Lipid Polymorphism

C.P.S. TILCOCK AND P.R. CULLIS

Department of Biochemistry
The University of British Columbia
Vancouver, British Columbia V6T 1W5, Canada

INTRODUCTION

While it is well established that the vast majority of the combined lipids within any given biological membrane form bilayer (lamellar) structures, it is clear that the bilayer structure of membranes is not immutable. For example, the phenomenon of membrane fusion, whether intercellular (as in fertilization, myogenesis, or the formation of bone polykaryocytes), intracellular (as in the lysosomal degradation of phagocytic vacuoles or the release of vesicles from the Golgi), or occurring at the level of the plasma membrane (during such processes as the release of secretory products, endocytosis, or cell division), requires the transient, controlled destabilization of bilayer structure at the fusion site.

Within this context it is of interest that many of the lipids of biological membranes, both singly and also in mixtures with other lipids, can adopt nonlamellar (*e.g.*, hexagonal H_{II}, inverted micellar, etc.) structures in response to physiologically relevant variables such as pH, ionic strength, or the distributed presence of divalent cations and proteins. In addition, exogenous lipophilic agents such as local anesthetics and (of particular relevance to this symposium) short- and long-chain alcohols can also influence lipid polymorphism. These structures may be of relevance to membrane contact and fusion as well as the packing properties of lipids in bilayers.

In this article we provide an overview of the polymorphic phase behavior of lipids.

NUCLEAR MAGNETIC RESONANCE DETERMINATION OF LIPID PHASE STRUCTURE

Individual phospholipids can adopt a variety of phases upon hydration including lamellar (bilayer), hexagonal H_I and H_{II}, micellar, inverted micellar, or cubic, dependent upon factors such as the nature of the lipid headgroup, the unsaturation or degree of side-branching of the lipid acyl chains, water content, temperature, pH, ionic strength, or the presence of divalent cations, other lipids, or polypeptides and proteins.[1] The essential structural features of the familiar lamellar (bilayer) and hexagonal H_{II} phases are shown in FIGURE 1 together with representative ^{31}P NMR spectra and also freeze-fracture electron micrographs. The H_{II} phase is comprised of hexagonally packed lipid cylinders in which the lipids are oriented with their headgroups towards central aqueous channels (~20 Å diameter).

While X ray and neutron diffraction are the definitive techniques for the determination of lipid phase structure, both ^{31}P NMR and ^{2}H NMR may be used to conveniently monitor lipid phase behavior. A detailed discussion of any of these techniques lies beyond the scope of this article. However, see REFERENCE 1 and the many review articles cited therein for a discussion of methodology. Each technique possesses advantages and disadvantages. For example, NMR does not tell of structure directly, but rather provides information concerning the motional properties of the

ensemble, which may then be correlated with structure. NMR is therefore used in an extrapolative manner, based upon direct structural determination by X ray or neutron diffraction techniques. As examined by ^{31}P NMR, all hydrated phosphodiester lipids in large (> 400-nm diameter) bilayer structures exhibit an asymmetric line shape with a low-field shoulder and high-field peak separated by 40 to 50 ppm. The actual

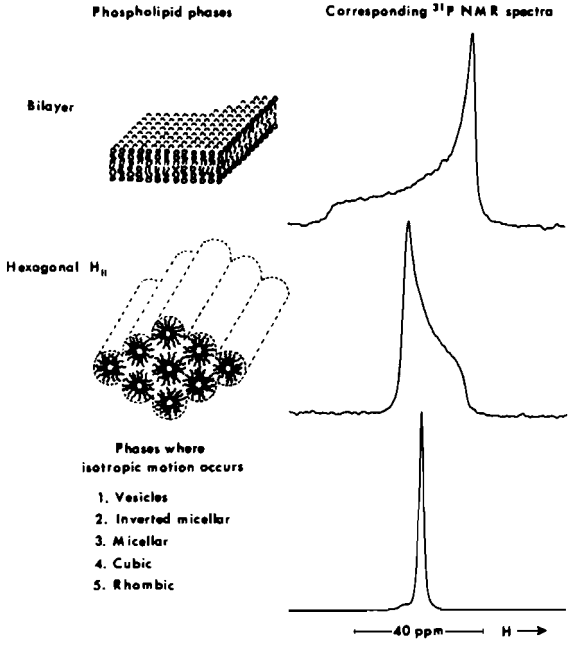

FIGURE 1. ^{31}P NMR and freeze-fracture characteristics of phospholipids in various phases. The bilayer ^{31}P NMR spectrum was obtained from aqueous dispersions of egg yolk phosphatidylcholine, and the hexagonal H_{II} phase spectrum from phosphatidylethanolamine (prepared from soybean phosphatidylcholine). The ^{31}P NMR spectrum representing isotropic motion was obtained from a mixture of 70 mol% soya phosphatidylethanolamine and 30% egg yolk phosphatidylcholine after heating to 90° C for 15 min. All preparations were hydrated in 10 mM tris-acetic acid (pH 7.0) containing 100 mM NaCl, and the ^{31}P NMR spectra were recorded at 30° C in the presence of proton decoupling. The freeze-fracture micrographs represent typical fracture faces obtained from bilayer and H_{II}-phase systems as well as structures giving rise to isotropic motional averaging. The bilayer configuration (total erythrocyte lipids) gives rise to a smooth fracture face, whereas the hexagonal H_{II} configuration is characterized by ridges displaying a periodicity of 6 to 15 mm. Common conformations that give rise to isotropic motion are represented in the bottom micrograph: (1) bilayer vesicles (~100 nm diameter) of egg phosphatidylcholine and (2), structures containing lipidic particles (egg phosphatidylethanolamine containing 20 mol% egg phosphatidylserine at pH 4).

separation is dependent on the lipid species, temperature, and other factors. This line shape is characteristic of axially averaged motion on the NMR time scale, due to rotation of the phospholipid about its long axis.[2] The resonance position of the high-field peak of an unoriented system (*e.g.*, a lipid vesicle dispersion) corresponds to bilayers lying parallel to the magnetic field, whereas the resonance position of the

shoulder corresponds to an orientation of bilayers perpendicular to the field. The asymmetry of the line shape reflects the greater probability of finding bilayers in unoriented systems lying parallel rather than perpendicular to the field.

In the hexagonal H_{II} phase, lipids experience additional motional averaging due to translational diffusion of lipid molecules around the walls of the lipid cylinders on the NMR timescale. This results in a decrease in the width of the spectrum by a factor of two and also a reversal in the sign of the chemical shift anisotropy, which is due to the fact that in the hexagonal H_{II} phase, the probability of finding a lipid cylinder parallel to the field (in which all the lipid directors are normal to the field) is less than finding a lipid cylinder normal to the field. Alternatively, in phases where motion is isotropic (*i.e.*, the lipid samples all possible orientations with respect to the applied field on the NMR timescale), ^{31}P NMR gives narrow, symmetric resonances. This occurs for lipids in structures such as small bilayer vesicles (diameter < 200 nm), micelles, inverted micelles, or phases such as cubic or rhombic.

Corresponding freeze-fracture micrographs are also shown in FIGURE 1. Lamellar phases give rise to extended smooth fracture faces, whereas hexagonal H_{II} phases exhibit rippled fracture planes. FIGURE 1 also illustrates the freeze-fracture replicas obtained from small unilamellar vesicles and from lipidic particles.[3]

^{2}H NMR may also be used to determine lipid phase structure, as illustrated in FIGURE 2 for dioleoyl phosphatidylethanolamine (PE) labelled at the C_{11} position of both acyl chains. Each deuteron gives rise to a doublet whose separation in an unoriented system depends upon the average orientation of the deuterium nucleus with respect to the applied field.[4] As the lipid undergoes a lamellar to hexagonal H_{II} transition, the additional motional averaging due to lateral diffusion of the lipid around the lipid cylinders causes a reduction in the (quadrupolar) splitting by a factor of two (or more). Particular advantages of ^{2}H NMR compared to ^{31}P NMR are that ^{2}H NMR allows quantification of order in the acyl chains and that it is possible to monitor the phase behavior of a single ^{2}H-labelled lipid in a mixed system.

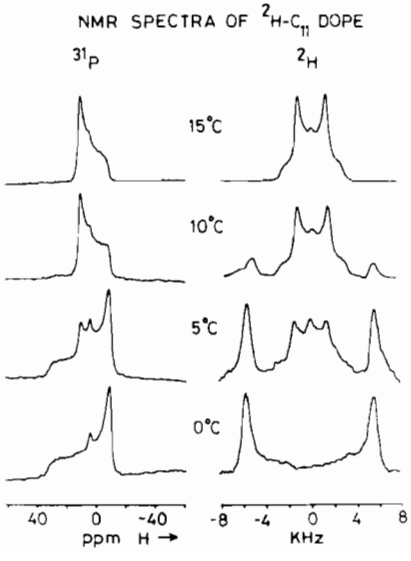

FIGURE 2. ^{31}P and ^{2}H NMR spectra as a function of temperature of fully hydrated dioleoyl phosphatidylethanolamine (DOPE), which is ^{2}H-labelled at the C_{11} position of the acyl chains ([C_{11}-$^{2}H_{2}$]DOPE). The ^{31}P NMR spectra were obtained at 81.0 MH_2 in the presence of proton decoupling, whereas the ^{2}H NMR spectra were obtained at 30.4 MHz.

TABLE 1. Lamellar to Hexagonal H_{II} Transition Temperatures for Various Synthetic and Naturally Derived Phosphatidylethanolamines

Species	Phase	Conditions
Diacyl species		
20:0/20:0	H_{II}	96° C
18:0/18:0	H_{II}	100° C
16:0/16:0	H_{II}	109–123° C
14:0/14:0	H_{II}	85° C
16:0/18:1_c	H_{II}	75° C
18:1_c/16:0	H_{II}	70° C
18:1_c/18:1_c	H_{II}	60° C
18:1_c/18:1_c	H_{II}	10° C
18:2/18:2	H_{II}	−15° C
18:3/18:3	H_{II}	−15° C
20:4/20:4	H_{II}	−30° C
22:6/22:6	H_{II}	−30° C
Egg	H_{II}	25–35° C
	L	pH 8.5, pressure
From egg PC—	H_{II}	40–45° C
E. coli	H_{II}	55–60° C
Human erythrocyte	H_{II}	8° C
Porcine erythrocyte	L + H_{II}	20–40° C, 10–90% water
Rat liver e.r.	H_{II}	7° C
Rabbit s.r.	H_{II}	0° C
Soya bean	H_{II}	−10° C
Rat mitochondrial	H_{II}	10° C
Dialkyl species		
18:1/18:1	H_{II}	80° C
16:0/16:0	H_{II}	86° C
14:0/14:0	H_{II}	93° C, excess water
		78° C, salt NaCl
12:0/12:0	H_{II}	100° C, excess water
		70° C, low water
Effect of acyl chain linkage		
Vinyl ether	H_{II}	30° C
Alkyl ether	H_{II}	53° C
Acyl ether	H_{II}	68° C

THE POLYMORPHIC PHASE BEHAVIOR OF INDIVIDUAL LIPID SPECIES

The lamellar to hexagonal H_{II} transition temperature (T_{bh}) for a variety of phosphatidylethanolamines (PE) of both synthetic and natural origin are shown in TABLE 1. Several general conclusions may be drawn. First, many species of naturally occurring PE preferentially adopt a hexagonal H_{II} phase at physiological temperatures. It is evident that for di-unsaturated species, T_{bh} increases with increasing saturation or the presence of trans-unsaturated acyl chains. For di-saturated dialkyl (ether linkage) or diacyl (ester linkage) species, T_{bh} decreases with increasing chain length. Decreasing the water content or increasing the ionic strength results in a

TABLE 2. Polymorphic Phase Preferences of Liquid Crystalline Unsaturated Lipids

Lipid	Phase Preferences	
	Physiological Conditions[a]	Other Conditions
Phosphatidylcholine	L	H_{II} low hydration and high temp
Sphingomyelin	L	
Phosphatidylethanolamine	H_{II}	L, pH 8.5 low temp
Phosphatidylserine	L	H_{II}, pH 3.5
Phosphatidylglycerol	L	H_{II}, high temp, high salt conc.
Phosphatidylinositol	L	
Cardiolipin	L	H_{II}, divalent cations, pH 3, high salt
Phosphatidic acid	L	H_{II}, divalent cations, pH 3.5, high salt
Monoglucosyldiglyceride	H_{II}	
Diglucosyldiglyceride	L	
Monogalactosyldiglyceride	H_{II}	
Digalactosyldiglyceride	L	
Cerebroside	L	
Cerebroside sulfate	L	
Ganglioside	M	
Lysophosphatidylcholine	M	
Cholesterol		Induces H_{II} phase in mixed lipid systems
Unsaturated fatty acids		Induce H_{II} phase

[a] L = lamellar, H_{II} = hexagonal, M = micellar.

decrease in T_{bh}. Alkaline pH stabilizes PE in a lamellar phase, whereas acidic conditions favor hexagonal H_{II} and cubic phases.

Many other species of lipid can also adopt nonlamellar phases under various conditions. The polymorphic phase preferences of many of the major classes of phospholipid found in biological membranes is shown in TABLE 2. It is clear that many lipids, under the appropriate conditions, in isolation adopt the hexagonal H_{II} phase. For example, low pH induces the hexagonal H_{II} phase for unsaturated phosphatidylserine (PS),[5] or the addition of calcium converts cardiolipin from a lamellar to a hexagonal H_{II} phase-preferring species.[6]

POLYMORPHISM IN MIXED LIPID SYSTEMS

While of great intrinsic interest, studies upon individual lipids are far removed from the complexity of a biological membrane. It is therefore of interest to examine the phase behavior of lipids in mixtures in order to determine how the lamellar/nonlamellar phase preferences of such systems may be modulated by physiologically relevant variables.

Phosphatidylcholine (PC) can stabilize unsaturated PEs in a lamellar phase in a manner that is dependent upon various factors including the acyl chain unsaturation, temperature, and also the molar ratio of PC to PE.[7,8] In general, increased unsatura-

tion, high temperatures, and the presence of less than 20–25 mole percent PC are all factors that favor destabilization of lamellar structure and formation of hexagonal H_{II}, cubic, or inverted micellar phases. This effect of PC is quite general in that any lipid that adopts a lamellar phase in isolation will stabilize a lamellar phase in mixtures with PE. This is illustrated in FIGURE 3 for mixtures of PE with 15–30 mole percent of various acidic phospholipids. Also shown is the ability of calcium to induce lamellar to hexagonal H_{II} transitions in those systems. For PE/PS mixtures, dependent upon the acyl chain unsaturation, calcium induces a lateral phase separation of the PS component into an anhydrous lamellar Ca^{2+}/PS complex, leaving the PE free to revert to the hexagonal H_{II} phase it preferentially adopts in isolation.[9] For PE/PG mixtures, calcium reduces the ability of PG to stabilize a lamellar phase without inducing a lateral phase separation, both lipids in the mixture participating in the lamellar-H_{II} transition.[10] The mechanism in the case of PE/PI mixtures is more equivocal, but there may be partial phase separation.[11] Since cardiolipin alone, in the presence of calcium, adopts a hexagonal H_{II} phase, addition of calcium to PE/CL results in a lamellar-H_{II} transition for both species.

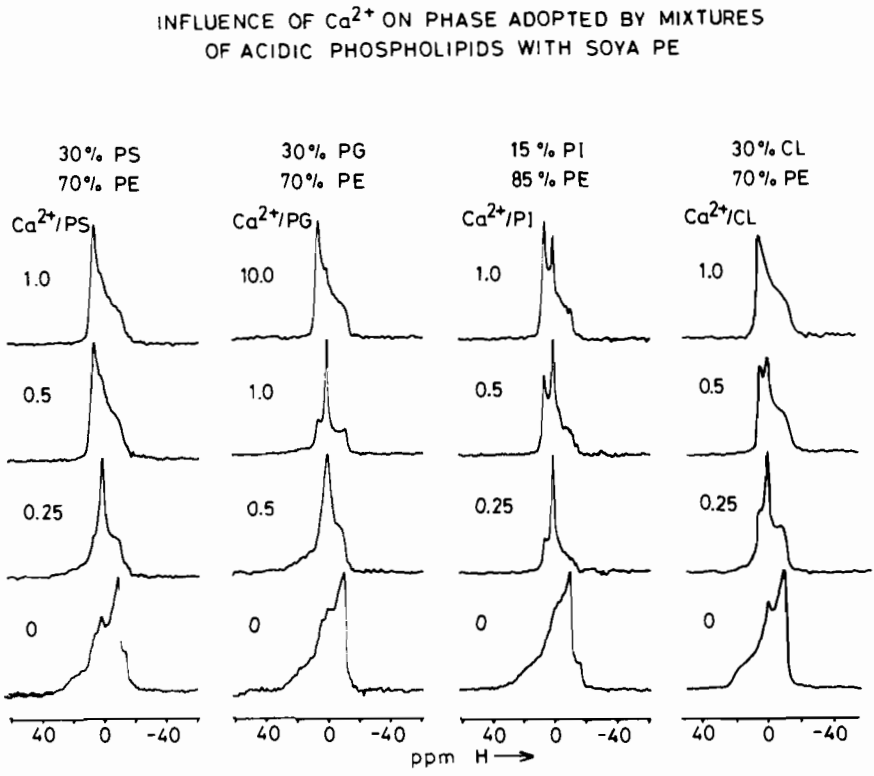

FIGURE 3. ^{31}P NMR arising from mixtures of acidic phospholipids with soya PE in the presence of various molar ratios of calcium.

EFFECTS OF CHOLESTEROL

In addition to its known ability to inhibit the formation of gel-state lipid and to decrease the permeability of lamellar systems, cholesterol is also able to destabilize lamellar structure and promote hexagonal H_{II} structure in a variety of unsaturated lipid mixtures. This is illustrated for a dioleoyl PE/dioleoyl PC (4:1) mixture in FIGURE 4. It can be seen that 20 mole percent cholesterol completely induces hexagonal H_{II} structure and that even as little as 2 mole percent can perturb the phase behavior. This figure also shows that ^2H NMR spectra from [C_{11}-$^2H_2$]-DOPC/DOPE (1:4) and DOPC/[C_{11}-$^2H_2$]-DOPE (1:4) mixtures are very similar, indicating that the DOPE and DOPC partition equally amongst the lamellar, hexagonal H_{II}, and isotropic phases, *i.e.*, on the NMR time scale, cholesterol does not exhibit a preferential association with either lipid component. The presence of cholesterol in PE/PS systems is also known to modify the response of such systems to divalent cations. First, the lamellar phase is destabilized at lower molar ratios of calcium to PS than in the absence of cholesterol. Second, magnesium can also induce hexagonal H_{II} phase structure, an effect not observed in the absence of cholesterol, and third, cholesterol inhibits the ability of divalent cations to induce lateral phase separations.[9,12] The bilayer destabilizing effects of cholesterol are quite general in that various sterols such

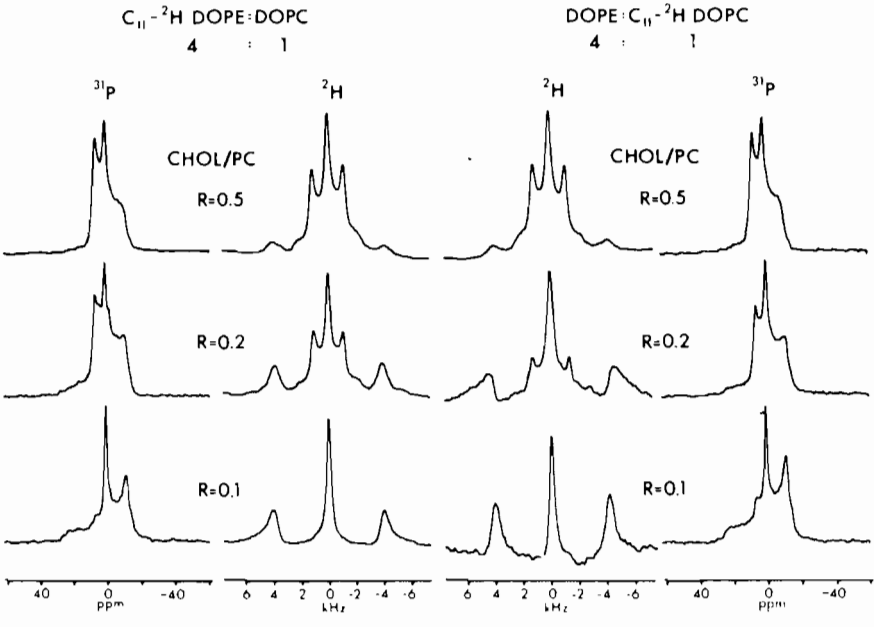

FIGURE 4. 81.0-MHz ^{31}P NMR and 30.7-MHz ^2H NMR spectra at 30° C arising from aqueous dispersions of mixtures of DOPE, DOPC, and cholesterol (CHOL) at a DOPE/DOPC molar ratio of 4:1 where either the DOPE is ^2H-labelled at the C_{11} position ([C_{11}-$^2H_2$]DOPE) or the DOPC is ^2H-labelled at the C^{11} position ([C_{11}-$^2H_2$]DOPC). The ratio R refers to the molar ratio of cholesterol to DOPC.

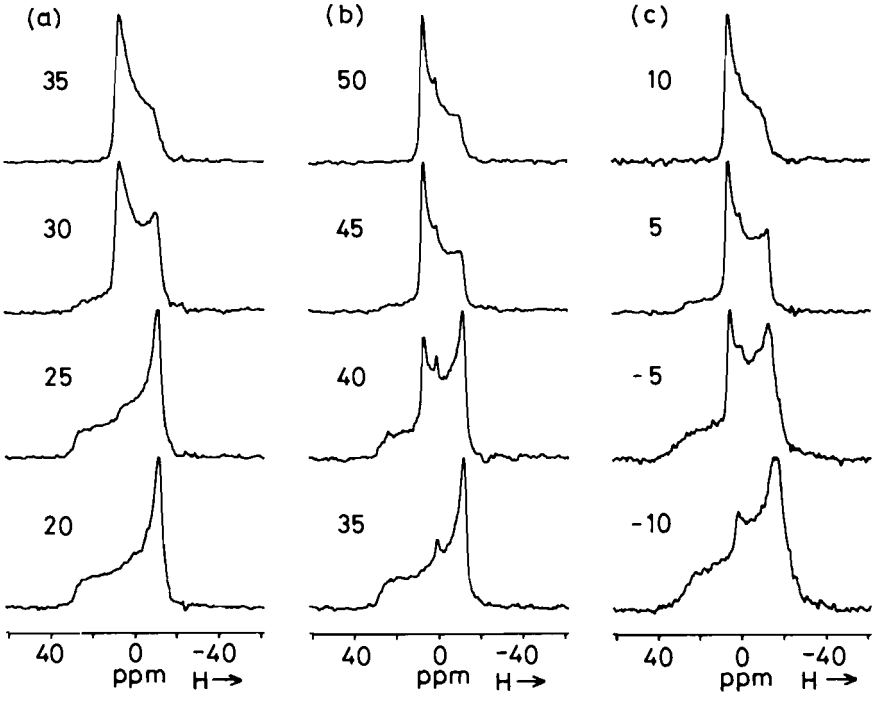

FIGURE 5. 81.0-MHz ^{31}P NMR of egg PE at the indicated temperatures, (**a**) in the absence of alcohols, (**b**) in the presence of ethanol (ethanol to lipid molar ratio = 4.5), and (**c**) in the presence of decanol (decanol to lipid molar ratio = 0.45).

as ergosterol, coprostanol, epicoprostanol, stigmasterol, and androstanol can all induce the same effects (Cullis and Tilcock, unpublished observations).

EFFECTS OF ANESTHETICS AND ALCOHOLS

The local anesthetics, dibucaine and chlorpromazine, can induce hexagonal H_{II} phase structure in mixtures with unsaturated cardiolipin or phosphatidic acid.[13,14] Alternatively, in mixtures with unsaturated PE, chlorpromazine, dibucaine, tetracaine, and procaine all stabilize lamellar structure.[15] The amount of anesthetic required to induce these effects depends upon the lipid composition, since the presence of acidic phospholipids can markedly increase the partition coefficient of positively charged anesthetics.

The effect of ethanol and decanol on the phase behavior of egg PE is shown in FIGURE 5. In the absence of alcohol, egg PE exhibits a T_{bh} of approximately 30° C. Addition of ethanol to give an ethanol/phospholipid molar ratio of 4.5 caused an increase in T_{bh} to about 42° C, whereas addition of decanol to a molar ratio of 0.45 resulted in a decrease in T_{bh} to 5° C. The effect of various alcohols upon the T_{bh} of egg PE is shown in FIGURE 6. Shorter chain alcohols ($C \leq 4$) may be considered to stabilize

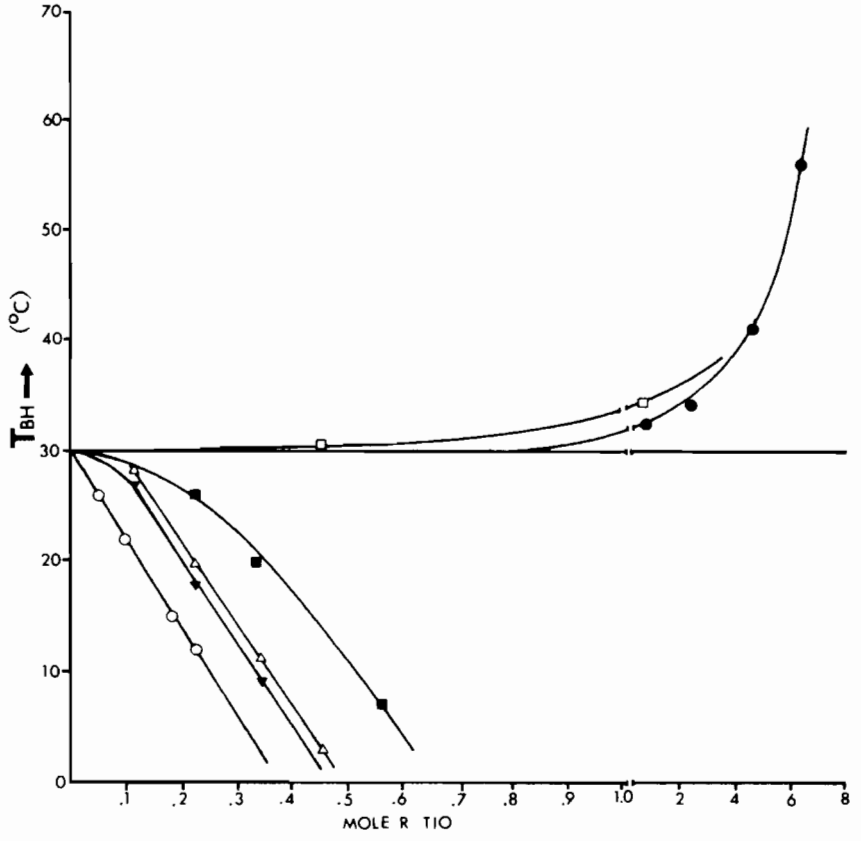

FIGURE 6. Influence of varying amounts of normal alcohols on the bilayer to hexagonal H_{II} phase transition temperature (T_{bh}) of egg PE. T_{bh} is estimated as that temperature where 50% of the lipid is in a lamellar organization and 50% is in the hexagonal H_{II} phase: ●-ethanol; □-butanol; ■-hexanol; ▼-octanol; △-decanol; O-lauryl alcohol.

lamellar structure in that they cause an increase in T_{bh}, whereas longer chain alcohols ($C > 6$) promote formation of the hexagonal H_{II} phase at lower temperatures.

LIPIDIC PARTICLES

Mixtures of lipids such as PC, which preferentially adopts a lamellar phase in isolation, and PE, which adopts the hexagonal H_{II} phase, often exhibit narrow isotropic ^{31}P NMR spectra that are associated with the appearance of lipidic particles as visualized by freeze-fracture techniques.[16] Lipidic particles are thought to represent intrabilayer inverted micellar structures, as illustrated in FIGURE 7, which have been

suggested to arise as intermediates in the lamellar-H_{II} transition. Such lipidic particles have been postulated to occur during membrane fusion, as illustrated in FIGURE 8.

THE MOLECULAR BASIS OF LIPID POLYMORPHISM

To a first approximation, the phase behavior of lipids may be rationalized in terms of their dynamic molecular shape, as illustrated in FIGURE 9. It may be considered that

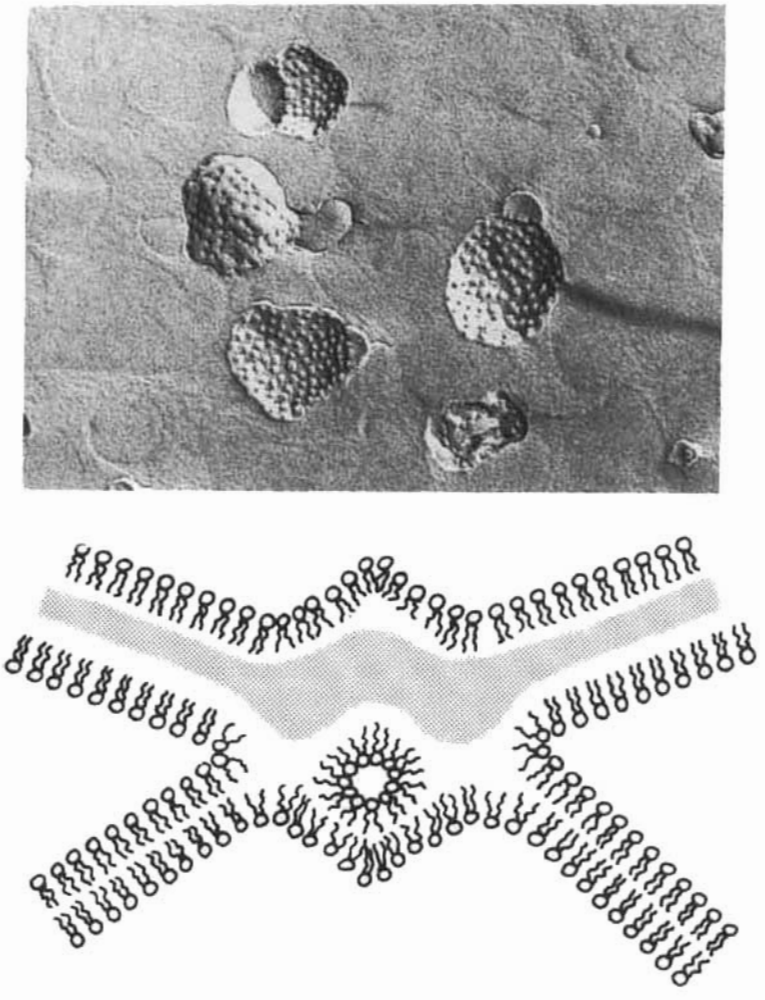

FIGURE 7. Freeze-fracture micrograph of lipidic particles induced by calcium in a cardiolipin/soya PE (1:4) mixture. A model of the lipidic particle as an inverted micelle is depicted *below* the micrograph. The *shaded area* represents the fracture region.

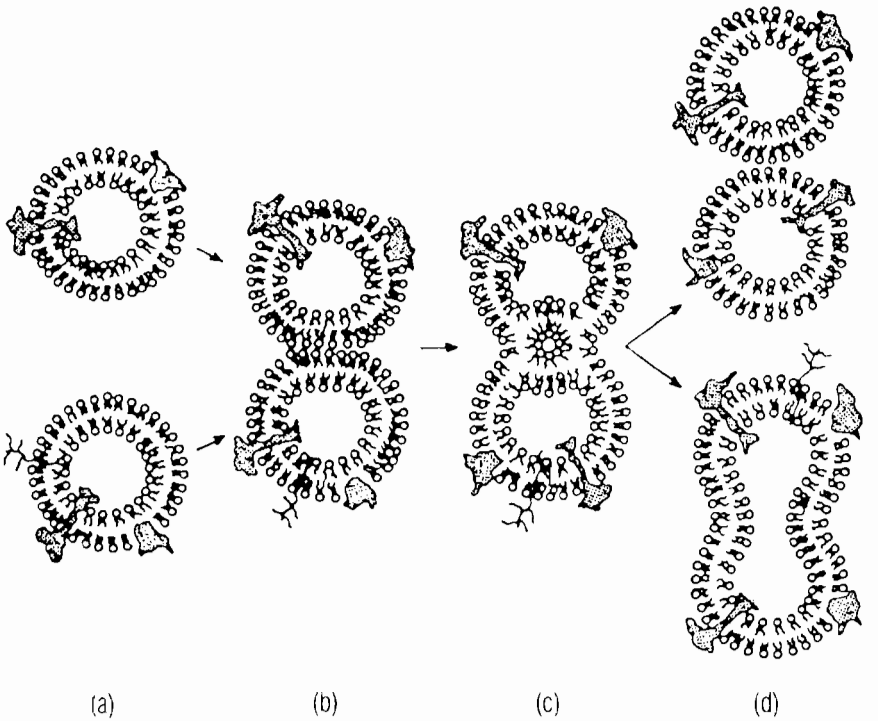

FIGURE 8. Proposed mechanism of membrane fusion proceeding via an inverted cylinder or inverted micellar intermediate.

lipids such as lysophosphatidylcholines possess a dynamic cross section that is inverted conical, the area subtended by the headgroup at the membrane/water interface being greater than that subtended by the acyl chains. On purely geometric grounds one might expect that such lipids would pack into micellar or hexagonal H_I aggregates so as to minimize hydrocarbon/water contacts. Lipids such as phosphatidylcholine or phosphatidylserine at neutral pH may be considered to possess a cylindrical dynamic cross section and would thus pack most readily into lamellar assemblies. Alternatively, lipids such as PE or PS at pH 3 (where the charge on the headgroup would be suppressed), may be considered to possess a conical dynamic cross section with the area subtended by the headgroup being less than that of the acyl chains. Such lipids would most readily adopt inverted structures such as inverted micellar or hexagonal H_{II}.

This simple rationale provides a qualitatively reasonable explanation for lipid phase behavior. In the case of PEs, increasing temperature leads to increased entropic splay of the acyl chain region, *i.e.* the swept hydrophobic volume increases relative to the headgroup, an effect that would favor a lamellar-H_{II} transition. Similarly for PS or phosphatidic acid, low pH results in protonation of the headgroup and an effective decrease in the effective headgroup area.

One prediction of this shape hypothesis is that mixtures of lipids that adopt the hexagonal H_{II} phase in isolation, and those that adopt micelles in isolation, should form

lamellar structures. This has been demonstrated to occur for mixtures of egg PE and various detergents,[17] as illustrated in FIGURE 10.

It is important to recognize that the dynamic shape is a consequence of many interrelated factors such as the size and motion of the lipid headgroup and acyl chains, the extent of headgroup hydration and charge, temperature, counterion binding, hydrogen bonding associations, and various other factors. Also the dynamic shape is an

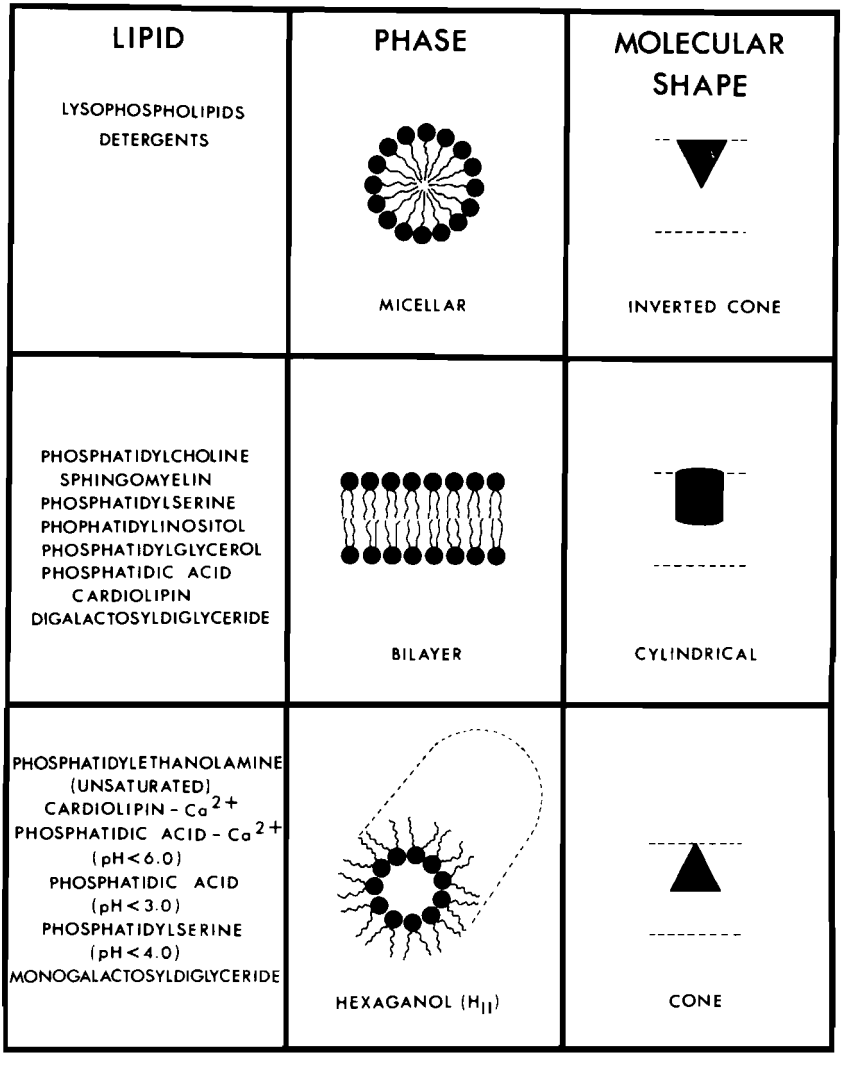

FIGURE 9. Polymorphic phases and corresponding dynamic molecular shapes of component lipids.

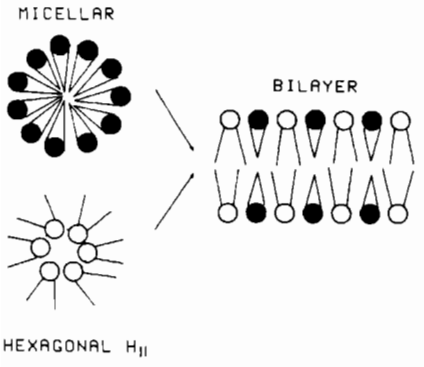

FIGURE 10. A net bilayer structure arising from mixtures of cone-shaped (H_{II} phase) lipids and inverted cone (micellar) lipids due to shape complementarity effects.

ensemble property, not an intrinsic property of a given lipid, and is thus modulated by interaction with surrounding lipids. This is illustrated in FIGURE 11, which shows the effect of dioleoyl PE on the quadrupole splitting (ΔQ) of deuterium-labelled C_{11}-dioleoyl PC. With increasing PE content in the mixture, ΔQ increases, indicating increased hydrocarbon chain order, at least at the C_{11} position. This effect may be rationalized by considering that the acyl chains of the "conical" PE molecules

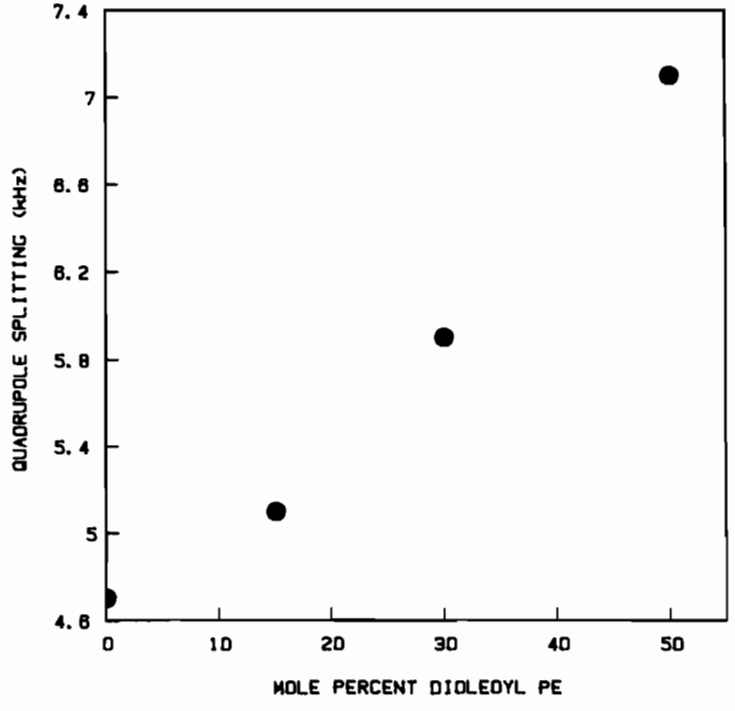

FIGURE 11. Effect of dioleoyl PE on the quadrupole splitting of $^2H_2$-C_{11}-dioleoyl PC at 30° C.

compress the surrounding PC acyl chains, in order to minimize water penetration into the hydrocarbon region that would otherwise be exposed.

SUMMARY

The phase behavior of phospholipids may be monitored using ^{31}P or ^{2}H NMR techniques, which provide information concerning the motional properties of the lipid ensemble, which may then be correlated with structure.

The lamellar/nonlamellar phase preferences of many lipids, either synthetic or naturally derived, may be controlled by factors such as variation in temperature, hydration, or of greater physiological relevance, pH, ionic strength, the presence of divalent cations such as calcium, or the presence of lipid soluble agents such as anesthetics and alcohols. The ability of short-chain alcohols to stabilize a bilayer structure for egg PE may be rationalized in terms of the packing of lipids whose dynamic shapes are complementary, as illustrated in FIGURE 11. On this basis, short-chain alcohols would partition preferentially at the membrane/water interface and would thereby stabilize a lamellar structure. Larger-chain alcohols may partition deeper into the hydrophobic acyl chain region in order to minimize hydrocarbon/water contact and so may perturb the acyl chain packing, increasing the effective swept volume of the chains and so promoting hexagonal H_{II} phase formation.

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DISCUSSION OF THE PAPER

B. CHANCE (*University City Science Center, Philadelphia, PA*): There is a transition in mitochondrial oxidative phosphorylation below 15 degrees. At about 12 degrees they refuse to accept adenosine diphosphate, for example. Have you tried to correlate structure and function?

CULLIS: We have tried to do that in the case of membrane fusion. The ability of lipids to adopt these different structures, particularly different phase structures such as the hexagonal phase, is mainly related in terms of function to processes such as fusion where you have membrane contact.

L. L. M. VAN DEENEN (*State University of Utrecht, Utrecht, The Netherlands*): Why are the effects of ethanol and decanol on PE of opposite nature? You did not give an explanation for this. Could these differences be explained by differences in the shapes of these molecules? The opposite effects of ethanol and decanol may be tied in with your explanation for lipid polymorphism.

CULLIS: These differences are quite compatible with the shape explanation. The ability of ethanol to reside just inside the membrane/water interface would be consistent with its having approximately an inverted cone shape. This would stabilize the bilayer, whereas the octanol would partition further into the membrane, increasing the hydrocarbon chain area thereby promoting hexagonal H_{II} phase organization.

D. B. GOLDSTEIN (*Stanford University School of Medicine, Stanford, CA*): Your diagram of membrane fusion (FIG. 8) shows that the phospholipids from the external membrane monolayer form the hexagonal H_{II} phase. PE is usually located in the inner monolayer. Does that bother you and do you have an explanation for this?

CULLIS: If one is worried about fusion in an intracellular context, then it doesn't bother me, because the PE is on the right side of the membrane. With regard to cell-cell fusion, if one had a process that put two cells in very close apposition, that would entail removing water from that region. Such dehydration promotes nonbilayer structure.

GOLDSTEIN: So you wouldn't need PE for that?

CULLIS: No, you wouldn't. The presence of cholesterol could be quite sufficient.