Observation of Inverted Cubic Phase in Hydrated Dioleoylphosphatidylethanolamine Membranes†

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ABSTRACT: We report the observation of an inverted *cubic* phase in aqueous dispersions of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) by small-angle X-ray diffraction. DOPE is a paradigm in the study of nonlamellar phases in biological systems: it exhibits a well-known phase transition from the lamellar (L_α) to the inverted hexagonal phase (H_{II}) as the temperature is raised. The transition is observed to occur rapidly when a DOPE dispersion is heated from 2 °C, where the L_α phase is stable, to 15 °C, where the H_{II} phase is stable. We report on the induction of a crystallographically well-defined cubic lattice that is slowly formed when the lipid dispersion is rapidly cycled between −5 and 15 °C hundreds of times. Once formed, the cubic lattice is stable at 4 °C for several weeks and exhibits the same remarkable metastability that characterizes other cubic phases in lipid–water systems. X-ray diffraction indicates that the cubic lattice is most consistent with either the *Pn*3*m* or *Pn*3 space group. Tests of lipid purity after induction of the cubic indicate the lipid is at least 98% pure. The cubic lattice can be destroyed and the system reset by cycling the specimen several times between −30 and 2 °C. The kinetics of the formation of the cubic are dependent on the thermal history of the sample, overall water concentration, and the extreme temperatures of the cycle. The presence of cubic phases may be a general feature of H_{II}-forming lipids and is discussed in terms of the spontaneous radius of curvature of lipid monolayers [Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665–3669].

A characteristic property of biological lipid–water systems is that they exhibit a variety of bilayer and nonbilayer phases as the temperature, pressure, and water content are varied. A well-known example of a non-bilayer-forming lipid is 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE;[1] also known as dioleoylphosphatidylethanolamine). DOPE–water dispersions are known to readily undergo a lamellar (L_α) to inverted hexagonal (H_{II}) thermotropic phase transition (Cullis et al., 1985; Gagne et al., 1985; Kirk & Gruner, 1985; Tate & Gruner, 1987).

More exotic nonlamellar lipid phases, such as cubic phases, are known from the early classic work of the Luzzati group [e.g., see Luzzati (1968) and Luzzati et al. (1987)]. Many such cubic liquid-crystalline phases are now known to consist of bicontinuous regions of water and hydrocarbon (Longley & McIntosh, 1983; Hyde et al., 1984; Charvolin, 1985; Rilfors et al., 1986; Luzzati et al., 1987). Because many of the cubic phases were seen in monoglycerides (Larsson, 1983), which are not majority constituents of most cell membranes, in exotic lipids extracted from thermophilic bacteria (Gulik et al., 1985; Luzzati et al., 1987), or at low water concentrations, cubic phases have received relatively little attention from most lipid researchers. When they have been observed, the cubic phases of the inverted (water in oil) type have always been seen to lie between the L_α and H_{II} phases (Seddon et al., 1984; Charvolin, 1985; Rilfors et al., 1986). This has led to several clever mechanisms for the L_α to H_{II} transition that invoke metastable cubic phases (Siegel, 1987). However, not every lipid that exhibited the L_α to H_{II} transition appeared to exhibit a cubic phase.

In this paper, we report the observation of cubic phases in aqueous dispersions of DOPE and several related lipids. Once formed, the cubic phases are stable for many weeks. The cubic phase in DOPE is most stable near the L_α–H_{II} transition temperature but has not been previously reported because induction of the cubic structure involves a complicated thermal history of the sample. The observation of a cubic phase in a well-studied lipid, such as DOPE, which has become a paradigm for lipids exhibiting the L_α to H_{II} transition, leads to the suggestion that underlying cubic states may be universal features of all lipids that normally exhibit inverted phases, such as the H_{II} phase. This has implications for both a thermodynamic understanding of inverted phases and the presence of biomembrane bilayer lipid species which, in pure form, exhibit inverted phases.

Materials and Methods

DOPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE), and 1,2-dioleoyl-*sn*-glycero-3-phospho-*N*-methylethanolamine (DOPE-Me) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The lipid was assayed by thin-layer chromatography (TLC) and found to be at least 98 mol % free of fatty acid and lysolipids. The lipid-containing chloroform stock solution

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[1] Abbreviations: DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPE-Me, 1,2-dioleoyl-*sn*-glycero-3-phospho-*N*-methylethanolamine; DEPE, 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine; TLC, thin-layer chromatography; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; L_α, lamellar liquid-crystalline phase; H_{II}, inverted hexagonal phase; d_c, unit cell spacing of cubic phase.

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was dried under a stream of dry nitrogen gas. The dry lipid was dissolved in cyclohexane (about 0.5 mg/μL), and the solution was then transferred to a previously weighed 1.5-mm X-ray glass capillary. The lipid solution was then lyophilized in the capillary and weighed. Deionized water was added directly to the lipid, the capillary was weighed to determine the lipid/water ratio, and the top of the capillary was sealed. The sample was cycled between −30 and 20 °C several times to ensure mixing of the lipid and water. Prior to each experiment, the sample was frozen deep into the gel state (−30 °C) as a fiducial starting state.

X-ray diffraction was performed with nickel-filtered Cu Kα radiation (wavelength 1.54 Å) generated on a Rigaku RU-200 microfocus generator. X-rays were focused by using Franks optics and slit collimation to provide a beam of dimensions 0.2 mm × 2 mm at the specimen. The diffraction pattern was recorded with image-intensified, slow-scan-TV two-dimensional detectors (Gruner et al., 1982). A two-dimensional X-ray diffraction pattern could be typically obtained in a 60–600-s exposure. The cubic phase space group assignments required very accurate determinations of very closely spaced X-ray diffraction lines. In these cases X-ray film exposures (Kodak DEF X-ray film, Rochester, NY) were acquired. Typical exposure times for 200-mm specimen to film distances were 50 h. Short TV-detector exposures before and after the film exposures demonstrated that the samples were stable over the course of the film exposures.

The specimen temperature was set by a home-built, programmable, thermoelectrically controlled specimen stage with a temperature slue rate of about 1 °C/s.

A typical protocol for thermally cycling specimens to induce cubic structures was as follows: A sealed X-ray capillary with the DOPE–water sample was placed in the specimen stage with the temperature set to 2 °C. A 60-s X-ray diffraction pattern was taken to ascertain that the lipid was in the L_α phase. The sample temperature was then set to −5 °C. After waiting for 2 min, the temperature was set to 15 °C, at which point another short X-ray exposure showed the lipid to be predominantly in the expected H_{II} phase. After waiting another 2 min, the temperature was reset to −5 °C. Calibrations performed with a thermocouple embedded in an X-ray capillary showed that, during the 2-min waiting period, the sample took 25 s to reach the set temperature. The thermal cycle was then repeated several hundred times. A 60-s X-ray exposure was taken at −5 °C every tenth cycle. Finally, the sample temperature was set to 2 °C, and extensive X-ray diffraction analysis was performed. We found that the pattern was stable at this temperature for at least several weeks.

The two-dimensional powderlike diffraction patterns recorded by the image-intensified detectors were azimuthally integrated to one-dimensional intensity vs scattering angle plots by integrating circular arcs within ±10° of a line perpendicular to the long dimension of the X-ray beam cross section. The patterns recorded on X-ray film were densitometrized by scanning along the same axis with a short strip aperture. The large cubic lattice spacings, calibrated against dry lead stearate, are estimated to be absolutely accurate to ±1 Å.

^{31}P NMR spectra were obtained by using a Bruker WP-200 spectrometer operating at 81 MHz for ^{31}P. Thermally cycled samples, sealed in X-ray capillaries, were placed in a 10-mm NMR tube, and 1 mL of ^2H_2O was added for the purpose of maintaining deuterium lock. Spectra were accumulated for up to 2000 transients employing a 19-μs 90° pulse, a 20-kHz sweep width, and 1-s interpulse delay in the presence of high-power (>5-W) broad-band proton decoupling. An exponential multiplication corresponding to 10-Hz line broadening was applied to the free induction decay prior to Fourier transformation.

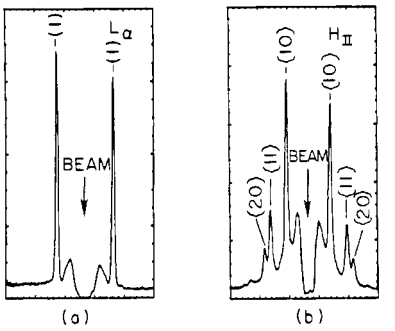

FIGURE 1: Azimuthal integrations of the two-dimensional X-ray diffraction patterns of aqueous DOPE (a) at 2 °C, when the system is in the L_α phase, and (b) at 15 °C, when the system is in the H_{II} phase. The arrow indicates the position of the unscattered X-ray beam, blocked by a beam stop. The first-order L_α peak indicated by the vertical lines in (a) is at (52.0 Å)$^{-1}$. In (b) lines indicate peaks whose positions index to an H_{II} lattice with first-order peak at (66.2 Å)$^{-1}$. The left–right asymmetry in peak intensity arises from the fact that no detector flat-field correction was applied to the data.

RESULTS

DOPE at −5 °C is initially in the lamellar (L_α) phase (Figure 1a). At 15 °C, if the sample is allowed to reach equilibrium, it goes completely into a hexagonal (H_{II}) phase (Figure 1b) (Kirk & Gruner, 1985; Tate & Gruner, 1987). When it is cycled between these two temperatures, initial X-ray exposures taken at −5 °C show diffraction patterns that consist of lines characteristic of the lamellar as well as an undercooled hexagonal phase. As the cycling is continued for several tens of cycles, new lines appear in the X-ray diffraction pattern along with broad features characteristic of specimen material that lacks long-range order (Figure 2a–c). As the cycling is continued further, the lamellar peaks decrease in intensity, and the new lines increase in intensity (Figure 2d,e) and sharpen. Eventually, the lines due to the H_{II} phase also disappear, leaving diffraction peaks that index to a cubic lattice (Figures 2f and 3a). The positions of the Bragg peaks are spaced in the ratios of $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}$, consistent with both the *Pn*3*m* and *Pn*3 space groups (Figure 3b) (*International Tables for X-ray Crystallography*, 1968). Although the cubic phase is stable at 4 °C for at least several weeks, stability on longer time scales is problematic because the lipids degrade. The sample can be reset by several freeze–thaw cycles to −30 °C. By contrast, when the cubic sample is heated to above room temperature it does not go completely to H_{II}.

Figure 4 shows the results of a phosphorous nuclear magnetic resonance (^{31}P NMR) study of a hydrated DOPE sample at 4 °C that was thermally cycled into a cubic phase. The spectrum reveals a narrow isotropic resonance of full width at half-height ~170 Hz, similar to signals seen with other phospholipids in cubic phases (Rilfors et al., 1986). ^{31}P NMR signals from phospholipids in the H_{II} or L_α phases are asymmetric and are broader than 4 kHz (Cullis et al., 1985).

After inducing a cubic phase, the purity of samples was checked in several ways. One sample was reset by deep cooling to −30 °C into the gel phase. This destroyed the cubic phase. The sample was then warmed to room temperature, and an X-ray diffraction pattern was taken. The resultant pattern was characteristic of an H_{II} phase with a unit cell spacing of 74 Å, which is 1 Å short of the equilibrium unit cell spacing of a freshly prepared, fully hydrated DOPE sample at 20 °C. Upon mechanical agitation with additional water, X-ray

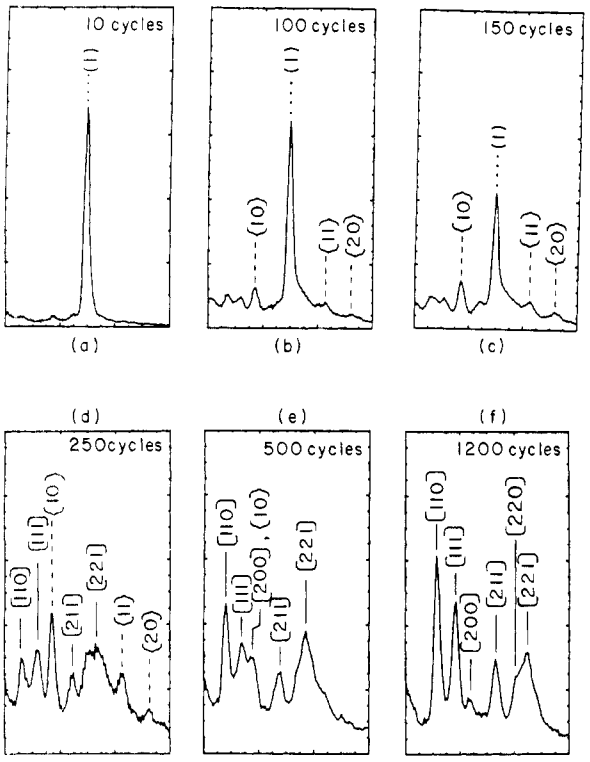

FIGURE 2: Sequence of azimuthal integrations of the two-dimensional X-ray diffraction patterns of aqueous DOPE after successive thermal cycling across the L_α–H_{II} transition, as described in the text. The primary, unscattered beam is past the left edge of each pattern. The patterns shown were all recorded at −5 °C after the number of cycles indicated in the figures. The dotted line indicates the position of the first-order L_α peak. Dashed lines indicate peaks that index to the undercooled H_{II} phase. Solid lines indicate peaks that index to the cubic phase (see Figure 3).

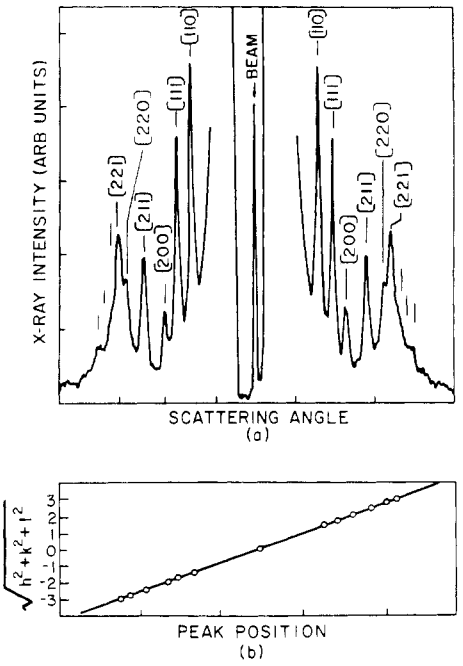

FIGURE 3: (a) Densitometrization of an X-ray film exposure of DOPE after 1200 thermal cycles across the L_α–H_{II} transition as described in the text. The vertical tic marks indicate the expected peak positions for the best fitting *Pn3m*/*Pn3* lattice of unit cell spacing 122 Å. (b) $(h^2 + k^2 + l^2)^{1/2}$ vs measured positions of the peaks in panel a. The spacing between the lines fall in the ratios of $\sqrt{2}$:$\sqrt{3}$:$\sqrt{4}$:$\sqrt{6}$:$\sqrt{8}$:$\sqrt{9}$ as would be expected of a *Pn3m* or *Pn3* cubic space group.

diffraction indicated the unit cell spacing returned to the expected value of 75 Å at 20 °C.

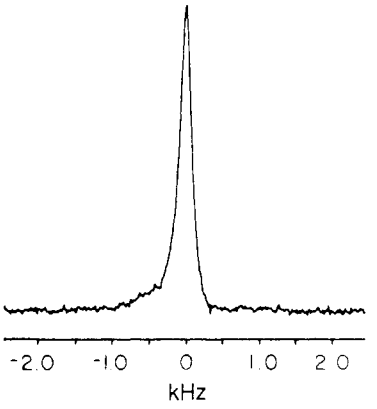

FIGURE 4: ^{31}P NMR spectrum of hydrated DOPE after successive thermal cycling across the L_α–H_{II} transition as described in the text. The ^{31}P NMR spectra were obtained at 81.0 MHz in the presence of proton decoupling. The line width at half-height is about 170 Hz. The presence of a narrow isotropic resonance is similar to ^{31}P NMR spectra of other phospholipids in cubic phases (Rilfors et al., 1986). Lipids in both H_{II} and L_α phases would exhibit asymmetric signals that are broader then 4 kHz (Cullis et al., 1985).

TLC performed on representative samples after formation of the cubic phase showed the lipids were at least 98 mol % pure. Calibration of the TLC procedure by the addition of known amounts of oleic acid and lysolipid showed that the TLC analysis cannot rule out a contamination with fatty acid or lysolipid of less than 2 mol %. The effect of a small amount (<2 mol %) of fatty acid on aqueous DOPE has not, to the best of our knowledge, been studied before. We have performed experiments in which 2 mol % each of fatty acid and lysolipid was deliberately added to DOPE dispersions, and the mixture did *not* spontaneously go cubic. Although we cannot rule out fatty acid and lysolipid contamination of <2 mol % due to lipid degradation in our samples, we believe that the presence of the cubic phase in our thermally cycled samples is not merely due to chemical contamination but is due to the presence of thermally generated physical defects (see Discussion). This does not, however, exclude the possibility that trace amounts of contaminants can alter the kinetics of the cubic transition.

The rate of conversion of the lamellar and hexagonal lattices into the cubic phase depends in a complex way on the water content of the sample. It appears that the greater the amount of water, the slower the rate of conversion. At 50% by weight of water, a distinct cubic phase was obtained after 500 cycles. When the amount of water was 65% by weight, the specimen appeared disordered, and even several thousand cycles were insufficient to obtain an unequivocal cubic phase. However, at 30% water by weight there was no indication of either progressive disorder or cubic phase formation. No systematic work has yet been done to determine the lowest water concentration at which a cubic phase may be obtained by thermal cycling.

Although the *Pn3m*/*Pn3* cubic diffraction pattern has been consistently obtained with many different DOPE specimens, the unit cell spacing varies from specimen to specimen. Even for single specimens, the unit cell spacing is seen to decrease slowly as the cycling is continued. The reasons for this variation in spacing and its dependence on the thermal history of the specimen are not understood.

Similar experiments were performed on DEPE and DOPE-Me (Gruner et al., 1988). Although the details of the cycling across the L_α–H_{II} transition were different, the qualitative features are similar in that thermally cycling these samples induced cubic phases. It is significant that, in the case

of DOPE-Me, small amounts of degraded lipid affected the transition kinetics.

Discussion

The existence of the previously unrecognized cubic phase reported above is surprising because DOPE is such a well-characterized and often-studied lipid. The cubic is most stable near the L_α–H_{II} transition region of the DOPE phase diagram, indicating that the free energy surface topography of DOPE in this region is considerably more complex than hitherto believed. The ease with which the L_α–H_{II} transition occurs suggests that a relatively small energy barrier separates these phases. By contrast, the difficulty involved in settling the system into or out of the cubic phase suggests that the corresponding free energy valley is isolated by relatively large kinetic barriers. If we assume that the topological genus of the lipid–water interfaces of the L_α, H_{II}, and cubic structures is each different, then one may speculate that the energy barriers are related to the energetic cost of tearing this interface and exposing hydrocarbon to water.

It has been demonstrated elsewhere that the L_α–H_{II} phase transition may be understood as a competition between the tendency for lipid monolayers to assume a spontaneous radius of curvature, R_0, and the way the lipid chains pack to fill the hydrocarbon volume (Kirk et al., 1984; Kirk & Gruner, 1985; Gruner, 1985; Tate & Gruner, 1987). Gruner et al. (1986) demonstrated that the spontaneous radius of curvature of a fully hydrated H_{II} phase is very nearly equal to the radius of the central water cores. It was shown that the energetic cost for modest variations of the mean radius of the water cores is proportional to the square of the difference between the actual and spontaneous curvatures. This is consistent with (at least the non-Gaussian term of) a functional form of the surface energy density suggested by Helfrich (1973), where the surface over which the energy density is to be integrated is near the lipid head groups. Therefore, the lipid monolayers in the H_{II} phase are nearly relaxed with respect to curvature, but due to the constraints of the H_{II} geometry, the lipid chains are in an energetically unfavorable configuration. Conversely, in the lamellar phase, the chain-packing energy is assumed to be low, but at the cost of a deformation of the head group surface away from its desired spontaneous curvature. Much of the mesomorphic behavior of lipid systems can be understood by the recognition that this balance of competing energies shifts toward favoring the H_{II} over the L_α geometry as the R_0 value of a system approaches zero (Gruner, 1985; Tate, 1987; Gruner et al., 1988).

However, as explained in Kirk et al. (1984), this does not exclude the possibility of geometries other than L_α and H_{II} which, for a given value of R_0 and chain characteristics, might be a more favorable compromise between curvature and hydrocarbon chain packing. What is sought is a geometry whereby the head group surface varies little from a curvature value of $1/R_0$, where the hydrocarbon–head group surface is continuously intact so as not to expose the chains to water, and whereby the thickness of the lipid monolayers varies little from a relaxed chain length. Recently, it has been shown that, in certain cubic phases with $Pn3m/Pn3$ symmetry, the chain-packing energy can be lower than for the H_{II} phase and the curvature energy of the lipid–water interface can be smaller than for the lamellar phase (Anderson et al., 1988). Therefore, there can exist a range of parameters for which the total free energy per lipid molecule, consisting of both curvature and chain-packing contributions, is lower in the cubic phase than in either the H_{II} or L_α phases. In these cases, stable cubic phases may be expected. However, certain cubic structures

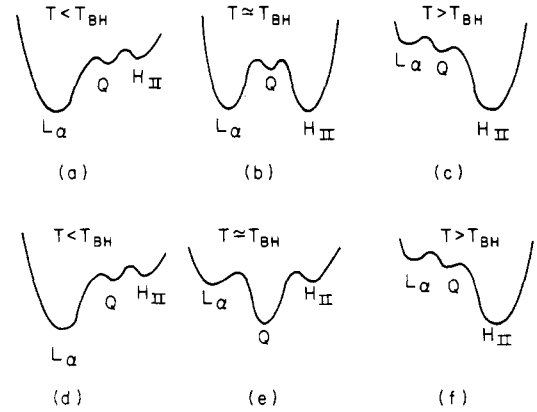

FIGURE 5: Schematic illustration of the Landau–Ginzburg picture of the free energy vs a generalized transition coordinate for a transition involving the three phases, L_α, cubic, and H_{II}, as the temperature is varied. Panels a–c illustrate the case when the cubic phase is never a global minimum in the free energy profile. Panels d–f indicate conditions under which the cubic phase is a global minimum.

may represent local minima in the free energy, but not global minima; in these cases, metastable cubic structures are expected.

The situation may be modeled under the hypothesis that a Landau–Ginzburg picture describes the free energy wells of the various phases (Landau & Lifschitz, 1969). In this picture, the free energy is assumed to depend on only one temperature-dependent parameter (such as R_0). Each phase is represented by a minimum in the free energy profile. The relative depths of the minima and the rates at which these depths change with temperature are a smooth function of R_0 and other parameters of the system, such as the length of hydrocarbon tails. For one set of parameters the progression of well depths may be as illustrated schematically in Figure 5a–c. Note that the cubic need not necessarily be a ground-state phase (i.e., a global minimum in the free energy) at any temperature and is only a metastable phase. For other values of the parameters, the situation is illustrated in Figure 5d–f. Here, as the temperature is raised, one has a succession of ground-state phases corresponding to L_α, cubic, and, finally, H_{II} phases. It is possible that the relative depth of the cubic well can be varied from the situation of metastability (Figure 5a–c) to stability (Figure 5d–f) by altering continuous parameters of the system that control the relative rates of change of the well depths with temperature. Investigations of the methylated analogues of DOPE have led us to suggest that R_0 is such a parameter (Gruner et al., 1988).

Methylating the head group of DOPE raises the L_α–H_{II} transition temperature and increases R_0. The relative magnitudes of R_0 are small for DOPE, intermediate for DOPE-Me, and largest for trimethylated PE or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). DOPE is stable in the H_{II} phase, and DOPC is stable in the L_α phase at room temperature. DOPE-Me, with values of R_0 intermediate between DOPE and DOPC, exhibits complex nonequilibrium disordered and cubic phases without extensive thermal cycling (Gruner et al., 1988). We note that a 3:1 mixture of DOPE/DOPC (wt:wt) behaves similar to DOPE-Me, suggesting that the value of R_0 may be adjusted by altering the mole fractions of PE and PC. This suggests that R_0 may be regulated by changing lipid composition in biological systems. A recent study of Lindblom et al. (1986) demonstrates that widely varying lipid compositions of *Acholeplasma laidlawii A* membranes exhibited nearly identical transition temperatures from the L_α to H_{II} or cubic phases. Further experiments are needed to measure R_0 of the lipids extracted from these bacterial membranes to test whether

R_0 is being held constant by the bacteria.

In the Landau–Ginzburg picture, the observation of three phases (stable and metastable) requires that the free energy profile have at least three minima. The situation may be more complicated than this, however. The disorder seen en route to the formation of the cubic, as well as the slow evolution of the cubic unit cell spacings, suggests that the well corresponding to the cubic state may itself be composed of many local subwells; i.e., the system exhibits glasslike behavior. Metastable trapping into these subwells may account for the complex history-dependent cubic behavior.

The formation of the DOPE cubic phase is sensitive to the rate and temperature extremes of the thermal cycling process across the L_α–H_{II} transition temperature. Siegel (1987) has suggested that cubic structures may arise from topological defects of the membrane surface that are generated during the L_α–H_{II} transition. We speculate that the average density of lipid–water interface defects grows as one dithers about the L_α–H_{II} transition, as long as the temperature excursion is not so extreme as to anneal out the defects. Eventually, the defects percolate to form a new lattice that corresponds to a cubic phase. The details of the intermediate structures and whether the defects do, in fact, percolate await further experimentation.

Conclusion

Speculations on the possible biological relevance of cubic phases have so far been restricted to conditions under which a lipid–water system actually exhibits the cubic phase in vivo. It has been speculated that cubic phases are involved in fat digestion and in the promotion of catalysis in the membranes of thermophilic bacteria (Luzzati et al., 1987). Therefore, the cubic phases may be functionally important in some metabolic processes.

Our work has shown that cubic phases can form in lipids that are common in mammalian membranes. This opens up speculation along the line that was advanced by Gruner (1985). Even if a lipid–water system is lamellar in vivo, if it goes nonlamellar at higher temperatures than encountered in vivo, *the interactions responsible for the phase transition are still present*, although with different magnitudes, at the physiological temperature. These interactions, which determine curvature and chain packing, for instance, affect the properties of the bilayer and may have physiological consequences. Along these lines, the recent demonstration by Lindblom et al. (1986) that widely varying lipid compositions of *A. laidlawii A* membranes exhibit nearly identical transition temperatures out of the lamellar phase to hexagonal or cubic phases may be an example of bacteria homeostatically regulating the spontaneous radius of curvature R_0, as proposed by Gruner (1985). If nature, indeed, does regulate this parameter, then it is likely that the value of R_0 has consequences for the bacterial bilayers at the growth temperature of the organism.

Added in Proof

After submitting this paper, we became aware of a report at the March 1988 meeting of the Biophysical Society (Veiro & Rowe, 1988) of the observation of an isotropic component, similar to our Figure 5, in the phosphorus NMR spectrum in thermally cycled DEPE sample.

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