component, and the ^{2}H spectrum now appears to contain two components of different LWHH. At 25 °C (Figure 5D), the proportion of isotropic component intensity in the ^{31}P spectrum has decreased, and the relative intensities of the two components in the ^{2}H spectrum have also changed. In this work, alkane and diglyceride ^{2}H NMR spectra in L_α- and H_{II}-phase samples were compared by using only data sets in which the corresponding ^{31}P NMR spectra contained either no isotropic components or components representing less than a few percent of the total phospholipid. Thus, ambiguity concerning the origin of isotropic ^{2}H NMR components in H_{II}-phase spectra was avoided.

Discussion

Low levels of alkanes and diglycerides are surprisingly effective at stabilizing inverted phases in phospholipid systems (Kirk & Gruner, 1985; Epand, 1986; Das & Rand, 1986; Tate & Gruner, 1987; Siegel et al., 1989). This study was designed to determine the location of alkanes and diglycerides within L_α- and H_{II}-phase lattices, in order to infer the mechanism of this stabilization by low levels of these dopants. The inferred location of these dopants within L_α- and H_{II}-phase lattices is depicted in Figure 6 and is discussed in detail below.

In the formalism of Gruner and co-workers, lipid impurities can stabilize the H_{II} phase in two ways (Kirk et al., 1984; Kirk & Gruner, 1985; Gruner, 1985). The first is by reducing R_0, the spontaneous radius of curvature of the host lipid system. R_0 is the radius of curvature that the lipid monolayers of a system would adopt in the absence of other constraints under given conditions. The principal constraint is that of having to pack the interstices between monolayer cylinders or spheres with hydrophobic moieties. In one-component systems, these interstices must be filled by entropically disfavored stretching of the phospholipid acyl chains. This is an unfavorable contribution to the H_{II}-phase chemical potential. Kirk and Gruner (1985) inferred that R_0 can be measured in H_{II}-phase-forming systems when the lipid is doped with low levels of alkane to relax the interstice packing constraint. Lipids that form stable lamellar phases have near-infinite values of R_0, while lipids that form the H_{II} phase have small values of R_0. The tendency of a system to form inverted phases is increased by additions of components or changes in conditions that decrease R_0. R_0 is often very nearly equal to the radius of the water channels

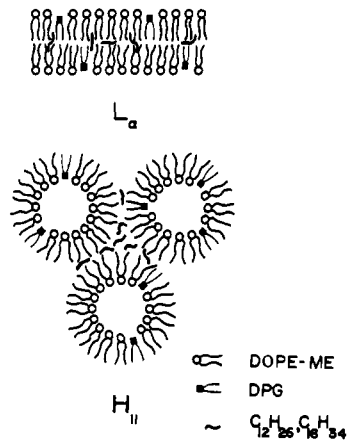

FIGURE 6: This drawing shows the inferred positions of low levels of long-chain alkanes and diglycerides in the L_α (top) and H_{II} (bottom) phases. Hexadecane, dodecane, and squalane tend to accumulate in the center of L_α-phase bilayers. Hexadecane and dodecane molecules intercalate between the lower portions of the phospholipid acyl chains to some extent and on the average are arrayed with their long axes roughly perpindicular to the plane of the bilayer. Squalane resides predominantly among the methyl terminals of the phospholipid acyl chains. 1,2-Dipalmitoylglycerol is in the monolayer leaflets with chains roughly parallel to the phospholipid chains. In the H_{II} phase, the alkanes reside at the periphery of H_{II} tubes and in the hydrophobic interstices between them. Alkanes may intercalate between phospholipid acyl chains to a smaller extent than in the L_α phase. Diglycerides remain at the lipid/water interfaces, roughly aligned with the phospholipid molecules. In this drawing, the mole fraction of alkane and diglyceride has been exaggerated.

in the H_{II} phase under these circumstances (Gruner et al., 1986). Therefore, changes in R_0 are reflected in changes in the H_{II}-phase lattice constant (the H_{II} tube diameter).

The second way in which hydrophobic impurities can stabilize the H_{II} phase is by partitioning into the interstices between H_{II} tubes. This relieves the entropically disfavored stretching of the phospholipid chains. If this formalism is a correct description of H_{II}-phase stability, traces of hydrophobic lipids that stabilize the H_{II} phase (reduce T_H) either should reside in the interstices of the H_{II} phase or should reduce R_0.

Dodecane, Hexadecane, and Squalane. The ^{2}H NMR data obtained from L_α-phase samples containing dodecane-d_{26} and hexadecane-d_{34} are consistent with the results of earlier studies (Pope et al., 1984; Jacobs & White, 1984a,b; Pope & Dubro, 1986; Sjölund et al., 1987) indicating that alkanes tend to reside within the central region of the bilayer, with the time-averaged orientation of the alkyl chains roughly parallel to the acyl chains of the phospholipid. The quadrupole splittings in spectra of dodecane-d_{26}-containing samples are somewhat larger than for hexadecane-d_{34}-containing samples at the same temperature (Figure 2D vs Figure 2A; Figure 3A vs Figure 3C). This may reflect a more effective exclusion of hexadecane from the region of the bilayer close to the headgroups of the phospholipids when the temperature is far above the chain-melting temperature of DOPC. Close to the headgroups, the order parameters of the phospholipid acyl chains are larger, and the motion of alkane chains should be more restricted. This relative exclusion of hexadecane is also consistent with the lower solubility of hexadecane in L_α-phase bilayers relative to dodecane [e.g., see Pope and Dubro (1986)].

The ^{2}H NMR powder patterns from samples of alkane-containing H_{II} phases are several times narrower than the powder patterns in comparable L_α phases at the same temperature (Figure 3). This difference is more pronounced for dodecane-d_{26} (ca. 6-fold reduction) than for hexadecane-d_{34} (ca. 5-fold reduction). In both cases, the reduction is con-

siderably more than the 2-fold reduction that accompanies the change from lamellar to cylindrical geometry on going to the H_{II} phase. The magnitude of the quadrupole splitting in these spectra is directly proportional to the local order parameters of the C^2H_2 groups of the alkane. The alkanes exist in a substantially more disordered environment in the H_{II} phase.

In this work, we assume that the time-averaged quadrupole splittings in our ^2H NMR spectra are directly proportional to the line widths and that changes in line widths reflect differences in the order parameters, not differences in the rate or type of motion of the C^2H_2 groups. However, if the C^2H_2 groups are undergoing intermediate time-scale motions (10–100 kHz), the line widths would not be related to the quadrupole splittings in a simple way. While we have no evidence that such intermediate time-scale motion is occurring, we cannot rule out this possibility.

The decrease in alkane order in H_{II}-phase samples is partly the result of a general decrease in the order parameters of phospholipid CH_2 groups in the H_{II} phase relative to the L_α phase (Hardmann, 1982; Sternin et al., 1988). The decrease in order parameter is greater the further the CH_2 group is from the phospholipid headgroup. Sternin et al. (1988) measured the order parameters of perdeuterated tetradecanol in POPE L_α and H_{II} phases. Tetradecanol is oriented parallel to the phospholipids in these phases, and presumably the order parameters of the different tetradecanol C^2H_2 groups reflect the order parameters of the phospholipid CH_2 groups that form their immediate environment. Sternin et al. (1988) showed that the H_{II}-phase quadrupole splittings of the deuterons on the 6th, 10th, and 14th carbons of tetradecanol are ca. 37, 32, and 24% of the L_α-phase values at the same temperature, respectively. [These values are obtained by multiplication of their values of $R(n)$ with the normalization factor $(S_H)_{max}/(S_L)_{max} = 1/2.4$. $R(n)$ is the ratio of splittings in each phase normalized to the maximum splitting in that phase. The normalization factor is the ratio of the maximum quadrupole splitting observed in the H_{II} and L_α phases, respectively.]

By comparison, the widths of the ^2H NMR powder patterns of dodecane-d_{26} and hexadecane-d_{34} that we observe in H_{II}-phase samples are only 15 or 20% of the widths in the L_α phase (Figure 3). The maximum (unresolved) splittings in these powder patterns must be reduced by a similar amount. Since only the CH_2 groups close to the methyl terminals of the phospholipid molecules suffer such a reduction in order parameter during the L_α/H_{II}-phase transition (Hardmann, 1982; Sternin et al., 1988), the perdeuterated alkanes must, on average, be localized to the perimeter of the H_{II} tubes and to the hydrophobic interstices between H_{II} tubes (Figure 6). Sjölund et al. (1987, 1989) also concluded that alkanes existed predominantly in these regions within H_{II} phases. This location of perdeuterated alkanes is consistent with their stabilization of H_{II} phases by the interstice packing effect described by Gruner and co-workers.

The difference in apparent order parameter of hexadecane-d_{34} between H_{II} and L_α phases is larger than the difference for dodecane-d_{26} (Figure 3). The widths of the ^2H NMR powder patterns of L_α- and H_{II}-phase samples containing these alkanes also indicate that hexadecane-d_{34} exists in a more disordered environment in both phases relative to dodecane-d_{26}. These data show that hexadecane-d_{34} partitions more extensively into the more disordered regions in the phase lattices. One would expect a bulky, branched molecule like squalane to partition even more extensively into the disordered regions of L_α- and H_{II}-phase lattices, as do isoprenoid lipids of similar structure [e.g., see Simon et al. (1977), Lai and Schutzbach (1986), and de Ropp et al. (1987)]. Figure 3E shows that squalane is in a more disordered environment than the other alkanes in the L_α phase, and Figure 3F shows that squalane is mostly in an environment as disordered as that of hexadecane in the H_{II} phase (compare with Figure 3D). It is therefore interesting to note that hexadecane and tetradecane are substantially more effective in reducing T_H on a volume-for-volume basis than dodecane (Table I) and that squalane is twice as effective as dodecane. These data imply that low levels of alkanes are more effective in stabilizing H_{II} phases the more extensively the alkanes partition into the most disordered regions of the L_α- and H_{II}-phase lattices.

This trend is easily rationalized in terms of the theory of Gruner and co-workers (Kirk et al., 1984; Kirk & Gruner, 1985). The hydrophobic interstices between H_{II} tubes are surrounded by the methyl terminal groups of the phospholipids, the most disordered region of the phospholipid acyl chains in this phase (Hardmann, 1982). Therefore, these regions (and the periphery of the H_{II} tubes) will be the loci of greatest methylene group disorder. In a series of different alkanes at low alkane concentrations, the alkane that partitions most extensively into the disordered regions of the H_{II} lattice should be most effective in filling these interstices and therefore most effective in reducing T_H. This is what we observe in the behavior of dodecane, hexadecane, and squalene.

The alkane chain length dependence of H_{II}-phase stabilization noted in this work is similar to the dependence reported by Sjölund et al. (1987, 1989). Our interpretation of the causes underlying this dependence is basically similar. Sjölund et al. used very large amounts of alkane (alkane/lipid ratios of 1/1 mol/mol or greater) to induce H_{II}-phase formation in lecithins. Lecithins do not form the H_{II} phase in the absence of alkanes. They also proposed that the alkanes in their systems induce H_{II}-phase formation by packing the interstices between H_{II} tubes. They reported that dodecane and hexadecane were generally about equally effective in stabilizing H_{II} phases but that longer chain length alkanes were less effective. However, Sjölund et al. (1989) also pointed out that, at the high levels of alkanes they employed, alkanes might stabilize H_{II} phases by a second mechanism, as well. Enough alkane might partition into the phospholipid bilayers to stabilize H_{II} phases by reducing R_0. In their systems, large amounts of alkane in an L_α-phase bilayer would expand the hydrophobic region of the bilayer with respect to the hydrophilic headgroup region, inducing each leaflet of the bilayer to "curl up" and form H_{II}-phase tubes. The solubility of long-chain alkanes in lecithin bilayers decreases with increasing chain length [e.g., see Pope and Dubro (1986)]. Longer chain length alkanes should be less effective at reducing R_0 in this manner, as they observed. Thus, we think that the different alkane chain length dependence they observed is due to this effect of alkanes on R_0 in their systems.

In contrast, in our system and under our conditions, it is clear that the alkanes do not stabilize H_{II} phases by reducing R_0. The data of Gruner et al. (1988) and the data in Table II show that low levels of alkanes slightly increase the H_{II} lattice constant in our phospholipid system, DOPE-Me (Gruner et al., 1988). This means that, if anything, R_0 is very slightly larger in the presence of alkanes, not smaller. DOPE-Me is different from lecithin in that it can form the H_{II} phase spontaneously in the absence of alkane. The small levels of alkanes that we add represent a very minor perturbation in phase structure. Hence, in our system and under our conditions, alkanes stabilize the H_{II} phase only by partitioning into the disordered lipid regions in H_{II}-phase lattices.

The increased effectiveness of longer chain length alkanes in our system (Table I) is consistent with this interpretation. The longer chain compounds are less soluble in the lipid monolayers of the two phases, more of the alkane is available to pack the interstices of the nascent H_{II} phase, and the alkane is more effective in reducing T_H on a volume-for-volume basis.

1,2-Dipalmitoylglycerol. The data in Figure 4 indicate that DPG-d_{62} molecules are oriented like phospholipids in the L_α phase, with acyl chains roughly parallel to those of the host phospholipid and glycerol moiety oriented at the lipid/water interfaces. The H_{II}-phase ^2H NMR powder pattern is qualitatively similar to the L_α-phase pattern, except that a greater percentage of the power is distributed close to the center of the pattern and the width of the resonance is only about 40% of the width of the L_α-phase pattern. These differences are consistent with DPG-d_{62} being oriented like phospholipid molecules in the H_{II} phase, as well. The powder pattern in Figure 4B resembles powder patterns of perdeuterated tetradecanol in H_{II} phases of POPE, in which tetradecanol would also be expected to orient in this manner.

Since the headgroups of DPG-d_{62} molecules reside at the lipid/water interfaces in both phases, DPG-d_{62} cannot stabilize H_{II} phases via the same mechanism as alkanes. The results of X-ray diffraction studies (data presented in Table II; Siegel et al., 1989; Gruner et al., 1988) indicate that low levels of dipalmitin and other diglycerides reduce the H_{II}-phase lattice constants of DOPE-Me/diglyceride systems relative to the constant of alkane-doped DOPE-Me at the same temperatures. The latter systems should have the equilibrium (temperature-dependent) values of R_0, because traces of long-chain alkane have removed the interstice packing constraint on R_0 (Kirk & Gruner, 1985). This implies that the diglycerides stabilize H_{II} phases by reducing R_0 (Kirk et al., 1984; Gruner, 1985).

Our X-ray diffraction data show that 2.0 mol % DPG-d_{62} reduces the H_{II} lattice constant of DOPE-Me by 0.2 nm relative to the value in dodecane-doped DOPE-Me (Table II). A similar reduction is observed when both DPG-d_{62} and alkane are added to the same sample of DOPE-Me (data not shown); 0.2 nm is a small reduction in lattice constant, but the water channel radius under these conditions is ca. 2 nm, so that this change might represent a 10% reduction in R_0. X-ray diffraction data show that other diglycerides induce larger changes [Table II; data from Siegel et al. (1989)]. The glycerol headgroup seems to be the moiety most responsible for this effect of diglycerides (Siegel et al., 1989).

Conclusions

Low concentrations of hydrophobic lipids and lipid metabolites can substantially lower T_H of phospholipid systems via two different mechanisms. These mechanisms are easily rationalized in terms of the formalism of Gruner and co-workers. Low concentrations of moderate- to long-chain alkanes stabilize nascent H_{II} phases by partitioning into the hydrophobic interstices between H_{II} tubes. The alkanes that are most thoroughly excluded from the more ordered regions of the phospholipid monolayers are the most effective at lowering T_H. Bulky, branched molecules like squalene (and, probably, polyisoprenoid lipids like dolichol) are particularly effective. Diglycerides stabilize H_{II} phases by reducing the spontaneous radius of curvature of the phospholipid interfaces, R_0.

Transient production of hydrophobic lipid metabolites in vivo may represent an important mechanism by which cells control the properties of biomembranes. Proximity to L_α/H_{II}-phase transitions has been shown to affect membrane fusion rates (Ellens et al., 1986, 1989) and may also affect other dynamic processes. Addition of 2 mol % of diglycerides like those produced via phosphoinositide hydrolysis in vivo lowers the T_H of some systems by 20 °C and greatly increases the rate of membrane fusion via a mechanism associated with inverted-phase formation (Siegel et al., 1989). Dolichol-induced inverted-phase formation in membranes [e.g., see Valtersson et al. (1985) and van Dujn et al. (1986)] may be related to dolichol activity as a saccharide carrier in protein glycosylation. Lipid metabolites can very substantially change the properties and dynamics of membranes even if they are present at concentrations of 1 mol % of the membrane lipid.

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Thermodynamics of Intersubunit Interactions in Cholera Toxin upon Binding to the Oligosaccharide Portion of Its Cell Surface Receptor, Ganglioside G_{M1}[†]

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ABSTRACT: The binding and the energetics of the interaction of cholera toxin with the oligosaccharide portion of ganglioside G_{M1} (oligo-G_{M1}), the toxin cell surface receptor, have been studied by high-sensitivity isothermal titration calorimetry and differential scanning calorimetry. Previously, we have shown that the association of cholera toxin to ganglioside G_{M1} enhances the cooperative interactions between subunits in the B-subunit pentamer [Goins, B., & Freire, E. (1988) *Biochemistry 27*, 2046–2052]. New experiments presented in this paper reveal that the oligosaccharide portion of the receptor is by itself able to enhance the intersubunit cooperative interactions within the B pentamer. This effect is seen in the protein unfolding transition as a shift from independent unfolding of the B promoters toward a cooperative unfolding. To identify the origin of this effect, the binding of cholera toxin to oligo-G_{M1} has been measured calorimetrically under isothermal conditions. The binding curve at 37 °C is sigmoidal, indicating cooperative binding. The binding data can be described in terms of a nearest-neighbor cooperative interaction binding model. In terms of this model, the association of a oligo-G_{M1} molecule to a B protomer affects the association to adjacent B promoters within the pentameric ring. The measured intrinsic binding enthalpy per protomer is −22 kcal/mol and the cooperative interaction enthalpy −11 kcal/mol. The intrinsic binding constant determined calorimetrically is 1.05×10^6 M^{-1} at 37 °C and the cooperative Gibbs free energy equal to −850 cal/mol. These studies provide the first direct thermodynamic description of the cooperative binding of a protein to its cell surface receptor and have allowed us to place energetics constraints on the putative changes in protein conformation triggered by the intrinsic receptor binding process.

Cholera toxin consists of a five-subunit ring, the B-subunit pentamer (M_r = 58 000), which surrounds the dimeric A-subunit (M_r = 27 000) (Gill, 1976). The B-subunit pentamer binds to ganglioside G_{M1} present on the outer surface of the cell membrane, and subsequently the A-dimer penetrates the cell membrane, where it activates adenylate cyclase. Ganglioside G_{M1} plays an active part in the processing of the toxin after binding. Previous studies in this laboratory have shown that the association of cholera toxin to ganglioside G_{M1} enhances the cooperative interactions within the B-subunit pentamer (Goins & Freire, 1988). In the absence of ganglioside G_{M1} the unfolding transition of intact cholera toxin or isolated B pentamer in aqueous solution is a process characterized by little or no intersubunit cooperative interactions. Upon binding to ganglioside G_{M1} micelles or to lipid membranes containing ganglioside G_{M1}, the unfolding process becomes highly cooperative and the pentameric B-subunit ring effectively behaves as a single cooperative unit. The molecular origins of this cooperative enhancement have not been identified and could be triggered either by the attachment of the toxin to the membrane or micellar surface or by intrinsic interactions arising from the binding of the oligosaccharide region of the ganglioside to the toxin molecule. To address this issue, we have isolated the oligosaccharide region of ganglioside G_{M1} and studied its association to cholera toxin as well as the effects of this association on the behavior of the protein. This experimental system allows one to dissect in-

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