Ann. Rev. Biophys. Biophys. Chem. 1985. 14 : 211–38

LIPID POLYMORPHISM: The Molecular Basis of Nonbilayer Phases

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CONTENTS

PERSPECTIVES AND OVERVIEW

The successful operation of biological systems depends on the synthesis of various component molecules and their subsequent assembly into functional units. Tremendous progress has been made in understanding the regulated expression, information content, and structure of DNA and

0084–6589/85/0615–0211$02.00

RNA. The process of folding newly synthesized polypeptides into functional proteins is less well understood, although we at least have good information about the structures that are formed. The biological membrane is perhaps the most complicated basic assembly found in the cell. In this case, our understanding of the forces governing composition, structure, and function is relatively primitive.

The first step in developing an understanding of the form and mechanism of biomembranes is to develop a coherent picture of the basic structural unit, namely, the lipid bilayer. Even this relatively limited objective presents enormous difficulties that arise from the chemical diversity of lipids and the number of forces that must be considered. The process has been further complicated by the range of physical variables (e.g. "fluidity") that have been used to characterize bilayers.

Given the difficulties involved, it is not surprising that increasing interest has recently focussed on understanding the rich variety of macroscopic structures adopted by relatively simple lipid mixtures upon hydration. The polymorphic phase preferences of lipids are exquisitely sensitive to the lipid species employed and have been shown to be modulated by biologically relevant parameters such as hydration, local ion composition, and membrane protein. This leads to the exciting possibility that a detailed molecular understanding of the polymorphism of pure and mixed systems will lead to an understanding of the interplay between diverse lipids in a bilayer environment; furthermore, nonbilayer structures themselves may play regulated roles in membrane-mediated events. In this work we discuss what is currently known concerning the molecular basis of lipid polymorphism. In particular, the statistical mechanics of lipids in bilayers is increasingly well characterized. However, little work has been done to extend such formalism to nonbilayer systems. In our view, such investigations are of basic interest and importance to a variety of disciplines.

TERMINOLOGY

Lipid-water mesophases are of interest to a diverse set of disciplines, including biology, chemistry, engineering, and physics. Prior to discussion of the mesophases, it is important to define some basic terms because words, such as "liquid crystalline," have different meanings in different disciplines. Lipid-water systems represent a subclass of lyotropic liquid crystals. The word "lyotropic" conveys the information that the systems have more than one component (e.g. lipid and water) and that the resulting structure depends on the ratios of the components. "Liquid crystalline" materials have the crystalline aspect of periodicity in one, two, or three dimensions, but the molecules are not rigidly locked in a lattice: The molecules exhibit

liquid-like diffusion, although, characteristically, the degrees of diffusional freedom are anisotropic. As an example, consider the L_α phase (Figure 1*a*), which consists of stacked lipid bilayers with intervening sheets of water. This structure is periodic, when viewed in a direction perpendicular to the bilayers, and exhibits repetitive successions of bilayers and water. However, within the plane of a bilayer, the lipid molecules are free to diffuse laterally. As the thermodynamic variables of the system, such as temperature or water concentration, are varied over a limited range, the structural dimensions of the aggregate will vary smoothly through a succession of equilibrium thermodynamic states while still maintaining a recognizable semblance to a single phase. For example, removing water from the L_α phase will result in a shorter one-dimensional repeat owing to thinner water sheets. However, if the thermodynamic variables are varied too far, there will often appear a distinctly different phase, representative of a cooperative, structural rearrangement in the system. For example, the removal of too much water from a L_α phase of certain phosphatidylethanolamines will result in the formation of the H_n phase (Figure 1*d*). The system is said to have undergone a phase transition and is exhibiting another mesomorphic

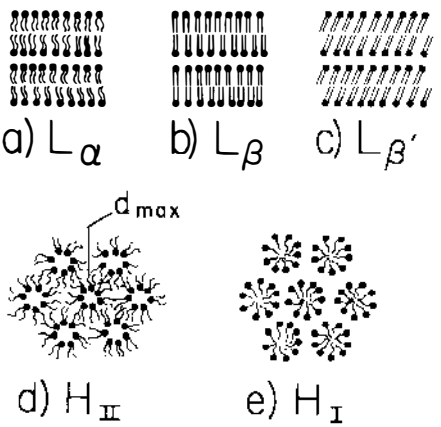

Figure 1 Cross-sectional views through a number of lipid-water phases. Lipids are represented by a dark circle for the headgroup attached to one or two hydrocarbon chains. Blank areas adjacent to the headgroups, in all cases, represent water areas. In the L_α phase (*a*), the chains are melted; this phase is most representative of the state of the lipids in biomembranes. If the temperature of a L_α lecithin phase is lowered, the chains will freeze and typically assume L_β and $L_{\beta'}$ forms (*b* and *c*, respectively). Phosphatidylethanolamine L_α phases convert to H_{II} phases (*d*) as the temperature is raised. In this form, the lipid forms long water-cored tubes that stack on a two-dimensional lattice. The reverse situation is observed with single-chain lecithins (*e*), in which the lipid tubes are surrounded on the outside with water.

phase or polymorph. The words "mesomorphism" and "polymorphism" both denote the fact that the system may assume several structurally distinct phases. For instance, diamond and graphite are different mesomorphic phases of carbon. In general, lyotropic liquid crystals exhibit extraordinarily rich polymorphic behavior.

In the context of lipids containing hydrocarbon chains, the words "liquid crystalline" are additionally used to specifically denote phases in which the hydrocarbon is melted, i.e. in which the chains contain many *gauche* rotamers. For instance, the L_β (Figure 1) to L_α transition is often called a gel (stiff chain) to liquid crystalline (melted chain) phase transition. This firmly entrenched usage is unfortunate because it would seem to imply that the frozen chain phase does not exhibit molecular diffusion characteristic of liquid crystals in a more general sense. In fact, molecular diffusion in many stiff chain mesomorphs (e.g. L_β) is still high compared to common crystals (56). Moreover, truly crystalline phases often represent yet additional mesomorphs (67).

General characteristics of lyotropic liquid crystals have been well reviewed by a number of authors (3, 18, 44, 89). The field is bedeviled by a lack of a consistent nomenclature. Especially good references with regard to nomenclature are Luzzati (47), Brown et al (3), and Tardieu et al (78).

A comment, and caution, on the meaning of the word "phase" is appropriate. A dictionary definition of a thermodynamic phase is a "portion of a physical system . . . that is homogeneous throughout, has definable boundaries, and can be separated physically from other phases" (42). X-ray diffraction, by virtue of the fact that diffraction involves many molecules in a periodic structure, is inherently sensitive to the thermodynamic phase of a system. Unfortunately, X-ray diffraction requires a lattice and is most applicable to purified systems in which a lattice structure has been induced. Biological membrane structures are heterogeneous and rarely periodic; consequently, the meaning of the state of "phase" of a biomembrane is often not clear. The power of NMR and electron microscope techniques is that they do not require a periodic lattice and, therefore, are more directly applicable to biologically relevant situations. The implicit resolution of the question of the "phase" of nonperiodic structures has been to apply the NMR and EM methods to periodic structures in which X-ray diffraction is applicable and the meaning of the physical phase is clear. One then extrapolates by asserting that, since NMR and EM are sensitive to the microscopic constraints of the structures and not to the lattice on which the structures are placed, these characteristic NMR and EM signatures may be used to indicate the presence of the microscopic structures even when no lattice is present. This procedure is certainly valid, provided that one is aware of its pitfalls and continually

applies tests for self-consistency. It does, however, lead to ambiguous terminology. For example, the "L_α phase" is used to refer both to isolated bilayers (e.g. "the cell wall is in a L_α phase") and to multilamellae (e.g. "at 70% concentration and 25°C egg lecithin forms a L_α phase"). This might be taken to imply that the interbilayer interaction energies contribute negligibly to the thermodynamic state of the bilayers. In fact, the interbilayer coupling in multilamellar systems is weak but by no means insignificant. Likewise, reference is often made to bilayer or H_{II} ^{31}P-NMR signals, even though the ^{31}P-NMR probe is lattice blind (see below). What is meant is that the signatures indicate structures, which, were they to be stacked on the appropriate lattice, would be recognizable by X-ray diffraction as a L_α or H_{II} phase.

STRUCTURAL TECHNIQUES

Experimental techniques that have been used to investigate the organization of lipid mesophases include methods that are directly sensitive to geometrical structure (e.g. X-ray and neutron diffraction and electron microscopy) and indirect methods from which structure or structural alterations may be inferred (e.g. NMR, IR spectroscopy, and calorimetry). We restrict our attention here to outlining the utility and limitations of the various methods.

X-ray Diffraction and Examples of Lipid Phases

X-ray diffraction has been fundamentally important in elucidating the structure of lipid phases. By this technique, a narrow, well-defined beam of X-rays is directed through the specimen, and the distribution and intensity of the X-ray scatter are recorded. A number of reviews have described X-ray diffraction studies of lipid-water systems (45, 47, 68). An excellent general reference, containing numerous tables and figures, is provided by Small (71). We will assume the reader is familiar with the basic theory and confine our remarks to those especially pertinent to "nonbilayer" lipids.

The first step in understanding the structure of a lipid system is to determine the phase diagram of the system. This involves mapping out the sequence of equilibrium phases that the system assumes as the thermodynamic variables (e.g. temperature and water concentration) of the specimen are varied. Different phases are often determined from the symmetry of the small-angle X-ray diffraction, which arises from periodic placement of the lipid molecules on a lattice. The periodically ordered structures that occur may be grouped depending on whether the structure is repetitive in one, two, or three dimensions.

One-dimensionally periodic phases (e.g. lamellar or smectic) consist of

locally planar, parallel bilayers separated by sheets of water of uniform thickness. In unoriented specimens, the characteristic low-angle X-ray diffraction pattern consists of a series of rings whose radii are in the ratio of 1:2:3:4.... Examples include the melted chain L_α and stiff chain L_β and $L_{\beta'}$ phases of lecithins (47, 78) (Figure 1*a–c*).

Two-dimensional mesomorphs consist of rod-like lipid structures laterally placed on a two-dimensional lattice. Examples (Figure 1*d–e*) include the hexagonally packed tubes of the H_{II} (water core; inverted) and H_I (hydrocarbon core; noninverted) phases of phosphatidylethanolamines and lysolecithin, respectively (47). The ratio of the radii of the characteristic X-ray orders of the hexagonal packing is $1:\sqrt{3}:2:\sqrt{7}$. . . . Rippled bilayers, such as the $P_{\beta'}$ phase (36, 54), may also be classified as two-dimensional.

Three-dimensionally periodic structures usually exhibit cubic symmetry. In some cases, as in the C_{f-1} phase (18), the fundamental repeating unit is not composed of bilayers; in other cases, as in the cubic phases of glycerol monoolein (43, 46), the structure consists of bilayer walls that form complex lattice channels. It should be noted that the structures of cubic lipid phases are very difficult to determine unequivocally and should be accepted cautiously. The reason for this is that the number of possible lattices rises rapidly with the dimensionality of the periodicity, thus necessitating the observation of more diffracted orders for an unambiguous structural assignment. Unfortunately, disorder intrinsic to the liquid crystalline state often limits the observable orders to a small number. Information about the symmetry of the lattice is also often further reduced because oriented specimens are difficult to prepare and one is forced to deal with "powder" pattern diffraction.

A determination of the symmetry of the lattice does not fully describe the structure, because many molecular arrangements are possible within a given unit cell. For example, the structures of Figure 1*a–c* are all lamellar and would all yield small-angle diffraction consisting of equally spaced orders. Ideally, one wishes to use the information encoded in the relative intensities of the diffracted orders to compute the electron density distribution within the unit cell, as is done, for example, in standard crystallography. Unfortunately, the small number of available orders limits the electron density reconstructions to low resolution; atomic resolution has only been achieved for the few lipids that form true crystals with no liquid crystalline disorder [e.g. Hauser et al (28) and references therein]. Even low resolution electron density reconstructions have been carried out extensively only for lamellar structures. The literature contains a number of good references to electron density reconstruction of lamellar systems (30,

48, 61, 74, 90, 91). Electron density reconstructions of nonbilayer mesomorphs are rare, highly model dependent, and utilize a priori information, such as the lengths of the molecules and the constraints of the hydrophobic effect. For example, the structures of the H_I and H_{II} phases are understood largely by symmetry arguments, the concentration dependence of mesomorphs (47), and agreement with models of concentric cylinders (59). Additional information derived by thin-section (76) and freeze-fracture (14) EM and NMR (63) serves to bolster the basic picture to the point where the models shown in Figure 1 are well accepted. However, more detailed structural questions, such as whether or not the H_{II} water cylinders are truly cylinders as opposed to hexagonal rods with rounded edges, cannot be answered by current structural methods. This example emphasizes the importance of applying as many techniques as possible toward understanding the structure of lipid mesomorphs.

Recent technical advances that have improved X-ray diffraction experiments include better means of orienting the specimens [see Clark et al (5) and references therein], synchrotron radiation sources (73, 88), and electronic X-ray detectors (23, 26). In combination, these methods may make possible the study of lipid phase transition kinetics (4).

Neutron diffraction techniques have been successfully applied to the study of bilayer lipids (60, 90). This method utilizes the neutron scattering contrast of different nuclei, most notably of hydrogen vs deuterium. Extensive neutron diffraction studies of nonbilayer lipid polymorphism have not, to our knowledge, been undertaken. This, no doubt, has been because of the paucity of available neutron sources and the preoccupation with bilayer structure. The potential for nonbilayer experiments is enormous.

Electron Microscopy

Electron microscopy (EM) is unique in that it may be used to visualize individual structures without reliance on signals that derive from ensemble averages. Freeze-fracture EM has been especially important in elucidating lipid polymorphism. By this technique, the specimen is ultrarapidly frozen, cracked open in a high vacuum, and a thin metal film is evaporated at an oblique angle onto the freshly cleaved surface. The resulting metal replica is a durable cast of the specimen surface topography and may be examined at leisure using the electron microscope. When the frozen specimen is cracked open, the fracture tends to propagate preferentially along the interface between the opposed terminal methyl groups of the lipid hydrocarbon because this interface is normally held together by the weak van der Waals forces (14). The resultant surface is a readily identifiable

fingerprint of the lipid phase. For example, L_α bilayers yield smooth fracture faces, whereas the H_{II} phase face appears as a series of parallel, concentric ridges arranged in distinct layers (Figure 2).

Care must be taken to freeze the sample ultrarapidly to avoid artifacts, both owing to disruption by ice crystals and to thermotropic changes that may occur in the lipid when the temperature is lowered. To avoid the former, cryoprotectants (e.g. ethylene glycol, dimethylsulfoxide, etc) are sometimes added to the lipid suspension. These agents can themselves perturb the lipid structure (66) and affect the thermotropic behavior of the specimen (83). Ultrarapid freezing at rates greater than several thousand °C/sec without cryoprotectants can usually avoid the formation of disrupting ice crystals (6, 70) and can trap many high temperature phases (85). Combined X-ray and freeze-fracture EM studies (6, 14) have demonstrated phase trapping, although it is, in general, very difficult to rule out the possibility of some structural rearrangement in the freezing step.

Freeze-fracture EM has proven to be especially valuable in examining features that tend not to appear on a regular lattice. Good examples include lipidic intermembraneous particles (84) and transient states (2, 55). It must be emphasized, however, that the freeze-fracture method cannot resolve individual molecules; consequently, interpretation of the observed features in terms of the molecular placement is very difficult and often controversial. This is especially true for isolated features for which additional information is not available from alternative structural probes.

NMR

The last decade has witnessed a resurgence of interest in nonbilayer polymorphism. This has been catalyzed by the use of ^{31}P-NMR for the identification of nonbilayer structures. The ^{31}P-NMR spectra that typically result from various phospholipid assemblies are shown in Figure 2. The differences in the shape of the spectra may be understood as arising from the degree of diffusional freedom of the phospholipid molecule in the lipid aggregate (7, 40, 64).

Briefly, the diesterified phosphate phosphorus of common phospholipids experiences anisotropic shielding from the applied magnetic field owing to anisotropy in the local electron distribution. This results in a "chemical shift anisotropy," where the resonance position of the phosphorus is dependent on the angle between the magnetic field and the phosphate region of the lipid headgroup. In liquid crystalline lamellar systems the phospholipids experience rapid rotational and lateral diffusion. If the lamellar systems are large enough (diameter $\geq$2000 Å), the lateral diffusion does not produce significant motional averaging. However, the rapid axial rotation produces motional averaging, which results in the projection of the anisotropic

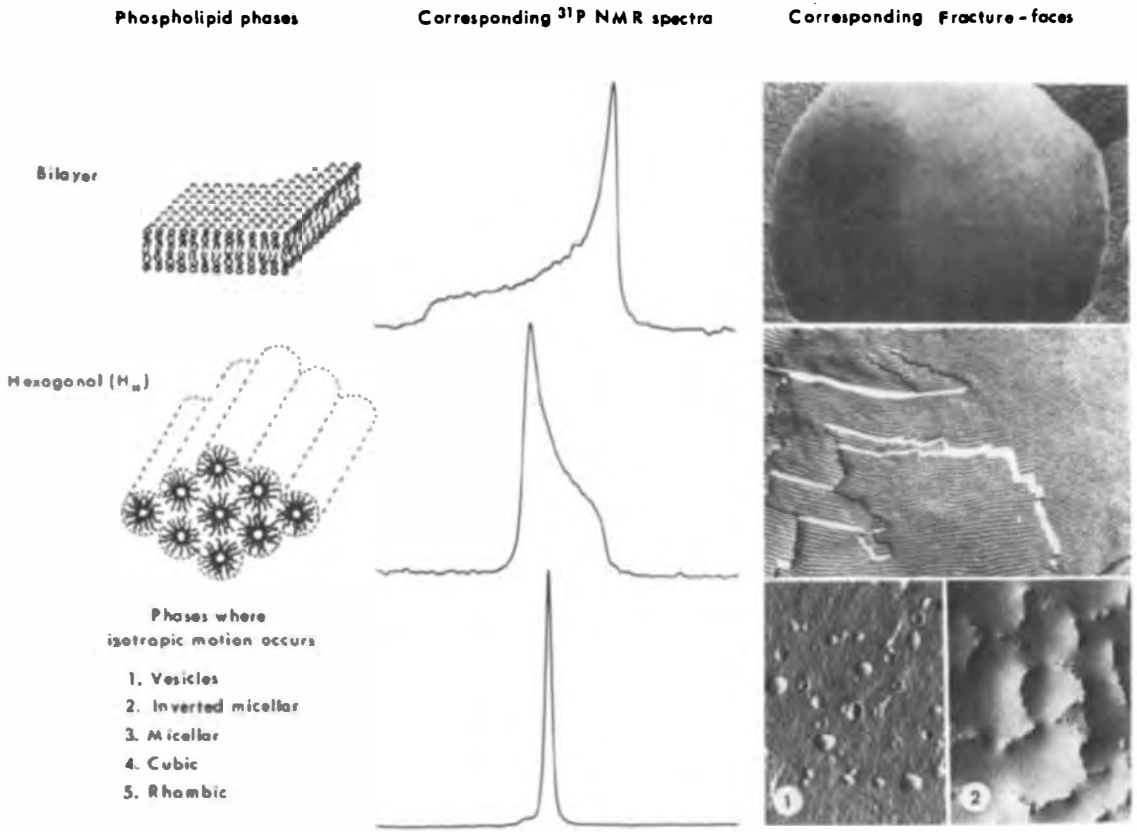

Figure 2 Various phospholipid structures (*left column*) are shown next to typical ^{31}P-NMR spectra (*center column*) and freeze-fracture faces (*right column*) observed for these structures. The lower-most, right-hand micrographs are for small bilayer vesicles (*1*) and lipidic particles (*2*). The latter consists of small vesicles of egg phosphatidylethanolamine and 20% egg phosphatidylserine, which have been prepared at pH = 7.0 and then incubated at pH = 4.0 for 15 min prior to freeze-fracturing. From Reference 10, with permission.

shielding along the axis of rotation. This effective chemical shift anisotropy results in the asymmetric ^{31}P-NMR lineshape associated with bilayer systems that is shown in Figure 2. In H_{II} systems, additional motional averaging occurs because of lateral diffusion of the lipid around the aqueous cylinders; this results in the narrower lineshape with reversed asymmetry (as compared to the bilayer ^{31}P-NMR signature) shown in Figure 2. Finally, if motion over all orientations is possible on the NMR time scale ($\sim 10^{-5}$ sec), this "isotropic" motional averaging leads to a narrow, symmetric ^{31}P-NMR resonance.

The shape of the ^{31}P resonance is indirectly related to the phospholipid phase. It is dependent on the local environment of the lipid phosphorus and the motion available in a given phase. This has led to valid concern over the reliability of using ^{31}P-NMR to identify lipid phases because it can be shown theoretically that the lineshapes taken as signatures of bilayer and H_{II} structure (Figure 2) can change and be incorrectly identified if the conformation of the phosphorus region is changed (53, 79), without any change in the phase. The important question, then, is how variable is the phosphorous region of phospholipids? A number of published experiments have investigated the phase assignments of various biologically occurring phosphodiester lipids by X-ray and ^{31}P-NMR and have found excellent agreement between the two procedures (1, 31, 50, 62, 81). Further, systematic X-ray and NMR comparisons in our laboratories (results in preparation) have also found excellent agreement. This strongly suggests that the local phosphorous conformation is highly invariant in phospholipids. Note that this kind of study can never prove that the ^{31}P-NMR probe will always yield correct assignments; rather, it serves to bolster confidence in the method. Definitive phase assignments, especially of novel lipids, must rely on direct structural methods, such as X-ray diffraction.

As previously indicated, the shape of the resonance is dependent to a large extent on the diffusional constraints imposed by the shape of the lipid assembly. Imprecise terminology has led to some confusion over the meaning of the ^{31}P signals. For example, spectra like that shown in the center of Figure 2 are sometimes referred to as "H_{II} ^{31}P-NMR signals" when what is meant is "^{31}P-NMR signals similar to those seen with phospholipids in the H_{II} phase." This is not splitting hairs. The former statement implies the existence of an H_{II} lattice while the latter suggests the presence of lipid in a cylindrical geometry. An example of nonhexagonally packed lipid cylinders is given by Kachar & Reese (37).

Deuterium NMR has also recently been used to help identify lipid structures (21, 80, 81). The bilayer and H_{II} phases, as distinguished by the magnitude of the quadrupolar splitting, are shown in Figure 3. As with ^{31}P-NMR, the magnitude of the splitting results from the averaging incurred as the molecules diffuse in the lipid assembly; consequently, the comments

already mentioned for ^{31}P-NMR also apply to ^{2}H-NMR. ^{2}H-NMR has the disadvantage of requiring the synthesis of specifically deuterated lipids, as opposed to ^{31}P-NMR, in which the natural abundance of ^{31}P in phospholipids is normally used. A great advantage involves the ability to detect the phase preference of a single lipid in a mixed system. The reader is referred to References 11, 63, and 65 for reviews on the theory and application of ^{2}H-NMR to lipids.

The advantage of both ^{31}P-NMR and ^{2}H-NMR as compared to X-ray diffraction is that the presence of nonbilayer features can be recognized, even if these features are not packed on a lattice. It is only necessary that the feature be present in sufficient concentration to yield respectable signal strengths. This is especially important with biomembranes, where the presence of protein may disrupt lattice structure. The disadvantage of NMR is that it requires relatively large quantities of lipid and yields limited

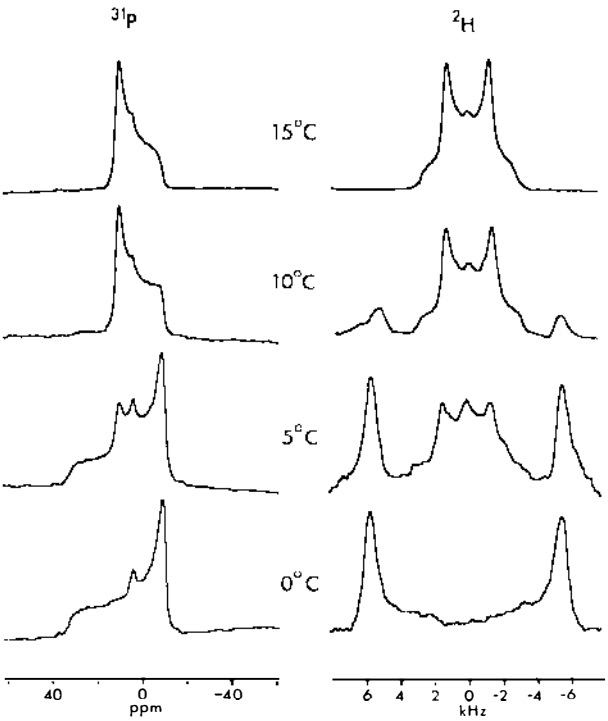

Figure 3 ^{31}P-NMR and ^{2}H-NMR spectra as a function of temperature of fully hydrated dioleoylphosphatidylethanolamine (DOPE) with a deuterium label at the C_{11} position in the acyl chains. The ^{31}P-NMR spectra were obtained at 81.0 MHz in the presence of proton decoupling, whereas the ^{2}H-NMR spectra were obtained at 30.4 MHz. DOPE is predominantly H_{II} at 15°C and L_{α} at 0°C. From Reference 10, with permission.

structural detail, i.e. the ^{31}P-NMR alphabet consists of only 3 letters (Figure 2).

Alternative Methods

The task of identifying phase transitions and of determining phase diagrams is considerably aided by methods such as differential scanning calorimetry, which can be used to pinpoint phase transitions. Newer spectroscopic techniques, such as infrared spectroscopy (49), yield promising signatures that may prove to be useful in identifying lipid phases.

PHOSPHOLIPID POLYMORPHIC PHASE PREFERENCES

The polymorphic phase preferences of pure and mixed lipids have recently been reviewed (10); some results for pure lipids are shown in Table 1. In this section we discuss the general polymorphic trends exhibited by lipid-water mixtures; readers interested in detailed information about specific systems are referred to Cullis et al (10).

Figure 4 shows that an increase in specific factors such as hydrocarbon unsaturation, temperature, etc, affects the preference of phospholipids for a bilayer or a H_{II} configuration. Qualitatively, the trends associated with each factor are consistent with the requirements of the hydrophobic effect and geometrical packing, i.e. minimization of water-hydrocarbon contact and avoidance of steric constraints leading to low density microvolumes in the hydrocarbon. To illustrate, consider the shape of the average volume available to each lipid molecule in the L_α and H_{II} phases of Figure 5. Consider the areas of a series of parallel cross sections, starting with the headgroup-water interface and then moving down through the hydrocarbon zone. In the L_α phase all these cross sections necessarily have the same area. In molecular terms, this means that the average L_α lipid must present the same cross-sectional area at the headgroup-water interface and in the hydrocarbon zone. In the literature, this attribute is often described by saying that L_α lipids are "cylinder" shaped (9, 33, 34). The word "cylinder" is not to be taken literally, but rather serves as a mneumonic for the uniform cross-sectional average molecular area. In the H_{II} phase (Figure 5), the average shape is tapered with a smaller cross-sectional area at the water interface than at the terminal methyls of the hydrocarbon tails. The average molecular area is said to be "cone" shaped.[1]

[1] Some authors (33) refer to the H_I molecular volume as "cone" shaped and the H_{II} volume as "inverted cone" shaped, whereas others (10) call the H_{II} volume cone shaped and the H_I inverted cone shaped. Obviously, the concept is the same in both cases.

Table 1 Polymorphic phase preferences of unsaturated lipids[a]

Lipid	Phase preferences: Physiological conditions[b]	Phase preferences: Other conditions
Phosphatidylcholine	L	H_{II}—low hydration and high temperatures
Sphingomyelin	L	
Phosphatidylethanolamine	H_{II}	L—pH ≥ 8.5 —low temperatures
Phosphatidylserine	L	H_{II}—pH ≤ 3.5
Phosphatidylglycerol	L	H_{II}—high temperatures and high salt concentrations
Phosphatidylinositol	L	
Cardiolipin	L	H_{II}—divalent cations —pH ≤ 3 —high salt
Phosphatidic acid	L	H_{II}—divalent cations —low pH ≤ 3.5 —high salt
Monoglucosyldiglyceride	H_{II}	
Diglucosyldiglyceride	L	
Monogalactosyldiglyceride	H_{II}	
Digalactosyldiglyceride	L	
Cerebroside	L	
Cerebroside sulphate	L	
Ganglioside	M	
Lysophosphatidylcholine	M	
Cholesterol		Induces H_{II} phase in mixed lipid systems
Unsaturated fatty acids		Induce H_{II} phase

[a] Reproduced, with permission, from Reference 10.
[b] Key: L, lamellar; H_{II}, hexagonal H_{II}; M, micellar.

Now consider each of the factors shown in Figure 4. For saturated 18-carbon tails, phosphatidylethanolamine in excess water exhibits a $L_\alpha \rightarrow H_{II}$ transition at $>100°C$ (27). As one and then two *cis* bonds per chain are introduced, the transition temperature drops to 10 and $< -15°C$, respectively (7, 8, 82). The effect of introducing unsaturated *cis* bonds is to introduce kinks in the hydrocarbon chains, thereby causing the tails to splay more widely. In shape terms, the tail cross-sectional area is increased, leading to a more cone-shaped molecule. At a given temperature, the more cone-shaped species is prone to be in an H_{II} phase. *Gauche* rotameric states of saturated bonds also introduce hydrocarbon disorder and may be

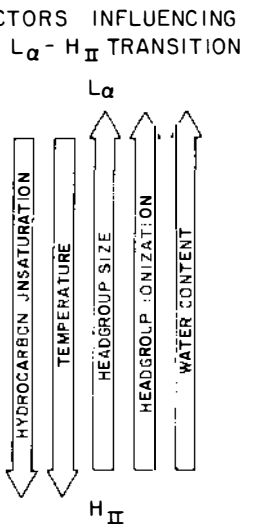

Figure 4 Factors that influence the shift between L_α and H_{II} phases are shown in the arrows. The directions of the arrows demonstrate the effect of increasing the factors. For example, increasing the temperatures shifts lipids from L_α to H_{II}, while increasing the headgroup ionization shifts lipids from H_{II} to L_α.

expected to cause the tails to splay. Since each *gauche* rotamer costs 0.5 kcal/mol (52), more rotamers are introduced as the temperature rises. This is consistent with the fact that the H_{II} phase always occurs at a temperature above the L_α phase. A factor that counterbalances an increase in the cross-sectional area of the hydrocarbon is an increase in the effective size of the headgroup. Thus, dioleoylphosphatidylethanolamine has a $L_\alpha \rightarrow H_{II}$ transition temperature of 10°C. Increasing the effective headgroup size by methylating one ethanolamine site increases this transition temperature by more than 50°C (38; G. L. Kirk, S. M. Gruner, C. P. S. Tilcock, and P. R. Cullis, in preparation). With successive methylations, the lipid is still in an L_α phase at 885°C (ibid). The word "effective" is used to emphasize that the headgroup "size" includes not only the hard-core steric size but also lateral forces that tend to keep the headgroups apart. Thus, lipid headgroups, which are ionized and repel one another and are in an L_α configuration, can often be made to go into the H_{II} phase by adjusting the pH so as to deionize the headgroups (Table 1). A final factor is the water content of the lattice. If one considers geometry only, a given amount of lipid in the L_α phase can accommodate an arbitrarily large amount of water: One need only make the water layers thicker. This is not the case for the H_{II} phase because as the water cylinders increase in radius, the point labelled "d_{max}" in Figure 1*d*

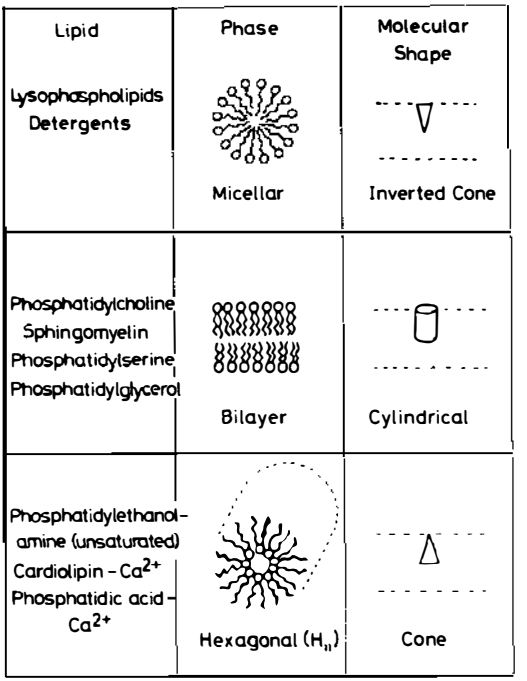

Figure 5 Polymorphic phases and the corresponding dynamic molecular shapes of the component lipids are shown for a variety of lipids. From Reference 10, with permission.

becomes further and further removed from the water cylinder surfaces. Eventually, it will exceed the length of a fully extended lipid tail, which is an entropically unfavorable circumstance. Note that the Gibbs phase rule allows a bulk free water phase to coexist with an L_α or H_{II} lattice. Thus, the actual amount of water that will be taken up by the lipid may be limited by factors in addition to geometrical constraints. These considerations are discussed in more detail in the next section.

MOLECULAR BASIS OF LIPID POLYMORPHISM

A full understanding of lipid polymorphism would allow quantitative prediction of mesomorphic behavior from the chemical formula of a lipid. By this measure, our understanding of lipid polymorphism is in its infancy. Even so, considerable growth during the last decade has occurred in the theoretical understanding of the physical basis of polymorphism. The thermodynamic problems inherent in lipid polymorphism are very complex and diverse; consequently, it is hardly surprising that this understand-

ing has arisen from a diverse set of scientific communities, each concerned with different aspects of the problem. Characteristically, each of these various approaches seeks to explain a limited subset of polymorphic behavior. None of these approaches, taken individually, can adequately account for the more complex lyotropic transitions, such as $L_\alpha \leftrightarrow H_{II}$. A synthesis of methods appears to be needed. Consequently, we will first identify and discuss some of the theoretical approaches that have been taken and then consider how these may be synthesized into more complex models. Four theoretical thrusts are considered: (*i*) monomer aggregation, (*ii*) hydrocarbon melting, (*iii*) elastic properties, and (*iv*) interaggregate forces.

Monomer Aggregation

Amphiphile solutions typically exhibit concentration-dependent aggregation, which differs from that of most polar or ionic molecules. At low concentrations the amphiphile exists dispersed in the monomer state. As more monomer is added, however, a "critical micelle concentration" (CMC) is reached, whereupon the monomeric units begin to aggregate in larger structures, such as micelles. The monomer solution is in equilibrium with the micelle solution; attempts to raise the concentration by addition of more monomer result in the formation of more micelles. This leads to dramatic changes in the concentration dependence of many physical parameters, such as the osmotic pressure, surface tension, and electrical conductivity. The basic reason why monomers aggregate is the hydrophobic effect (77): nonpolar, oil-like entities prefer to consolidate out of contact with water. An alkane of n carbon atoms can lower its chemical potential by an amount

$$\Delta\mu \approx 10.2 + 3.7n \text{ kJ mol}^{-1}$$

in moving from an aqueous to a hydrocarbon solvent (77). This is a manifestation of the peculiar self-bonding affinity of water: Segregation of nonpolar moieties decreases the surface-to-volume ratio of the nonpolar aggregate and thereby decreases the area of aqueous contact. Mixing entropy competes with aggregate formation. In so far as the system may be considered ideally mixed, equilibrium thermodynamics requires that the chemical potential of the coexisting aggregates be the same. If x_N is taken to be the mole fraction of assemblies of aggregation number N, then (33)

$$\mu_N^0 + \frac{kT}{N} \ln (x_N/N) = \mu_1^0 + kT \ln x_1, \qquad 1.$$

where μ_N^0 = chemical potential of a N-molecule assembly, k = Boltzmann's constant, and T = temperature.

The leftmost term refers to monomers. At the CMC this equation has a nontrivial solution for x_N, which obviously depends on the way μ_N^0 drops with increasing N. Thus, the CMC is the compromise between the requirements of hydrophobic coalescence of the nonpolar lipid tails and the entropy of mixing. The fundamental problem of amphiphile aggregation, then, is to understand how μ_N^0 varies for different molecules. This would explain why different lipids have different CMCs. Note that N molecules may be arranged in different shapes (e.g. spherical vesicles, rod-like, disk, and spherical micells, etc), so one would also like to understand how μ_N^0 varies with the shape of an aggregate. In particular, one would like to understand the molecular parameters that affect the shape dependence of μ_N^0.

A number of reviews discuss micelles and monomer aggregation (20, 33, 71, 87). Tanford (77) is an additional, invaluable reference. Not surprisingly, much of the literature comes from the surfactant community and has a strong emphasis on ionic detergents. Relatively few studies have dealt with micelle-forming, nonionic lipids (e.g. lysophosphatidylcholines and lysophosphatidylethanolamines) and even fewer with lipids that aggregate into non-micelle structures. The latter category includes common diacyl lipids such as phosphatidylcholines and phosphatidylethanolamines. These exhibit very low CMCs ($\sim 10^{-10}$ M vs 10^{-3} M for lyso-lipids) and spontaneously form bilayer and H_{II} assemblies. For these lipids, inter-aggregate forces cannot be ignored because vesicles and H_{II} domains often coalesce at low lipid concentrations.

If all amphiphiles produced spherical micelles at the CMC, then the problems of coalescence would be vastly simplified: Of all shapes, the sphere maximizes the surface area-to-volume ratio for the volume enclosed, as demanded by the hydrophobic effect. The fact that some lipids form nonspherical micelles, and others do not form micelles at all, demonstrates that molecular diversity places additional constraints on the functional form of μ_N^0. Tanford (77), Israelachvili et al (33–35), and Mitchell & Ninham (51) have emphasized the importance and interplay of the molecular shape and the molecular area of the hydrophobic-hydrophilic interface. The interfacial energy/molecule is approximately

$$\mu_N^0 = 2\gamma a_0 + \frac{\gamma}{a}(a - a_0)^2. \qquad 2.$$

Here γ is a positive interfacial energy/unit area, a is the actual area/molecule, and a_0 is an "optimum" area/molecule (33). This results from the competition between the interfacial energy and the expected form of a low order expansion of the collection of electrostatic, steric, van der Waals, and hydration energies. This equation may be understood as arising from

the net magnitudes and directions of the forces acting throughout the depth of a lipid layer (see Figure 4.1 of Reference 33); these forces, together, determine the average molecular shape. Israelachvili et al (34) define a dimensionless packing parameter $v/a_0 l_c$ to characterize this shape. Here, v is the volume/molecule and l_c is roughly the length of a fully extended hydrocarbon chain. Geometrical considerations of trying to pack various shapes into different types of aggregates, as discussed in the previous section, demonstrate that the magnitude of the packing parameter may be related to the aggregate shape that is allowed.

This concept of average molecular shape yields insight into the aggregates that can be expected to result from molecular diversity. Thus, in the language of Cullis et al (10), lyso-lipids are inverted cone-shaped ($v/a_0 l_c < 1/3$): the hydrocarbon volume has a small area relative to the headgroup area, so spherical micelle packing predominates. Adding two chains "widens" the hydrocarbon area ($1/2 < v/a_0 l_c < 1$) and leads to bilayer packing. If the effective headgroup area is small, such as in the case of phosphatidylethanolamines, the relative tail splay is wide, leading to a cone shape ($v/a_0 l_c > 1$) and H_{II} packing. As discussed in the previous section (Figure 4 and the associated text), many mesomorphic trends qualitatively conform to this picture.

Notions of molecular packing and shape are qualitatively important for understanding mesomorphic diversity. The word "qualitative" needs emphasis: a quantitative, a priori determination of the expected "molecular shape" from a given chemical formula is not possible at the present time. The "shape" is a composite of many complex, poorly understood, interrelated forces. A real understanding of the effective molecular shape must involve a good understanding of the various forces and a realistic statistical modelling of the cooperative effects that result.

Hydrocarbon Melting

An important segment of the mesomorphic literature deals with the melting of hydrocarbon chains in the L_β to L_α bilayer transition. The theoretical literature has been reviewed by Nagle (52) and Pink (57). Consequently, we will confine the discussion to general remarks.

Statistical mechanical models of the $L_\beta \leftrightarrow L_\alpha$ transition are important because they yield detailed insight into the cooperative behavior of the hydrocarbon. With few exceptions, theoretical models have considered the interplay of forces in lipid monolayers; the coupling of two monolayers into a bilayer is weak and generally introduced ad hoc. In retrospect, this is fortunate: the fundamental unit of all lipid mesomorphs is the lipid monolayer. The monolayer is wrapped into cylinders in the H_I and H_{II} phases, into spheres in micelles, and coupled back-to-back in bilayers.

Consequently, one hopes that the models introduced for the purpose of understanding bilayers can be modified to include cooperative hydrocarbon states in nonbilayer geometries (see below).

Generally, the approach taken is to write a Hamiltonian that explicitly characterizes the various energy contributions of importance to the monolayer. This is used to calculate a partition function from which thermodynamic properties may be derived via standard statistical mechanics. The catalog of known contributions to the Hamiltonian is long: van der Waals terms, translational, rotational, and vibrational excitations, excitation of *gauche* rotamers, steric effects, electrostatic interactions, plus a host of ill-understood polar energies all serve to make analysis of a complete Hamiltonian entirely too difficult. It is expedient to include only those energies deemed important to the transition; selection of these energies distinguishes many of the models from one another. Once the energies are chosen, there remains the difficult task of computing an explicit partition function. Again, approximations are made that are the source of much scientific dispute. Finally, the results are compared against the experimental data. Not surprisingly, different models often fit some experimental curves well and others poorly; indeed, interest in understanding particular experimental parameters is often the rationale used in selection of the energies going into the Hamiltonian. While it is clear that none of the published models is wholly satisfactory, it must be stressed that a great deal has been explained about the behavior of lipid monolayers (87) and that much of this knowledge has not been derived from any single article.

It is clear from examination of these models (if not from common sense) that the "shape" of a lipid molecule is not a property intrinsic to an isolated molecule. [But see the discussion in Day & Willis (12, 13).] The fluid nature of the melted hydrocarbon allows it to fill complex shapes, as it must do when embedded in a matrix of neighboring lipid. Each shape assumed is a compromise among competing energies. Moreover, the relative magnitudes of the competing energies vary with the molecule, with temperature, and with water content. It is not surprising that phase transitions involving discrete, cooperative changes in average shape (e.g. $L_\alpha \rightarrow H_{II}$) occur as these relative magnitudes vary. It is important to realize that the models of the $L_\beta \leftrightarrow L_\alpha$ transition necessarily impose the bilayer geometry as a constraint; alternative constraining geometries are feasible (39).

Elastic Properties

A time-honored method for dealing with complex molecular forces is to lump them into a small number of phenomenological constants that specify the elastic properties of the material. These constants ultimately are an

expression of the sum of microscopic forces as the molecular volumes are deformed from the relaxed shapes. Consider bending a relaxed sheet of material. The surface area on one side grows as that on the other side shrinks; consequently, the volumes available to those molecules, or parts of molecules, on the one side expand while those on the other side shrink. The curvature of the unstressed sheet need not be flat: If the material is composed of unidirectionally oriented "cone-shaped" molecules, then the relaxed state is curved in the same way as tapered stones placed side-by-side make up an arch. In this case, deformations that either bend or unbend the sheet cost elastic energy. One may even consider the case where the molecular parts on one side of the sheet have a different coefficient of thermal expansion than that on the other, as in a bimetallic strip. It is clear that the unstressed curvature would then vary with temperature.

Lipid layers may be considered as elastic sheets. This approach has long been used to characterize normal single component, thermotropic liquid crystals (75). Applications to bilayers were considered by Helfrich and coworkers (16, 29). Doniach (17) has applied ideas drawn from the elastic formalism to model the rippled or $P_{\beta'}$ phase of saturated lecithins. Evans & Skalak (19) have reviewed the elastic mechanics of bilayers in detail, and Kwok & Evans (41) have performed measurements to characterize the constants. Recently, Sornette & Ostrowsky (72) have examined the interplay of elastic properties and thermal fluctuations of bilayers. Since the lipid monolayer may also be considered as an elastic unit, in reading the literature one must note if the elastic constants refer to monolayers or bilayers. Most papers refer to bilayers because bilayers are usually the experimentally measured surfaces. However, in considering how molecular volumes lead to the elastic coefficients, the monolayer is the more fundamental unit. Elastic contributions resulting from molecular packing into monolayers are discussed by Israelachvili et al (33–35) and by Mitchel & Ninham (51). In this regard note that the rightmost term of Equation 2 is really an elastic term, since the elastic compressibility modulus, K_c, may be defined as

$$\text{elastic energy stored} = \frac{K_c}{2a}(a-a_0)^2. \qquad 3.$$

Very little work has been done in trying to derive elastic moduli from statistical mechanical models of lipid monolayers. The thermal coefficients of the headgroups and hydrocarbon zones of monolayers may be expected to differ, the latter responding more like a polymer chain. The resultant differential expansion may lead to a change of the equilibrium curvature of the monolayer (i.e. the curvature for which $a = a_0$ in Equation 3). If the two monolayers are constrained to lie flat and back-to-back in a bilayer, then

the thermal stress may build as the two monolayers desire to arch away from one another. One way to relax this stress is to allow the layers to curl up as in an H_I or H_{II} phase. In this way, energy may be stored in the stressed system, leading to a high elastic energy state that is relaxed entropically upon a cooperative shift in the local geometry at the phase transition. Some of this thinking is implicit in the models of the $P_{\beta'}$ phase [see, for example, Doniach (17)]. The cohesive forces of the headgroups may also be modulated isothermally by dehydration, ionic shielding, or interlayer forces, leading to a drift in the equilibrium curvature. This approach was used by Kirk et al (39) in modelling the $L_\alpha \leftrightarrow H_{II}$ transition. We know of no attempts to formally apply a similar procedure to the thermal dependence of bilayer to nonbilayer transitions. There is, obviously, rich ground for theoretical modelling here. We urge its exploration. The polymer literature is, no doubt, an untapped resource that could be helpful in expression of these ideas.

Interaggregate Forces

Strong forces result when polar surfaces are brought near to one another. If the intervening space is an aqueous fluid, then the dominant forces are electrostatic interactions of charged surfaces (as shielded by aqueous ions), van der Waals interactions, and hydration forces specific to water. The first two forces are addressed by DLVO theory (15, 86). A major advance occurred in the 1970s with the recognition that very strong "hydration" forces are present across thin ($\lesssim 20$ Å) water layers, even with electrically neutral surfaces or in the presence of strong ionic shielding (22, 32, 58). The work of Parsegian, Rand, and co-workers (see 58) has been especially important in characterizing these forces for lipids. At dimensions characteristic of lipid liquid crystals, hydration effects dominate the intersurface interactions for all but the most highly charged surfaces. Intersurface forces effect a coupling between layers. As noted in the preceding paragraph, these forces modulate the interactions between neighboring lipid molecules within the same layer, thereby causing a coupling between inter- and intralayer forces. The molecular shape at close dimensions is affected by this coupling. As an example, it is known that the area/molecule in bilayers decreases as the interbilayer water thickness is lowered [see Rand (58) for a discussion]. This molecular coupling is not well understood, so it has been largely ignored in considering mesomorphic transitions. It is an area in urgent need of study.

Synthetic Approaches

In the preceding sections we have tried to emphasize that the various theoretical thrusts are dealing with different, but interrelated, aspects of

lipid morphology. In attempting to understand the thermodynamics of complex transitions such as $L_\alpha \leftrightarrow H_{II}$, one rapidly recognizes that the drastic change in monolayer geometry at the transition and the three-dimensional nature of the aggregate demand a synthetic approach. This was the route used by Kirk and co-workers (38, 39) in a model of the isothermal $L_\alpha \leftrightarrow H_{II}$ transition. An attempt was made to construct a modular framework in which one first decides on the interactions that are most important in driving the transition and then attempts to assign explicit functional forms to the effects. This approach is important for two reasons: (*a*) there is little understanding of which interactions are important, and (*b*) explicit functional forms, because of computational complexity, will inevitably be based on simplistic approximations. In a modular approach, the functional forms can be refined piecewise by later workers while preserving the basic framework. Four contributions to the free energy were deemed important: elastic curvature, molecular packing, hydration repulsion, and electrostatic effects. It was shown that each of these can be expressed as a free energy per molecule, which is a function of the water concentration in each of three geometries: L_α, H_{II}, and C_{II} (C_{II} = close packed, inverted spherical micelles). A curvature term similar to Equation 3 was derived directly from a plausible partition function of the lipid monolayer. Molecular packing considerations stemmed from the observation that some parts of the hydrophobic volume are relatively inaccessible in the nonbilayer phases; tails that extend into these regions are anomalously stretched. This costs energy. Electrostatic and hydration terms were solved for each geometry.

The resultant curves and their sum are shown in Figure 6 for uncharged lipids. At low hydrations the H_{II} geometry is of lowest free energy (Figure 6*a*), and, therefore, is the stable phase. The main reason for this is the high curvature and hydration energy of the L_α phase (Figure 6*a* and *c*). As the water content is raised, the curvature and hydration energies for the three geometries approach one another, and packing energies (Figure 6*b*) cause the L_α phase to be of overall lowest energy. In all cases, the C_{II} phase is not of lowest energy, which suggests that it should not occur in binary systems. These overall trends conform to experimental observations. The exact numerical values derived were taken as approximate, since they may be expected to change as the functional forms are refined.

The synthetic approach is useful, in part, because it seeks to identify the component energies that drive polymorphism and suggest experiments. As an example, Kirk et al (39) pointed out that large H_{II} lattices are very sensitive to the packing stress. It was predicted that the addition of a small amount of oil could relax this stress. To test this, Kirk and Gruner (results in preparation) examined the effect of free alkanes (e.g. dodecane) upon

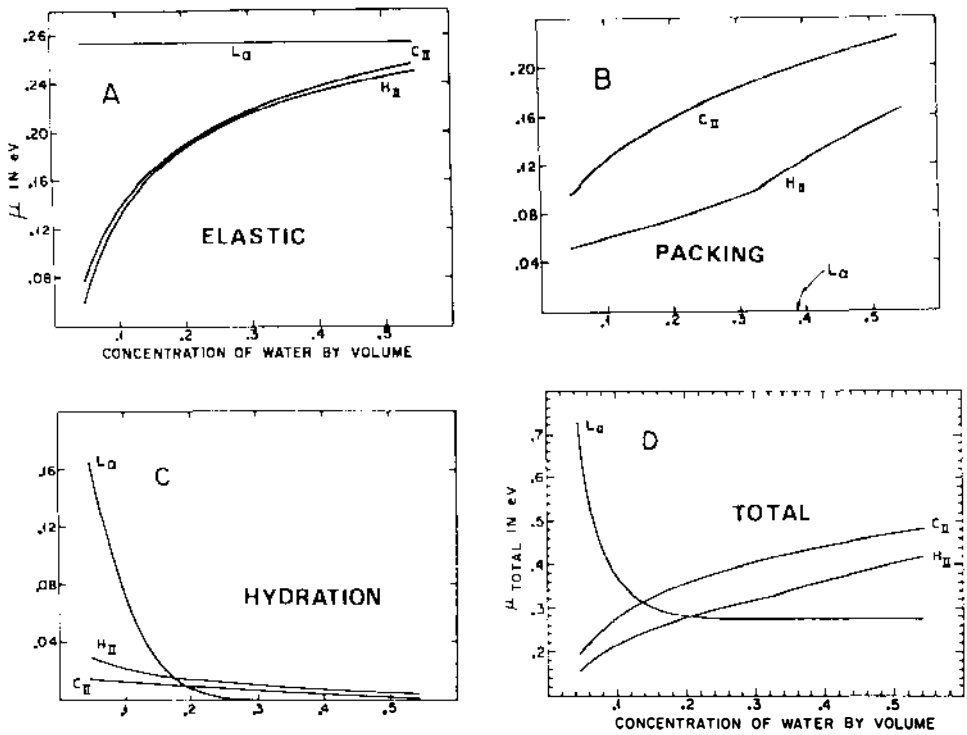

Figure 6 The free energy vs water concentration from various contributions to the overall energy are shown for a theoretical model of the L_α, H_{II}, and C_{II} phases of a zwitterionic diacyl lipid. The total free energy (*D*) is the sum of (*A*) elastic, (*B*) packing, and (*C*) hydration terms. From Reference 39, with permission.

the $L_\alpha \rightarrow H_{II}$ transition. Lipid mixtures were chosen that a priori could be expected to have large equilibrium radii of curvature and, therefore, would accept a high water concentration; in these cases, the H_{II} phase should be limited by packing stress. It was shown, for instance, in a dioleoylphosphatidylethanolamines: dioleoylphosphatidylcholine = 3:1 mixture in excess water, that the addition of only 5% dodecane dropped the transition temperature by 55°C and expanded the lattice repeat by 18 Å. H_{II} lattice repeats of almost 100 Å were obtained in some cases. The fact that these dramatic effects were predicted and observed to result from very small quantities of alkane confirms that packing stress is important.

In closing this section, we wish to emphasize future studies that need to be done. More work on synthetic approaches is clearly desirable. Also, very little is known about transition dynamics in these phase transitions. In this

regard, Borovjagin et al (2) and Siegel (69) are notable. Finally, the interaction of phase-separated domains in the phase coexistence regions of the phase diagrams is an open question. The curious domain structures that are observed may be used to model the growth of phase-separated regions (24, 25). Knowledge of domain growth is crucial to understanding bilayer-bilayer interactions in events such as fusion.

CONCLUDING REMARKS: CONNECTIONS BETWEEN BIOMEMBRANES AND MESOMORPHISM

Biophysicists study lipid polymorphism both to understand biomembranes and because it is intrinsically interesting. However, a pure, bulk lipid liquid crystal is not a biomembrane. It is important to discuss the differences between the two so as to recognize the dangers of extrapolating results obtained with one system to the other. Lipid liquid crystals are generally relatively homogeneous, bulk (i.e. three-dimensional) aggregates in which there is a well-defined water concentration. Biomembranes are extraordinarily heterogeneous and usually exist as asymmetric isolated bilayer walls with distinct inside and outside surfaces. Thus, three distinctions are immediately apparent: water concentration, heterogeneity, and bilayer asymmetry. Additionally, liquid crystals are usually examined as phases in thermodynamic equilibrium. Biomembranes are not equilibrium phases.

Biomembranes exist in excess water. In consequence, lipid liquid crystals in contact with excess water are studied as models for biomembranes. This, however, is an oversimplification of events that occur in cells. Even in excess water, unconstrained zwitterionic lipid bilayers maintain a fixed distance from one another, thereby fixing the local water concentration. This "equilibrium water gap" is the result of a balance between distance-dependent attractive and repulsive forces that act upon the bilayers (58). In the cell, biomembrane separations are usually controlled by proteins and the cytoskeletal framework. Neglecting, for the moment, any direct perturbations the proteins exert on the lipids, this means that the relevant water concentration is more accurately defined by the thickness of the water layer between bilayers than by the overall water content. This is the case because the predominant forces between the lipids (van der Waals, hydration repulsion, electrostatic, and steric) are of relatively short range and are weak beyond roughly 30 Å. Alternatively stated, a lipid molecule in a bilayer facing another bilayer across a water gap that is less than the equilibrium water separation feels nearly the same forces as would a lipid

molecule in a liquid crystal below the excess water concentration. The biomembrane lipid molecule has no way of "knowing" that the close opposition is externally enforced by the cytoskeleton and that there is a large pool of water nearby (39). Thus, the study of liquid crystals at below excess water concentration is relevant to understanding biomembranes in near contact. This is why the study of polymorphism is important to understanding biomembrane fusion.

Biomembrane heterogeneity arises from both nonlipid molecules in the membrane and the incredible diversity of lipid species in a typical biomembrane. The implications of the former are very difficult to assess because the understanding of, say, the protein-lipid interaction is very poor. The behavior of mixed lipid liquid crystals has been reviewed by Cullis et al (10). To a first approximation, most phospholipid mixtures exhibit behavior that is intermediate to that expected from the behavior of the pure components. For instance, adding lipid that is prone to be in the H_{II} phase makes the mixture more prone to adopt nonbilayer configurations. Addition of other lipids (e.g. cholesterol) can have more complicated effects. In general, considerable work remains to be done on lipid mixtures and alloys.

Most biomembranes appear to be asymmetric. In the cell, asymmetry arises from vectorially oriented membrane proteins, from different chemical (e.g. ionic) environments encountered on opposite sides of bilayer walls, and from differing lipid composition in the opposed monolayers composing the bilayer. In principle, bulk lipid liquid crystals can be made to include these asymmetries, but very little work has been done in these areas. The effects of lipid asymmetry may be particularly important. All biological membranes appear to contain substantial fractions of lipids that prefer the H_{II} phase in isolation under physiological conditions (10). It has been conjectured that, since such lipids pack well in nonbilayer configurations, they may somehow contribute to the nonbilayer geometries that necessarily must occur during processes such as the fusion of vesicles [see Cullis et al (10) for a review]. It must be remembered, however, that biological fusion is a carefully controlled event in which specific sides of bilayer organelles are brought into contact and fuse. To illustrate, consider the hypothetical case of approaching biomembrane vesicles. Let these vesicles have external monolayers of 100% phosphatidylcholine (PC) and inner monolayers of 100% phosphatidylethanolamine (PE), for an overall composition of 50% PC and 50% PE. In light of the previous discussion, we expect the local surface interactions at near contact to be highly dependent on the interacting surface molecules, which, in this case, are PC. Now consider a liquid crystalline system of the same overall composition (50% PC, 50% PE), but in which the two lipids are equally mixed in each monolayer of

each bilayer. Is there any good reason to expect the surface interactions to be the same? The question has not been explored. In principle, bulk liquid crystal systems composed of asymmetric bilayers can be made. We urge their study.

Despite the cautions that must be applied in extrapolating between the liquid crystal and biological regimes, it is obvious to workers in the field that we are entering on very exciting times. In part, this is due to a convergence of disciplines: surfactant chemists, physicists, and biologically oriented researchers are recognizing, more than ever before, that they have been working on different aspects of the same problem of amphiphilic aggregation and that there is much to be gained by exchange of ideas and problems.

ACKNOWLEDGMENTS

We have benefited from discussions with many colleagues, most notably Greg Kirk and Adrian Parsegian. We thank Don Small for allowing us to see an advance copy of his book.

S.M.G. is supported by grants from the NIH (GM32614) and the DOE (DE-AC02-765V03120). The research programs of P.R.C., M.J.H., and C.P.S.T. are supported by the MRC of Canada and the BS Heart Foundation. P.R.C. is an MRC scientist.

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