Structure of the inverted hexagonal (H_{II}) phase, and non-lamellar phase transitions of lipids

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I. Introduction

It is now nearly half a century since the pioneering X-ray diffraction work of Bear, Palmer and Schmitt [1,2] which established that hydrated natural lipids could form fluid lamellar phases, and which provided a firm support for the concept that the lipid bilayer forms the basis of biological membrane structure. The question naturally arises as to whether the lipid component alone dictates this structure, or whether it requires the active collaboration of the many other components of the membrane, such as the integral and peripheral proteins, to stabilise the bilayer configuration. The many studies that have been performed on lipid extracts, purified lipid components, and synthetic lipids have established that, under suitable conditions of temperature and hydration, lipids may indeed spontaneously form lamellar phases, consisting of one-dimensional periodic stacks of identical bilayers, each separated by a water layer of constant thickness. However, it was shown by the pioneering work of Luzzati and co-workers [3] that this lamellar phase was only one of a large variety of liquid-crystalline phases which may be adopted by hydrated lipid systems. To date, the most common of these non-lamellar phases, in biological lipid systems, is the inverted hexagonal (H₇) phase. It is the purpose of this review to summarise the structural properties of this phase, and to describe the thermodynamics, and geometric and topological aspects, of non-lamellar phase transitions. Particular emphasis will be placed on the role of interfacial curvature, since I believe that this is the most fundamental parameter which controls the polymorphic phase behaviour. I do not claim that hexagonal phases will be found as static structures in cells – in fact the evidence to date suggests the contrary (other than in certain pathological conditions), rather, the case I would like to argue strongly is that the physicochemical interactions which are operating in pure lipid systems, tending to drive the equilibrium state towards a non-bilayer configuration, are certainly also present in some biological membranes, and may have subtle, yet profound influences on many membrane-related processes.

The basic thesis of this review is then that non-lamellar phases of lipids are of fundamental relevance for many processes taking place in, on, or through most biomembranes. It has been argued [4–6] that this is the case irrespective of whether such phases, or their structural elements, actually exist as stable structures within biological systems. The fundamental observation in support of this view is that most membranes contain large amounts of lipids which, in isolation, will adopt non-lamellar phases, such as the inverted hexagonal H₇ phase, under quasi-physiological conditions. Such lipid systems are inherently under particular internal stresses under conditions (such as lowered temperature) where the fluid bilayer Lₑ phase is adopted. The reason for this is that the forces which induce the lamellar to non-lamellar phase transition are already present in the bilayer a long way away (thermodynamically speaking, e.g., temperature) from the actual transition. In general, the principle factor driving such transitions is a tendency for one or both monolayer halves of the bilayer to curl away from a planar configuration. This arises from an imbalance developing in the lateral stresses (pressures and tensions) from the headgroup region, the polar/non-polar interface, and the hydrocarbon chain region of the bilayer. These different contributions to the lateral stress in a monolayer are illustrated schematically in Fig 1.

For an isolated monolayer to stay flat, these lateral interactions must be in balance across the monolayer. If the lateral pressure distributed in the headgroup region outweighs that in the chain region, there will be a tendency for the layer to curl towards the hydrocarbon region. For the opposite case of the pressure in the chain region being dominant, then the curvature will tend to develop towards the aqueous region. As shown in Fig 2, we take these situations to correspond to positive and negative mean curvature, respectively (see subsection IV-C).

However, for a bilayer, since the two halves are hydrophobically coupled, a deformation of one side is inevitably strongly influenced by the response of the apposing monolayer to such a deformation. When one monolayer tends to adopt a positive curvature (i.e., curving towards the hydrocarbon chain region), and the other tends to adopt a negative curvature (i.e., curving towards the aqueous exterior), then the bilayer can...
lower its internal stress simply by bending, for example, towards a cylindrical geometry. This can then lead to the bilayer rolling up into a cylindrical spiral (such as myelin sheath). However, for a symmetrical bilayer (with identical conditions on each side), the two halves of the bilayer both want to curve in the same way (either positive or negative curvature), and so counteract each other since they are oppositely oriented back-to-back. This state of balance in which the internal stresses across the whole bilayer stabilize a planar bilayer configuration is an example of a state of physical frustration, whereby each monolayer is prevented from adopting an equilibrium curvature, by the coupling to the other. It should be emphasized that such a bilayer membrane is fundamentally different from one in which each monolayer has no tendency to curvature. In the latter case, the interfacial tension in each monolayer is internally balanced by lateral pressures both in the headgroup and in the hydrocarbon chain region. In the former case, however, it is possible for the interfacial tension to be balanced predominantly by either the headgroup or the chain regions, the lateral pressures in the other region having a relatively low value. These two situations correspond, respectively, to a tendency to form ‘normal’ or ‘inverted’ non-lamellar phases (see subsection II-A). An experimentally accessible parameter which gives some measure of this tendency is the value of interfacial area per molecule small, or large, relative values correspond, respectively, to the lateral pressure being concentrated in the chain, or headgroup, regions (the chain lateral pressure is a strong function of the area per molecule). The distribution of lateral forces on an embedded molecule (such as an integral membrane protein) at different depths in the bilayer will depend upon how the balance of stresses is achieved. In the region of the water/hydrocarbon interface, the interfacial tension will exert lateral stretching forces on the embedded molecule. In a bilayer containing ‘HII’ phase forming lipids, there will also tend to be a relatively large lateral pressure in the bilayer interior, and so any integral molecules will experience compressive forces in this region. Conversely, a more hydrophilic bilayer will have larger lateral pressures in the headgroup region, giving rise to compression of this part of the embedded molecule. One can thus see that, for example, the energetics/kinetics of transport across membranes may in principle be modulated by the internal lateral stresses across the lipid bilayer, which in turn is a direct function of the tendency of the lipid component to adopt non-lamellar phases.

Thus the presence in a bilayer of a proportion of ‘HII’ phase forming lipids may modify many of the properties of the bilayer such as its thickness, permeability, bending stiffness (deformability), and may affect the rates of rotation and lateral self-diffusion both of the lipid and of any associated protein components. Recent osmotic swelling experiments on lipid vesicles, where the ‘HII’ forming phosphatidylethanolamines were found to have unusually large values of membrane elastic modulus [7] support this view.

Indeed, there is strong evidence that the function of membrane proteins can be affected by modifying the properties of the lipid matrix in which they are embedded. It appears that the activity of reconstituted systems is strongly influenced by the particular lipid used for the reconstitution. For example, the $^{32}$P$_i$-labelled ATP exchange activity of reconstituted mitochondrial proteins was enhanced when the lipid vesicles contained increasing amounts of PE [8]. Similarly, the activity of Ca-ATPase, reconstituted into liposomes composed of lipid mixtures, was found to increase upon increasing the amount of PE [9], or cholesterol [10] in the proteoliposomes. Although no...
evidence for non-lamellar structures was found in the reconstituted systems. PE is known to promote $H_{11}$ phase formation in the pure lipid systems.

The possible role of non-bilayer lipid structures in dynamic processes in membranes is difficult to prove or disprove. Since many such processes, such as fusion, necessarily involve a destabilization of the bilayer structure, it is very tempting to assume that Nature must have evolved so as to take advantage of the extraordinary polymorphism of lipids, as a means of lowering the activation barriers. However, such processes need only occur in highly localised regions of the membrane, and for short times (ms), and will thus be very difficult to detect by any spectroscopic or structural technique. Ultra-fast cryofixation electron microscopy might conceivably manage to capture such events.

This review will concentrate on the physicochemical aspects of the $H_{11}$ phase and non-lamellar phase transitions, with regard to biological lipid systems. Little will be said about the fascinating and very important topic of cubic phases [11,12], since a review of this subject has recently appeared [13]. Biological and biochemical aspects of the $H_{11}$ phase and of lipid polymorphism have been extensively reviewed in recent years [4,14–20], and these articles should be consulted for much detail which will not be repeated here. A number of previous reviews have dealt with structural aspects of lipid phases, both in the dry [21–23] and the hydrated states [3,11,24–34]. A number of reviews deal with surfactant association into micelles [35], and liquid-crystalline phases [36–41]. A review of the self-organization properties of polymeric liquid crystals has recently appeared [42]. A number of reviews have dealt with spectroscopic studies of lipids such as NMR [28,43,44], ESR [45,46] and infrared spectroscopy [47]. The thermoelastic properties of membranes have been discussed in detail [48]. The physical chemistry of lipids and membranes with regard to the molecular interactions involved in bilayer stability has been extensively reviewed [49–56]. A number of reviews have dealt with lipid phase transitions and phase diagrams [57–63]. The role of electrostatic interactions in lipid phase transitions has been reviewed by a number of authors [55,64–68]. The current view of the role of hydration interactions in determining the structural properties of lipid systems has been discussed in a number of proceedings and reviews [69–73]. The role of hydrogen-bonding in lipid polymorphism has been reviewed a number of times [74–76]. Finally, excellent overviews of the current status of the physics of lyotropic mesophase structure and stability have recently appeared [77–79]. The possible implications of lamellar to non-lamellar transitions for biological processes have been extensively discussed [4,14–19,80,81]. Finally, the possible role of lipid polymorphism in the mechanism of membrane fusion has been reviewed a number of times [68,82–88].

Historically, it is often forgotten that when non-lamellar phases were first discovered, many physical chemists doubted their very existence. In fact some decades previously, a similar controversy had occurred over the existence of micelles. To some extent an analogous situation occurs today, with many biologists being unwilling to accept that any structure other than bilayers are in any way relevant to biological membrane structure. Whilst it is indeed likely that non-lamellar phases per se will only be found in very limited or unusual circumstances in biological systems, nonetheless the same physico-chemical factors which determine the bulk phase behaviour of pure lipid systems will still be operative in real membranes at the molecular level and will thus influence many of the microscopic properties of the membrane.

II. Structural polymorphism of lipids

II-A Topology

It is crucial to realize that most lyotropic mesophases exist as symmetric pairs, a 'normal' (type I) oil-in-water version, consisting of lipid aggregates in a continuous water matrix, and a topologically 'inverted' (type II) water-in-oil version, in which water/hydrated headgroup aggregates are arranged within a continuous non-polar matrix composed of the fluid hydrocarbon chains. The simplest example of this is the pair of hexagonal phases $H_1$ and $H_{11}$, as shown in Fig 3.

There is a fundamental dysymmetry between the $H_1$ and $H_{11}$ phases, when one considers the effects of varying the water content of the phases (by whatever means). The $H_1$ phase can in principle swell without a significant change in the interfacial area per molecule, whereas swelling the $H_{11}$-phase lattice inevitably causes the interfacial area per molecule to increase, as demonstrated in Fig 4.

The situation will of course be the other way round if one considers swelling the phases by addition of a non-polar solvent.

A further dysymmetry is that for the $H_1$ phase, the water continuum is a true solvent, in the sense that, although a structured fluid, it is able to freely fill all of the polar volume unoccupied by the lipid headgroups. For the $H_{11}$ phase, this situation is not necessarily the case, since the hydrocarbon chains are pinned at one end to the polar interface by the headgroups, and the conformational state of the hydrocarbon chains in part determines whether the hydrophobic region can be uniformly filled, and hence the $H_{11}$ phase allowed to form. It has been suggested [89–91] that the free energy cost of some of the chains stretching in order to completely fill the hydrophobic volume can inhibit $H_{11}$ phase formation. This idea is strongly supported by the observed inverse chain-length dependence of the $L_n$-$H_{11}$ phase...
transition temperature (see subsection V-B) and by the fact that addition of small amounts of alkane relieves the hydrocarbon chain packing stress and facilitates the \( L_a \rightarrow H_{\|} \) transition [92–94].

The normal hexagonal \( H_1 \) phase of lipid/water systems was first found by McBain [95] in hydrated dodecylsulfonic acid (23–70\% in water), and its structure elucidated by Luzzati and co-workers [96]. The \( H_1 \) phase had in fact first been recognized by Bernal [97] in a study of the structure of aqueous solutions of tobacco mosaic virus (TMV). This normal type of hexagonal phase is very widespread in biological systems, being found in solutions of DNA, polypeptides and polysaccharides [98], and in muscle, and possibly in collagen [99]. In fact any biopolymer which adopts a rod-like shape (for example an \( \alpha \)-helix) will be expected to form a hexagonal phase under certain conditions, since this is the natural packing for circular cylinders. Although the \( H_1 \) phase is not normally observed for diacyl phospholipids, it is formed by lyso-lipids for example, egg lysoPC forms the \( H_1 \) phase at 37\°C over the range 22–52 \% water [100], and similar results are found for chemically homogeneous lysoPCs [101]. It is possible that \( H_1 \) phases will also be found in the future for short-chain diacyl PCs: a 35 mM solution of diheptanoyl PC in water (D_2O) forms (polydisperse) rod-like micelles of mean length 257 Å [102], which suggests that an \( H_1 \) phase might occur at lower water contents.

The \( H_{\|} \) phase of phospholipids was discovered, and its structure deduced, by Luzzati and co-workers [103,104], in a lipid extract from human brain containing 52\% PE, 35\% PC, and 13\% PI, at 37\°C and at water contents below 22 wt\%. Independently, Ekwall and co-workers identified the same structure in the ternary surfactant system sodium caprylate/decanol/water [105].

The question naturally arises as to whether it is possible to determine unambiguously the topology of the hexagonal phases (i.e., whether type I or type II) directly from the X-ray diffraction patterns. A problem arises here due to Babinet’s principle: complementary structures (e.g., in the optical analogy, an opaque screen with a lattice of holes, vis-a-vis a clear screen with the same lattice, but of opaque spots) give rise to identical diffraction patterns at all (observable) angles. For a hypothetical hexagonal phase consisting of a lipid of uniform electron density in water, at a volume fraction \((\phi_0/(\phi_0 + \phi_1)) \) of 0.5 (i.e., equal volumes of water and lipid), the topology would be impossible to determine from any single diffraction experiment. In reality, of course, these conditions are not frequently encountered. Nonetheless, determining the topology is not necessarily a trivial problem. The approaches used in practice are described in subsection III B.

In terms of the hypothetical binary phase diagram shown in Fig 7, the natural location for the \( H_1 \) and \( H_{\|} \)
phases is on either side of the lamellar phase, which, owing to its zero curvature, may be considered to occupy a central position in the phase diagram. We consider the normal hexagonal phase $H_1$ to have a positive mean curvature, $+1/2R$, in that the interface bends towards the oil, or non-polar, region, conversely, the inverted hexagonal phase $H_{12}$ has a negative curvature, $-1/2R$, in that the interface bends towards the water region. The value of cylinder radius, $R$ (and hence also the value of interfacial area per molecule), depends upon where the interface is chosen to be, and thus is to some extent ill-defined, particularly if the headgroups are large, and there is considerable interpenetration of the water and the headgroups. However, for many systems, it is reasonable to adopt one (or more) of the following protocols in order to estimate $R$ (and hence also the interfacial area per molecule) from diffraction data.

1. In the Luzzati approach, the water and lipid components are taken to occupy distinct regions within the mesophase; i.e., the interface is smooth and sharp. In the case of inverted phases such as $H_{12}$, the water component is assumed to form idealized circular rods, lined with the hydrated lipid headgroups and with the remaining volume filled by the fluid hydrocarbon chains. For 'normal' phases such as $H_1$, the situation is the opposite, with the circular lipid aggregates being embedded in a water continuum. Note that this approach assumes for simplicity that the symmetry of the structure elements (circular cylinders for hexagonal phases) is higher than that of the lattice. It is conceivable that the cylinders in some systems might deviate from circular, for example, by distorting towards having a hexagonal cross-section. This would partly relieve the packing stress due to the lipid chains having to reach into the deepest part of the hydrocarbon region in order to fill all space (free volume is energetically very costly).

2. A certain number of waters are considered to be 'strongly bound', giving rise to an interfacial region of thickness, $t$, consisting of the headgroups and this associated water. In this case, there will be two radii, $R$ and $(R+ t)$, and two values of curvature (and interfacial area).

3. The water and headgroups are taken together to comprise a polar region, the interface then being at the polar–non-polar boundary.

In all of these protocols, it is (to first approximation) assumed that the aggregates are regular in shape, e.g., in the hexagonal phases, the cylinders are taken to be circular in cross-section.

II-B Description of non-lamellar phases

The current situation with regard to the well-established non-lamellar phases of lipids is summarised in Table I. The spacegroup symbol nomenclature (short Hermann-Mauguin form) is that of the International Tables for Crystallography [106]. For consistency with all previous literature, the old notation for the cubic phases is retained.

The proposed structures of the type II versions of some of these phases are shown in Figs 5 and 6. For the cubic phase $Pn3m$, the structure is shown as rod-like to emphasize the symmetry elements of the phase. In reality, for the examples known to date, the structure would be more accurately represented by draping a

TABLE I

Description of the known non-lamellar phases of lipids

Abbreviations used: K-, Na-, Ca-soaps potassium, sodium and calcium salts of long-chain carboxylic acids. SDS, sodium dodecyl sulphate. Cytochrome c, MO monooleoyl glycerol, OA, oleic acid, 2-D, 3-D, two and three dimensional.

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Spacegroup symbol</th>
<th>Spacegroup number</th>
<th>Type I example</th>
<th>Type II example</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D</td>
<td>M (oblique)</td>
<td>p2</td>
<td>2</td>
<td>SDS</td>
<td>K-soaps (dry)</td>
</tr>
<tr>
<td>2-D</td>
<td>P (centred rectangular)</td>
<td>cmm</td>
<td>9</td>
<td>SDS/decanol</td>
<td>PC</td>
</tr>
<tr>
<td>2-D</td>
<td>H (hexagonal)</td>
<td>p6m</td>
<td>17</td>
<td>lysoPC</td>
<td>PE and PC</td>
</tr>
<tr>
<td>3-D</td>
<td>O (body-centred orthorhombic)</td>
<td>Pmmm or P222</td>
<td>47 or 16</td>
<td></td>
<td>Na-soaps (dry)</td>
</tr>
<tr>
<td>3-D</td>
<td>T (body-centred tetragonal)</td>
<td>I422</td>
<td>97</td>
<td>SDS</td>
<td>Ca-soaps (dry)</td>
</tr>
<tr>
<td>3-D</td>
<td>R (rhombohedral)</td>
<td>R3m</td>
<td>166</td>
<td>SDS</td>
<td>PC</td>
</tr>
<tr>
<td>3-D</td>
<td>Q (cubic)</td>
<td>P432, Pm3n, Pn3m</td>
<td>212, 223, 224</td>
<td>lysoPC</td>
<td>Cyt-c/MO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PE</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>OA/MO</td>
</tr>
<tr>
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<td></td>
<td>PE</td>
</tr>
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<td></td>
<td></td>
<td>K-soaps PC and PE</td>
</tr>
</tbody>
</table>

The examples, which are by no means a complete list, are taken from Refs 3, 12 and 107–110. Except where specifically excluded, a water component (of unstated amount) is also present.
b) P_{u}, centred rectangular

A

Vertical

II-C Phase equilibria and phase diagrams

For binary systems, phase transitions may be induced either by varying the composition (water content) or the temperature. For certain systems, for example many single-chain ionic amphiphiles, the former variable tends to be the most important for inducing transitions between fluid phases, and the phase boundaries are close to vertical. This is shown schematically in Fig. 7.

Conversely, for many non-ionic or zwitterionic double-chain systems such as phospholipids, temperature is the more important variable, and many of the phase boundaries can be closer to horizontal than to vertical. In this case, increasing temperature plays a qualitatively similar role to that of decreasing water content.

Most binary systems studied to date conform to this scheme, in terms of the relative positions of the phases (although a given system will not in general display all of the phases shown). Thus the phases are arranged in Fig. 7 in their 'natural' sequence, and we must enquire into the underlying factors which determine this phase behaviour. A crucial pair of parameters is the mean and Gaussian curvatures of the interface, which will be discussed further in subsection IV-C.

The case where the transitions are driven primarily by changes in water content is found in many ionic (charged) lipid systems, e.g., the alkali carboxylic soaps. In reality, there is usually some temperature dependence, and so the phase boundaries deviate from vertical. Interestingly, the phase diagrams so far published for lyso-phospholipids also tend to come into this category, although they are uncharged. For example, Fig. 8 shows the binary phase diagram for the system lyso-oleoyl phosphatidylcholine/water. It thus seems that such phase diagrams can be found when the lipid only has a single chain, and is thus considerably less hydrophobic than diacyl compounds.

The other case, where the transitions are driven predominantly by temperature, is more typical of certain diacyl zwitterionic phospholipids. Fig. 9 shows the phase diagram for the saturated C_{20} diacyl lipid di-arachidonylphosphatidylethanolamine. The L_{α} phase is only stable over a short range of temperature, and the H_{II} phase is seen to occur at high temperatures, at all water contents out to the excess water region.

Exchanging the headgroup ammonium group by a trimethylammonium group has drastic effects on the phase diagram. For example, Fig. 10 shows the phase diagram for the C_{14} saturated diacylphosphatidylcholine dimyristoyl phosphatidylcholine (DMPC) in water. In this case, and for most other chainlengths, only
Fig 6 Examples of the structures of three-dimensional inverse mesophases (A) $Q^{224}_{11}$ (Pn3m), (B) $Q^{229}_{11}$ (Im3m) (C) $Q^{230}_{11}$ (Ia3d), (D) $R_{3m}$ (R3m), and (E) $T_{11}$ (I422)
fluid lamellar phase is seen when the chains are melted, except at very low water contents.

Varying the chain length can have profound effects on the phase diagrams. For example, for short chain-length phosphatidylethanolamines, additional phases appear between the lamellar and inverse hexagonal phase, as shown in Fig. 11 for the C\textsubscript{12} dialkyl lipid didodecyl phosphatidylethanolamine. These phases include a number of bicontinuous cubic phases Q\textsuperscript{230} (Ia3d), Q\textsuperscript{224} (Pn3m); Q\textsuperscript{229} (Im3m), but other as yet unidentified non-lamellar phases probably also occur.

The structures of the normal hexagonal H\textsubscript{I}, the lamellar bilayer L\textsubscript{a}, and the inverse hexagonal H\textsubscript{II} phases are fairly well established. However, the many...
intermediate phases which may be found in locations a, b, c, and d of Fig. 7 are much less well understood. The majority of these phases (detected to date) are cubic those occurring in regions c and d being of type I (oil-in-water), whereas in regions a and b the structures are inverse, type II (water-in-oil). Furthermore, the cubic phase(s) in region d are—at least in part—based on an ordered packing of anisotropic micellar aggregates [13], whereas cubic phases detected to date in regions b and c tend to have structures based upon bicontinuous interfacial headgroup regions (phases Q\textsuperscript{212} and Q\textsuperscript{227} are exceptions to this, being based partly on closed aggregates, and partly on a continuous lipid monolayer [12]). Cubic mesophases in region a might be expected to consist of ordered lattices of inverse micellar aggregates, although no such structure has as yet been deduced.

A great deal of effort has been spent in recent years in trying to understand even the qualitative features of such lyotropic phase diagrams. In part this is hampered by the difficulties in establishing the structures of the intermediate phases, but perhaps the major problem lies in identifying and quantifying the important molecular interactions which are responsible for mesophase stability.

III. Physical techniques

III-A Optical and electron microscopy

The optical textures of liquid crystals under a polarizing microscope in some cases provide a very simple means of phase identification. This technique is routinely used in the study of thermotropic liquid crystals [114,115], and has also been employed to investigate phase diagrams of simple surfactant systems [36,116–119] and biopolymer solutions [98]. However, it has been little employed in studies of phospholipid systems, in part perhaps due to problems of sample inhomogeneity.

Although electron microscopy is one of the most direct ways of obtaining structural information, there are a number of severe problems which have hindered its use in the study of lipid phase structures. Obviously, untreated samples cannot be studied, since the water will be lost and the structure destroyed by the vacuum inside the microscope. Chemical fixation and staining in general may also destroy the labile phase structures.

The first visualization of a non-lamellar phase by electron microscopy was by Stoeckenius, who used positive staining electron microscopy to study a hydrated human brain phospholipid extract (containing approx 52% PE, 35% PC and 13% phosphonolipids) at a composition of 3 wt% water, fixed at 37°C with osmium tetroxide [104]. One of the micrographs is reproduced in Fig. 12. The electron micrographs clearly showed the hexagonal arrangement of the phase, the cylinder–cylinder separation of 42–45 Å being in excellent agreement with a complementary X-ray study on this system (without osmium fixation), which showed the formation of an H\textsubscript{II} phase at 37°C at water concentrations below 20 wt% [103].

Subsequent work used negative staining to visualize hexagonal phase structures in fully hydrated phosphatidylethanolamines, rat liver extract [120], and pig erythrocyte extract [121]. The former authors found that an H\textsubscript{II} phase was formed at 55°C, which had a lattice parameter of 60–80 Å, but that on incubation much larger values were observed. Even more surprisingly, the latter authors observed, in addition to an H\textsubscript{II} phase with a lattice parameter of 56 Å, an apparent H\textsubscript{I} phase with a lattice parameter of 61 Å. These results were clearly artefactual, due either to pH changes induced by the stain, decomposition of the lipids, or contamination with charged lipids.

A much more successful method has been found to be that of freeze-fracture or freeze-etching electron microscopy. The work of Gullik-Krzywicali and co-workers [122,123] and Verkleij and co-workers [84,124] has been particularly significant in this area. Unfortunately, in many of the published studies it is not possible to assess the extent to which the original phase structure has been preserved by the freezing procedure. One solution to this problem is to take the diffraction patterns of the samples before and after the freezing process, which allows a critical assessment of the perturbations introduced by the freezing [123,125,126].

Retinal rod outer segments are one of the few cases
Fig 12 Electron micrograph of the H_{11} phase formed by a phospholipid extract from human brain [104]. The water content was 3 wt% upon osmium tetroxide fixation at 37°C. The lattice parameter is in the range 42–45 Å. (Reproduced from the J Cell Biol (1962) 101, 221–229 by copyright permission of the Rockefeller University Press.)
Fig. 13 Freeze-fracture electron micrograph of an H₁₁ phase inclusion embedded within the lamellar array of rod outer segment disk membrane.

[128] The scale bar is 1000 Å. (Reproduced with permission from the I. Millonig, 1986, 141, 277-290.)
III-B X-ray and neutron diffraction

The principles behind these two techniques are very similar, the main difference being that X-rays are scattered by the electrons in the sample, whereas neutrons are scattered by the nuclei. Since the nuclei are so small (circa $10^{-15}$ m) that they act as point scatterers, the neutrons are scattered isotropically from a given atom, and this simplifies the analysis relative to X-rays, whose scattering from a given atom is angular-dependent because the atomic electrons occupy a volume comparable in dimension to that of the X-ray wavelength (typically 1.54 Å). A more important advantage of neutron diffraction is that different isotopes of a given element may have very different scattering powers. For example, hydrogen has a neutron scattering length of $-0.374 \cdot 10^{-12}$ cm, whereas for deuterium, the value is $+0.667 \cdot 10^{-12}$ cm. The fact that the value for hydrogen is actually negative means that its scattering is out of phase with, and hence tends to cancel out the scattering from other neighbouring atoms such as carbon, nitrogen, phosphorus, etc. The scattering contrast of specific regions of lipid mesophases can thus be altered either by specific deuteration of the lipid, or by exchange of $H_2O$ by $D_2O$.

The characterisation of lipid mesophases by diffraction [3] is based firstly on symmetry. Information on the long-range organization of the phase is contained in the low-angle region of the diffraction pattern the long-range translational ordering of the lipid/water aggregates (bilayers, cylinders, micelles, etc.) onto one-, two- or three-dimensional lattices gives rise to Bragg reflections whose reciprocal spacings ($s_{hk} = 1/d_{hk}$) are in characteristic ratios, for example:

1. Lamellar $s_l = l/d$
2. Hexagonal $s_{hk} = 2(h^2 + k^2 - hk)^{1/2}/\sqrt{3}a$
3. Cubic $s_{hl} = (h^2 + k^2 + l^2)^{1/2}/a$

(Ratios 1, 2, 3, 4 ...) (1, $\sqrt{3}$, 2, $\sqrt{7}$, 3, $\sqrt{12}$, $\sqrt{13}$ ...)
(1, $\sqrt{2}$, $\sqrt{3}$, 2, $\sqrt{5}$, $\sqrt{6}$, $\sqrt{8}$, 3 ...)

In these equations, $d_{hk}$ is the spacing of the set of lattice planes $(h, k, l)$, characterised by the Miller indices $h, k$ and $l$.

A list of the well-established non-lamellar mesophases is given in Table I.

Not all of the reflections may be observed for a particular sample; some may be absent for additional symmetry reasons (for example a body-centred cubic phase only gives reflections for which $h + k + l = 2n$ (i.e., even), additional symmetry elements within a particular crystallographic space group may give rise to other systematic absences). Furthermore, a symmetry-allowed reflection may nonetheless have zero intensity because the unit cell transform happens to pass through zero at that particular diffraction angle (vide infra). It is thus of paramount importance in structural studies of lipid mesophases to ensure that the symmetry of the phase is correctly identified. Unfortunately, this is often not so trivial as it appears, because usually only a few diffraction orders are detected, due to the large thermal disorder inherent in liquid-crystalline phases. There are many examples in the literature where incorrect assignments appear to have been made. Note that if the $\sqrt{3}$ and $\sqrt{7}$ reflections of a hexagonal phase are too weak to be observed, the pattern might be incorrectly indexed as lamellar. Similarly, a cubic phase whose $\sqrt{2}$ [110] reflection was weak or missing could be mistaken for a hexagonal phase, if only the first three reflections were detected (it is by no means unusual for so few reflections to be observed). One way to guard against such errors is to explore around a particular point in the phase diagram, checking that the diffraction data index consistently as the temperature and composition are altered within the single-phase region. In addition, other techniques such as polarizing microscopy and NMR can be invaluable for corroborating the phase identification.

There are two further aspects to analysing the diffraction data from unoriented samples of hydrated lipid mesophases; firstly, to analyze the positions of the Bragg peaks ($d$-spacings), secondly, to account for their intensities. If certain assumptions are made about the shape of the hydrated lipid aggregates and the densities of the components within the phase, a great deal of useful structural information can in fact be deduced simply from the positions of the diffraction lines, in conjunction with the chemical parameters such as the lipid molecular weight, $M_L$, the water and lipid partial specific volumes $\bar{v}_w$ and $\bar{v}_l$, and the lipid weight con-
concentration \( c_L \) (lipid/(lipid + water)) The volume concentration (fraction) of lipid is then

\[
\phi_L = \left[ 1 + \left( \frac{\phi_w}{\phi_L} \right) \left( 1 - c_L / c_L \right) \right]^{-1}
\]

(1)

and the volume concentration of water is \( \phi_w = 1 - \phi_L \).

For the \( H_{II} \) phase, the lattice spacing (see Fig 14) is related to the \( d \)-spacings (the \([10] \) Bragg peak) by \( a = (2/\sqrt{3}) d \), and the diameter of the water cylinders is given by

\[
d_w = \left[ (2\sqrt{3}/\pi)(1 - \phi_L) a^2 \right]^{1/2}
\]

(2)

The lipid layer thickness along the line connecting the cylinder axes is then \( d_L = a - d_w \). The half thickness \( d_L/2 \) gives the minimum length of a lipid molecule, \( l_{min} \), in the \( H_{II} \) phase. Similarly, the distance from the interface to the centre of the hydrophobic region represents the maximum length \( l_{max} \), and this is given by \( (a/\sqrt{3}) - d_w/2 \).

The area per molecule at the lipid/water interface is given by

\[
S = 2\pi d_w \sigma_l \delta / (\sqrt{3} a^2 \phi_L N_a)
\]

(3)

A problem arises in assigning the interface between the aqueous and the lipid regions of the phase; there is simply no unambiguous way of doing this, since there is a partial interpenetration of the polar lipid headgroups with some of the water. The Luzzati operational approach, which we will adopt in the following equations, assumes:

(1) The aggregates consist of flat bilayers and circular cylinders in the lamellar and hexagonal phases, respectively.

(2) All of the water is segregated from the lipid within each phase.

(3) The densities of the components within the phase are equal to their bulk values at the same temperature and pressure.

All of these assumptions are clearly only approximations to the real situation, but the approach has nevertheless proved to be extremely useful.

It is obvious that the first assumption will break down under certain circumstances, for example for the \( H_{II} \) phase at low hydrations. More subtly, the competition between chain configurational entropy and the need to fill all of the non-polar volume of the \( H_{II} \) phase with hydrocarbon might cause the circular cylinders to deform slightly towards a hexagonal cross-section [92].

In order to establish the topology of a hexagonal phase, a number of approaches may be employed [3].

(1) For the \( L_a \) and \( H_{II} \) phases of surfactants, such as potassium soaps, log/log plots of interfacial area per molecule \( S \) versus the molar ratio of (polar headgroups/water) fall on the same straight line for all chainlengths between \( C_8 \) and \( C_{22} \) [137]. Repplotting the hexagonal phase data with the (incorrect) assumption of an \( H_{II} \) structure yields complex dependences of the curves.

(2) When data for the interfacial area per molecule are plotted versus water concentration, \( S \) often stays rather constant for a type I hexagonal phase, but increases dramatically for a type II structure. This may be understood qualitatively from examination of Fig 4. A lattice of lipid rods in a water matrix can (in principle) swell at constant surface area (as can the lamellar phases), an inverse phase such as \( H_{II} \) must necessarily undergo an increase in \( S \) as increasing amounts of water penetrate the rods comprising the polar groups. Plots of \( H_{II} \) data, assuming the incorrect type I structure, give unreasonably large values for \( S \), and more importantly, show a strong decrease with increasing water content. This last feature breaks a strong rule, obeyed for almost every surfactant or lipid system, that the area \( S \) always increases or stays constant, but never decreases, with increasing water content. This rule is essentially simply an expression of the fact that the favourable interaction between water and the amphiphile polar headgroup normally causes a lateral expansion of the headgroups.

(3) Observation of a hexagonal phase in coexistence with an excess aqueous phase is by itself strong evidence in favour of an inverted type II structure, all type I hexagonal phases (of simple lipid systems) so far studied invariably break up into micellar solutions beyond a certain limiting water content.

(4) The value of interfacial area \( S \), measured at the (effective) water/lipid interface, is normally lower for an \( H_{II} \) phase than for an adjacent \( L_a \) phase, both when the transition is driven by varying the composition, and by varying the temperature. On the other hand, \( H_{I} \) phases normally have larger values of \( S \) than \( L_a \).

(5) Analysis of the intensities of the Bragg peaks with those calculated from models of the structure, for a range of water contents, will usually provide unambiguous evidence for one or the other topology (see Fig 17).
Neutron contrast variation techniques provide a powerful method for determining topology, although as yet this has been little employed in structural studies of lipid polymorphism. For example, it has been shown for microemulsions that the interfacial curvature can be measured from the extrapolated zero-angle scattered intensity as a function of the scattering contrast between the aqueous region and the interfacial and hydrocarbon regions [138]. For the particular system studied (SDS/toluene/n-butanol/brine) it was found that a well-defined interfacial film of zero mean curvature exists when the volume fraction of the aqueous region was 0.5. This lends strong support to the idea that bicontinuous phases are based upon minimal surfaces (see Section IV).

Boden and co-workers have proposed an approach for analyzing diffraction data from ternary amphiphile/water/solubilize lamellar mesophases, to permit the effect on the interfacial area of varying any one of the components to be deduced [139,140].

### Scattered intensity

The incident and scattered X-ray or neutron beams are described by their wavevectors \( k_i \) and \( k_s \), whose moduli are equal, and given by \( |k| = 2\pi/\lambda \), where \( \lambda \) is the wavelength of the radiation. The geometry of the diffraction experiment is shown in Fig. 15. The Ewald sphere, which defines all of the possible scattered wavevectors \( k_s \), is of radius \( 2\pi/\lambda \), and is centred on the sample. It is convenient to express the direction of the scattered beam in terms of the 'scattering vector' \( \mathbf{Q} \), where \( \mathbf{Q} = (k_s - k_i) \), and has modulus \( |\mathbf{Q}| = 4\pi \sin\theta/\lambda \), where \( 2\theta \) is the angle between the incident and scattered beams. The function \( F(\mathbf{Q}) \) is the amplitude of the wave of scattering vector \( \mathbf{Q} \), and is the Fourier transform of the sample structure (electron density or neutron scattering length density distribution). The transform \( F(\mathbf{Q}) \) is centered on the surface of the Ewald sphere, at the point where the undiffracted beam emerges. The orientation of the sample (defined by the function \( \rho(r) \)) and its transform \( F(\mathbf{Q}) \) are coupled together rotating the sample about its centre has the effect of rotating the transform about its centre by the same amount. The intensity scattered in a direction defined by some particular value of \( \mathbf{Q} \) is given by

\[
I(\mathbf{Q}) = |F(\mathbf{Q})|^2
\]

Thermal disorder does not affect the sharpness of the Bragg peaks, but decreases their intensities progressively towards higher diffraction angles. In calculations, this may be allowed for by scaling the structure factors by a Debye-Waller (temperature) factor

\[
\exp\left[-\alpha Q^2\right]
\]

Effects due to factors such as surface roughness of the water/lipid cylinders may be absorbed into this factor.

Because of the short-range liquid-like disorder within (truly) liquid-crystalline mesophases such as \( \text{L}_a \) and \( \text{H}_{11} \), the Bragg peaks are largely confined to the low-angle region and the scattered intensities are insensitive to the precise atomic configurations. It is therefore appropriate to follow the approach adopted in solution small-angle scattering [141–146], whereby the particles are modelled by idealized regions or shells of uniform scattering density. For a particle of a defined shape and dimensions, it is then possible to calculate the scattered amplitude \( F(\mathbf{Q}) \) (the form factor). In general, one must also take into account the orientational distribution of the particles, and the positional/orientational correlations between particles (interparticle interference terms) to calculate the scattering. However, in the case of the \( \text{H}_{11} \) phase, inasmuch as it can be modelled as an infinite hexagonal lattice of cylindrical aggregates, the problem is much simpler, as will now be outlined.

The scattering from cylindrical particles has been discussed by a number of authors [141,143,147–150]. The scattered amplitude from a solid cylinder of length \( 2L \), diameter \( 2r \), volume \( V \), and electron density \( \rho \), embedded in a uniform solvent of density \( \rho_s \), is given by

\[
F(\mathbf{Q}) = F(Q, \beta) = 2(\rho - \rho_s) V \left[ \sin(QL \cos \beta)/QL \cos \beta \right] J_1(Qr \sin \beta)/Qr \sin \beta
\]

where \( \beta \) is the angle between the cylinder axis and the scattering vector \( \mathbf{Q} \), and \( J_1 \) is the first-order Bessel function of the first kind. This function is essentially the Fourier transform of the cylinder, which to a first
approximation, as indicated in Fig 16, has roughly the form in reciprocal space of a flat ‘pancake’, aligned with its symmetry axis parallel to the real space cylinder. In terms of the surface at which the transform first passes through zero, the diameter of the ‘pancake’ is roughly \((7.7/r)\), and its thickness is roughly \((2\pi/L)\), in reciprocal space (Subsidiary non-zero regions exist beyond this surface) It is the intersection of this function with the Ewald sphere which yields the diffraction pattern of a cylinder (see Fig 15).

Now, in the case of the hexagonal H II phase, we may assume that the cylinders are very long, i.e., we let \(L\) tend to infinity in Eqn 6. The effect of this is to put the first term in square brackets to zero, except for \(\beta = \pi/2\), when it is equal to unity, i.e.,

\[
F(Q,\pi/2) = F(Q) = 2(p \rho_w - \rho_{pol})V \left| J_1(Qr_w)/Qr_w \right|
\]

In other words, for an infinitely long cylinder aligned along \(z\), the transform only exists in the \(x\)-\(y\) plane, i.e., the ‘pancake’ becomes infinitely thin.

To generate a hexagonal phase of such solid cylinders, we simply mathematically convolute the single cylinder with a two-dimensional (2D) hexagonal lattice of points (delta functions) aligned in the \(x\)-\(y\) plane (see Fig 16), which has the effect of setting down a cylinder at each of the lattice points. We now use the Convolution theorem, which states that the Fourier transform of the convolution of two functions is equal to the product of the individual transforms, to deduce the transform of the hexagonal phase. The transform of a 2D hexagonal lattice of points in the \(x\)-\(y\) plane, of lattice parameter \(a\), is another 2D hexagonal lattice (rotated by 30° around the \(z\) axis), but of infinite lines extending along \(z\). The transform of the hexagonal phase is thus the product of this with the thin ‘pancake’ transform of the single cylinder, i.e., it consists of a 2D hexagonal lattice of points, but whose amplitudes \(F_{nl}\) reflect the value of the underlying continuous cylinder transform at that position.

The fact that the water/lipid cylinders of the H II phase are not actually of uniform electron density may be taken into account by dividing up the cylinder into a series of concentric cylindrical shells, whose thicknesses and electron densities may be estimated from the chemical knowledge of the components and the measured dimensions of the phase. This treatment assumes that the matter is uniform within each shell, that the shells are circular, and that the electron density of the hydrocarbon region is uniform. At the low resolutions yielded by these mesophases, these assumptions are probably usually reasonable.

For the H II phase of phospholipids, one can adopt a two-shell model: an inner cylinder of water with a radius \(r_w\) and an electron density \(\rho_w\), and a cylindrical shell of headgroups of outer radius \(r_{pol}\) and electron density \(\rho_{pol}\), the remaining volume being filled by hydrocarbon chains of electron density \(\rho_{par}\). The scattered amplitude per unit length then becomes

\[
F(Q) = 2\pi r_w^2 (\rho_w - \rho_{pol}) \left| J_1(Qr_w)/Qr_w \right| + 2\pi r_{pol}^2 (\rho_{pol} - \rho_{par}) \left| J_1(Qr_{pol})/Qr_{pol} \right|
\]

An equivalent expression has been extensively used by Luzzati and co-workers in their crystallographic analyses, not only of hexagonal phases, but also of cubic phases. An example of the use of this equation to analyze data is shown in Fig 17. The parameters were deduced from the published data for DDPE and DAPE [108].

Although the fit is not quantitative, the agreement is quite good considering the simplicity of the model structures. Two important points emerge from this analysis. Firstly, at low water contents the first-order (10)
Bragg peak has a much larger intensity than the higher order peaks, which may thus be unobserved. Secondly, as the water content increases, the zeroes in the continuous transform move to lower Q (i.e., angle) more rapidly than do the positions of the Bragg peaks, putting their intensities to zero as they coincide with them in Q value. Thus at a water content where the (11) reflection has zero intensity, the structure could be mistaken for lamellar, if only the (10) and (20) reflections were observed. The literature on lyotropic liquid crystals contains a number of examples where such incorrect assignments have probably been made.

An alternative approach is to use one of a variety of methods for deducing the phases of the observed diffraction peaks, and then to explicitly calculate the low-resolution density map by Fourier transformation of the phased amplitudes. An example of an electron density map of an HII phase of phosphatidic acid/water \([151]\) is shown in Fig 18.

The map was calculated using the seven observed structure factors (it is unusual to be able to detect as many Bragg reflections as this from phospholipid hexagonal phases), and agrees very well with the model structure shown, whose dimensions were calculated from the composition, lattice spacing, and densities.

### III-C. Differential scanning calorimetry

Phospholipid lamellar - inverted hexagonal transitions were first studied calorimetrically in unsaturated phosphatidylethanolamines \([152,153]\). This was then extended to include the corresponding saturated systems \([154–157]\). The measurements showed that the L\(_\sigma\)-HII transition is invariably of low enthalpy, typically 5–15\% that of the gel–fluid transition. This result indicated that, notwithstanding the massive structural rearrangement occurring at the transition, this is achieved without major energetic changes. Of course, it is possible that some cancelling effects might be present, for example the positive contribution to the enthalpy from activation of increased chain disorder, might be partially cancelled by a negative enthalpy change from the interfacial region, if the headgroup packing were to become tighter in the HII phase (vide infra).

It has been pointed out by several authors that the low enthalpy of the L\(_\sigma\)-HII transition has important consequences for the sensitivity of the transition temperature to perturbations. To a first approximation, the magnitude of such shifts should be inversely proportional to the transition entropy, and thus should be relatively large for non-lamellar transitions. This implies...
that it should be possible to induce non-lamellar transitions isothermally by small changes to the physicochemical conditions, such as hydration, pH or salt concentration. It is indeed usually found that such transitions tend to be much more sensitive to such perturbations than the chain-melting transition (see Section V).

It is interesting to note that a scanning densitometry study [158] found a 2.4% change in volume at the gel–fluid transition of DMPE, but showed no detectable change in the volume coefficient of expansion at the $L_a$-$H_{II}$ transition of either fully-hydrated egg PE, or dioleoyl PE. However, the fact that pressure raises the $L_a$-$H_{II}$ transition temperature (see subsection VI-H) implies that there must also be an increase in specific volume – albeit small – at this transition. One important finding that emerged from the calorimetric studies of the saturated phosphatidylethanolamines [157,155] was that the $L_a$-$H_{II}$ phase transition temperature has an inverse dependence on chain length, dropping steeply until for long enough chain lengths it merges with the (rising) gel–fluid transition temperature (See subsection V-B).

### III-D Spectroscopy

**Magnetic resonance spectroscopy (NMR and ESR)**

NMR and ESR have been used both diagnostically, to identify non-lamellar phases, and also (to some extent) to probe the segmental chain conformation in hexagonal $H_{II}$ phases. The former application is based on the fact that the translational diffusion of lipid molecules around the cylinder axis in both type I and type II hexagonal phases can give rise to an additional averaging mechanism, compared with the lamellar phase, for spectroscopic properties such as ESR hyperfine splittings and NMR chemical-shift anisotropy or quadrupolar splittings. However, in the case of phospholipid $H_{II}$ phases, the rate of lateral diffusion (circa $2 \times 10^{-8}$ cm$^2$ s$^{-1}$ in $L_a$ [159]) is too slow on the ESR timescale (ns) to observe this effect; it has, however, been demonstrated to occur in a soap/water $H_1$ phase, where the rate of lateral diffusion, $D = 1.5 \times 10^{-6}$ cm$^2$ s$^{-1}$, is some two orders of magnitude faster [160]. On the other hand, the NMR timescale of $\mu$s is slow enough to allow complete averaging to occur, leading to a halving [161] of the chemical shift anisotropy (e.g., $^{31}$P-NMR) or the quadrupolar splittings ($^2$H-NMR). Furthermore, the NMR experiments involve little or no perturbation to the mesophase structure, since they are performed either using naturally present nuclei ($^1$H, $^{31}$P, $^{13}$C), or in the case of $^2$H-NMR involve merely the substitution of deuterium for hydrogen. In fact, the $^2$H experiment may be performed by deuterating either the phospholipid or the water, the latter approach, which is of course far easier, can also be used because the water which is partially bound to the lipid headgroups also experiences an additional averaging mechanism by the diffusion around the cylinder axis.

Although $^{31}$P-NMR has been very extensively and successfully used to study $H_{II}$ phases in phospholipid systems, in particular by Cullis, De Kruijff and co-
workers, some caution is called for, since the NMR spectrum does not directly reveal the symmetry of the phase. It has been shown [162] that a change in headgroup conformation within a lamellar phase could in principle give rise to the same characteristic halving of the chemical shift anisotropy as a transition to a hexagonal phase. It was claimed that aqueous dispersions of phosphatidylglycerol and of phosphatidylcholine give rise to "hexagonal-like" $^{31}$P-NMR line-shapes, although the lipids were organised in bilayers [163]. However, a subsequent X-ray study showed that these systems did indeed form the HII phase, at 59 and 54°C, respectively [164]. On the other hand, an NMR study of hydrated sphingomyelin [165] did indicate the presence of an HII phase, where in reality the structure was lamellar. Such mistakes can arise if the $^{31}$P-NMR spectrum contains a mixture of "bilayer" and "isotropic" signals, attention should be paid to the chemical-shift positions of the peaks in the spectrum, in addition to the shape of the spectral envelope. The validity of $^{31}$P-NMR phase identifications has been reviewed by comparison with X-ray results [166].

ESR and 2H-NMR may be used to measure the orientational order of specific chain or headgroup segments of phospholipids in different phases. This is conveniently described in terms of so-called order parameters $S = 1 - 2/3(cos^2\theta) - 1$, where $\theta$ is related to the instantaneous orientation of a particular segment with respect to the normal to the aggregate surface.

ESR has been used to investigate the changes in chain conformation that occur at Lα-HII transitions. One study [167] found little difference between spectra from the lamellar phases of egg PC and sodium cardiolipin, and that from an HII phase of calcium cardiolipin. Another study, of the system phosphatidic acid/water [151], using a stearic acid spin label with the nitrooxide group attached to the C4 carbon, found that a plot of the order parameter against temperature showed no discontinuity at the Lα-HII transition, but only a change of slope. This result indicated that not only are these chain motions occurring on a rapid time-scale ( < ns) very similar in both the Lα and HII phases, but also that the rate of lipid lateral diffusion in the HII phase cannot be much faster than in Lα. A further ESR study found an increased chain disorder at the Lα-HII transition, which was larger towards the terminal methyl ends of the chains [168]. A similar study, where headgroup spin-labeled lipids were also employed, found that the increase in molecular mobility at the transition for the chain region, changes over to a decrease in the headgroup region (Seddon, J M, Eibl, H., Sachse, J H., Watts, A. and Marsh, D., submitted) This reflects directly the inverse nature of the HII phase, and was shown to correlate with the lateral area per molecule calculated at different distances away from the water/lipid interface.

2H-NMR studies have shown that the orientational order of the entire acyl chain of PE bilayers is larger than for PC [169,170]. Incorporation of increasing amounts of PE in bilayers of PC, 2H-labelled at the C-11 position, caused a progressive increase in order of the labelled CD2 group [4]. A study of the Lα-HII transition of DEPE demonstrated an increase in chain conformational disorder at the transition [171]. In addition, the variation with carbon position n, of the hydrocarbon chain orientational order parameter $S(n)$, has been shown to be different in the HII phase from that of the Lα phase [170,172]. The order parameter falls more rapidly with n in the case of the HII phase, consistent with the increased chain conformational disorder. These results are in contrast to those obtained for the (oil-in-water) HII phase, whose order parameter profile was very similar to that of the Lα phase [173]. This indicates that the statistical:thermodynamic approaches developed for type-I systems [174–177], will need modification to allow for additional effects peculiar to inverse phases such as HII.

2H-NMR has also been used to study headgroup orientation in hydrated glycolipid systems [178–180]. For β-ditetradecyl glucopyranosylglycerol, it was found that at the Lα-HII transition there is a reorientation of the sugar ring, and that its orientational order parameter decreased slightly from a value of 0.45 to 0.38. The latter point implies that there is no large increase in the amplitude of headgroup motion on entering the HII phase. Different behaviour was observed for the anomeric α-form of the glycolipid. In this case the sugar ring is almost parallel to the bilayer surface in Lα, it has a larger order parameter of 0.56, and does not reorient on entering the HII phase.

Infrared spectroscopy

Infrared spectroscopy has been used to shed valuable light on lipid polymorphism [147,181,182]. In studies of natural phosphatidylethanolamines, it was found that the frequency of the CH2 symmetric stretching mode (close to 2850 cm$^{-1}$) increases by approx. 1 cm$^{-1}$ at the lamellar - hexagonal HII transition, consistent with an increase in the concentration of gauche bonds in the acyl chains. The corresponding shift at the gel-fluid transition was only twice as large, which is surprising because undoubtedly there is a far larger increase in the conformational disorder at the latter transition than at the former. The carbonyl C=O stretch band showed a very interesting, but unexplained, temperature behaviour. The frequency dropped by 3.5 cm$^{-1}$ at the gel-fluid transition, but returned to the gel-phase value in the HII phase. No significant changes were seen in the infrared bands arising from the phosphate and ethanolamine groups, and thus was interpreted as implying that the headgroup conformation, hydrogen-bonding, and hydration are essentially the same in the lamel-
lar and the hexagonal phases. However, diffraction studies performed on saturated PEs as a function of concentration [108], indicate that the latter point is not in general the case, and furthermore, show that the hexagonal H$_{II}$ phase can form at all hydrations, even down to the anhydrous compound, which is hardly conceivable without some change in headgroup conformation occurring.

**IV. Thermodynamics of non-lamellar phase transitions**

### IV-A Lipid self-assembly and the hydrophobic effect

The basic idea behind the concept of lipid self-assembly is that the aggregate structures which form the various lyotropic mesophases arise from an interplay between the hydrophobic effect, which ensures a segregation of hydrocarbon chains away from contact with water, and various geometric packing constraints [49,51]. For example, no point within an aggregate can be further from the interface than the fully extended length of the molecule. Similarly, for different shapes of aggregates, specific geometric relationships will hold between the molecular volume, the molecular surface area at the interface, and the molecular length. Such concepts originated from early investigations by workers such as Hartley and Tartar [183,184].

A monomeric solution of an amphiphile such as a phospholipid spontaneously aggregates into micelles or liquid-crystalline phases as the concentration is increased above the cmc (critical micelle concentration), and these aggregates then coexist with a critical micellar concentration of monomers in the solution. For normal, zwitterionic phospholipids, the cmc has a very low value in the region of $10^{-10}$ M, whereas single-chain lipids typically have larger values lyso phospholipids, cmc $= 10^{-7}-10^{-6}$ M, surfactants, cmc $= 10^{-4}-10^{-1}$ M. The driving force behind lipid aggregation is the hydrophobic effect [49], whereby the molar free energy cost of transfer of an hydrocarbon chain of length $n$ from an oil to an aqueous environment is

$$
\delta \mu = 10 \times 3.7 \, n \, (kJ \cdot mol^{-1}) = 2.44 + 0.88 \, n \, (kcal \cdot mol^{-1})
$$

By forming into closed aggregates, the extent of chain-water contact is minimized and the chemical potential of the chains is thereby reduced. The hydrophobic energy is in fact directly proportional to the area of hydrocarbon-water contact, with a value of approx. 10.5 kJ·mol$^{-1}$ per nm$^2$ (25 cal·mol$^{-1}$ per Å$^2$), which implies an interfacial hydrophobic free energy density of $\approx 35 \times 10^{-20}$ J·nm$^{-2}$. This value is comparable to the interfacial tension of an oil/water interface $\gamma = 5 \times 10^{-20}$ J·nm$^{-2}$.

The hydrophobic effect on its own would favour complete phase separation of the lipid from the water, however, the requirement that the polar headgroups be hydrated prevents this and forces the system to form aggregates in which all of the headgroups can be in contact with water.

For an $N$-aggregate (i.e., one composed of $N$-lipid molecules) to be in equilibrium with lipid monomers in a binary lipid/water solution requires that the chemical potentials of monomeric and aggregated lipids be equal [49,51]

$$
\mu_X^N + kT/N \ln(X_X/N) = \mu_X^1 + kT \ln x_1
$$

where $\mu_X^N = \text{standard part of free energy per molecule of lipid in } N\text{-aggregate}$, $X_X = \text{mole fraction of } N\text{-aggregates in solution}$, $\mu_X^1 = \text{standard part of free energy per molecule of lipid monomers free in solution}$, $x_1 = \text{mole fraction of monomeric lipid in solution}$.

$N$ B Interactions between monomers and between aggregates are assumed to be negligible, when they are not, activity coefficient terms must be added to each side of this equation.

When the aggregates are very large, i.e., $N \to \infty$, the equation reduces to

$$
\mu_X^N - \mu_X^1 = kT \ln(\text{cmc})
$$

which relates the free energy of micellization to the cmc.

The equation for the $N$-aggregate can be rewritten in the form of a distribution function for aggregate size

$$
X_X = N \chi^X \exp[-N(\mu_X^N - \mu_X^1)/kT]
$$

It is the variation of $\mu_X^N$ with $N$ which dictates the aggregation behaviour. In the absence of free energy considerations, i.e., $\mu_X^N = \mu_X^1$ for all $N$, this equation reduces to

$$
X_X = N \chi^X
$$

which, since $\chi^X > 1$, shows that most of the lipid molecules will be in the monomeric state ($N = 1$).

A necessary condition for aggregation to occur is for $\mu_X^N$ to decrease with increasing $N$, or to have a minimum at some particular value of $N$; it is the variation of $\mu_X^N$ with $N$ (and with aggregate geometry) which determines the aggregation behaviour. Most thermodynamic treatments of lipid aggregation into micelles and mesophases reduce to consideration of the form of $\mu_X^N$ in terms of the molecular interactions involved. The following contributions to the free energy per molecule have been considered [49-52,174,175]

$$
(\mu_X^N(G) - \mu_X^1) = f_b(G) + f_a(G)
$$

where $f_b(G)$ is a surface term arising from the lipid
headgroup region, and $f_h(G)$ is a core term arising from the hydrocarbon chain region. There are two principal contributions to $f_h(G)$, one from the interfacial tension arising from residual water-hydrocarbon contact, due to incomplete shielding by the polar headgroups, and a term (normally repulsive) containing the sum of all interactions of the polar headgroups with themselves, with nearby chain segments, and with surrounding water and ions. These latter interactions contain contributions from steric, electrostatic, hydrational, hydrogen-bonding, and Van der Waals interactions, and are extremely difficult to quantify phenomenological forms are usually adopted for the $f_h(G)$ term. The dependence of the surface term on the geometry of the aggregate is fully accounted for by the interfacial area of the surface of the aggregate, and the simplest form proposed for it is

$$f_h(G) = \gamma A + \frac{C}{A'} = \gamma \left[ A + \left( \frac{A_h}{A'} \right) \right] \quad C = \text{constant} \quad (15)$$

$$\gamma = 35 - 50 \, \text{mN} \cdot \text{m}^{-1} \, (\text{dyn/cm})$$

is the effective interfacial tension of the water/hydrocarbon interface (it is unnecessary to explicitly allow for the fact that some fraction of the area of hydrocarbon is shielded by the headgroups, so long as this shielded area is independent of the total area).

$A = \text{area per lipid molecule at the hydrocarbon/water interface}$

$A' = \text{area per molecule at the centre of the headgroup repulsion}$

$A_h = (C/\gamma)^{1/2} = \text{value of } A \text{ which minimizes } f_h \text{ for a planar layer ("optimal area per headgroup")}$

In general, the centre of the headgroup repulsion will be displaced some distance $d$ ($d < 0$) along the normal to the water/hydrocarbon interface, and only for planar layers (i.e., principal curvatures $c_1$ and $c_2$ both equal to zero) will the two areas $A$ and $A'$ be equal. From Eqn. 28 we see that the area per lipid molecule at these two parallel surfaces are related by

$$A'(d) = A\left[1 - (c_1 + c_2) \frac{d}{c_1 + c_2} \right] \quad (16)$$

and thus, up to quadratic terms in $c_1, c_2, d$

$$f_h = \gamma A \left[1 + \left( \frac{A_h}{A} \right)^2 \right] \left[1 + (c_1 + c_2) \frac{d}{c_1 + c_2} + (c_1^2 + c_2^2 + c_1 c_2) \frac{d^2}{c_1 + c_2} \right] \quad (17)$$

N.B. $A' = A \left[1 - d/R \right]$ for cylinders

$$A' = A \left[1 - 2d/R + d^2/R^2 \right] \text{ for spheres} \quad (18)$$

The hydrocarbon core term $f_c(G)$ has been treated in quite different ways by different authors, although one common feature is the assumption that the chains are (volume) incompressible.

In the approach of Tanford [49], and that of Israelachvili and co-workers [51], $f_c(G)$ is taken to be constant, independent of geometry, so long as certain packing criteria are met. These conditions are, firstly, that no hydrocarbon chain should protrude into the aqueous exterior; secondly, that no part of the interior of any aggregate can be further away from the water/hydrocarbon interface than some critical length $l_c$. Thus (at least) one radius $R$ of the hydrocarbon region of any aggregate must satisfy $R \leq l_c$. Both of these criteria are due to the hydrophobic effect. Tanford took the critical length to be equal to the average chainlength of a liquid alkane. $l_c = \langle l \rangle \approx 0.7 \, l_{max}$, where $l_{max}$ is the all-trans length of the chain. Israelachvili and co-workers, on the other hand, assumed that in the optimal geometry the interfacial area per molecule $A$ equals the optimal area per headgroup $A_{h}$, and that the chains will allow this geometry at no free energy cost so long as $R \leq l_c = l_{max}$. The preferred geometry, when more than one solution is possible, is taken to be the one which corresponds to the smallest aggregates, since these are entropically favoured.

For simple aggregate shapes such as spheres, cylinders and bilayers, the following definite relationships exist between the aggregate hydrophobic radius, $R_{par}$ (half-thickness for bilayers), the hydrocarbon/water interfacial area per molecule $A$, and the chain volume per lipid molecule $v_{par}$.

$$v_{par}/(A \cdot R_{par}) = \frac{1}{3} \quad \text{spheres}$$

$$v_{par}/(A \cdot R_{par}) = \frac{1}{2} \quad \text{cylinders}$$

$$v_{par}/(A \cdot R_{par}) = 1 \quad \text{bilayers}$$

These relationships are illustrated in Fig. 19. Note that for cylinders, the average volume available per lipid molecule will in fact tend to be wedge-shaped, since one of the principal curvatures is zero.

Although spheres are always entropically favoured, they are only allowed if $R_{par} \leq l_{max}$, i.e., if $\left( v_{par}/A \right)/\left( l_{max} \right) \leq 1/3$.

Thus the Israelachvili model predicts, in the absence of interactions between aggregates, the following preferred geometries for different values of the 'critical packing parameter' ($v_{par}/A \cdot l_{max}$).

<table>
<thead>
<tr>
<th>$(v_{par}/A \cdot l_{max})$</th>
<th>Preferred geometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1/3</td>
<td>spheres</td>
</tr>
<tr>
<td>1/3–1/2</td>
<td>cylinders</td>
</tr>
<tr>
<td>1/2–1</td>
<td>bilayers</td>
</tr>
<tr>
<td>&gt;1</td>
<td>inverse structures</td>
</tr>
</tbody>
</table>

Fig 19 Hydrocarbon chain packing relationships for different aggregate shapes (a) spheres, (b) cylinders, and (c) bilayers.
Whilst the Tanford and Israelachvili approaches have been immensely useful in understanding many features of lipid polymorphism, they are necessarily incomplete in that, for example, they fail to predict the formation of the intermediate bicontinuous cubic phases. Leaving aside for the moment the neglect of interactions between aggregates, a major defect of these ‘molecular shape’ approaches is that they totally neglect the geometry dependence of the chain contribution \( f_c(G) \) to the free energy. The recent statistical thermodynamic approaches of Gruen, and Gelbart and co-workers [174–177, 185, 186] have attempted to evaluate \( f_c(G) \). They calculate the geometry-dependent part of the chain contribution as

\[
\int \tau(z) A'(z) \, dz
\]

where \( A'(z) \) is now the molecular area at a distance \( z \) from the interface, and the chain lateral pressure profile \( \tau(z) \) is evaluated from a statistical thermodynamic treatment which adjusts the chain conformational probability distribution function so as to minimize the free energy per chain.

They make the important point that the real optimum value, \( A_0 \), of the area per molecule at the polar/non-polar interface is the value of \( A \) which minimizes \((f_h + f_c)\), i.e., the sum of the headgroup and chain contributions to the free energy. The value of \( A_0 \) will in general be intermediate between \( A_h \) and \( A_c \) (the optimal values of \( A \) for headgroup and chain packing, respectively).

The major conclusion which arises from these studies is that the hydrocarbon chains do not passively fill any volume accessible to them, rather, they maintain a nearly identical average conformational state at a given temperature, independently of the shape of the aggregate. This result is fully in agreement with NMR measurements of the state of disorder along the chains in aggregates of different shape [187]. However, as noted in subsection III-D, this may not be true for inverted (type II) phases.

**IV.B Intermolecular interactions**

The equilibrium phase formed under a given set of conditions is the one which minimizes the overall free energy of the whole system, including the aqueous region. The first problem lies in identifying which free energy contributions are important in determining mesophase stability. The simplest approach is to ignore the interactions between the aggregates, i.e., to consider the system to be a dilute solution. Most of the approaches to date have attempted to determine the relative stabilities of certain simple aggregate shapes, such as spheres, cylinders, or planar bilayers. They thus make no direct predictions as to the equilibrium phase expected for a given set of conditions, and do not allow for the possible formation of more complex non-lamellar structures such as the cubic, tetragonal, or rhombohedral phases. However, a recent statistical mechanical treatment which considers an assembly of nonintersecting random fluid films as a simplified model of an amphiphile/water system does predict regions where bicontinuous cubic phases are stable [188].

The first attempts to provide a quantitative description of non-lamellar phase transitions in lyotropic systems involved the lamellar-normal hexagonal \( H_1 \) transition observed either in soap/water systems with increasing water content [189], or in soap/fatty alcohol/water systems with decreasing alcohol content [190]. Both authors treated the transitions as arising from a change in the balance between an interfacial free energy due to the hydrophobic effect, and an electrostatic free energy arising from the charged headgroups of the soap molecules. With the packing constraints considered previously, the hexagonal \( H_1 \) phase has characteristically an interfacial area per molecule greater than that of the lamellar phase, and hence has a higher value of interfacial free energy than the lamellar phase. Parsegian showed that the electrostatic free energy in the hexagonal phase, calculated using an approach developed for cylindrical polyelectrolyte molecules [191], was lower at all water contents than that in the lamellar phase. Both the hydrophobic and the electrostatic contributions were found to increase with increasing water content for both phases, but the plots of the total free energy for each phase as a function of water content gave a crossover point within the experimentally determined transition region. In the case considered by Mather, the fatty alcohol molecules ‘dilute’ the interfacial charge, thereby reducing the electrostatic contribution to the free energy, and hence shifting the equilibrium in favour of the lamellar phase.

Both of these treatments implicitly assumed a constant degree of dissociation of the charged headgroups, which will not in general be the case. Ohki and Aono [192, 193] extended the Parsegian approach to consider the relative stability in aqueous solution of phospholipid bilayers, cylindrical micelles, and spherical micelles as the headgroup charge varied from zero to two units.

Such ‘dilute’ treatments have been extended by including additional terms in the free energy, for example in the work of Nagarajan and Ruckenstein [194], but will not be considered further here, since we are primarily interested in the stability of inverse phases such as \( H_1 \), in which the close-packed aggregates are intrinsically not dilute, even in the presence of a large excess of solution.

Jonsson and Wennerstrom [195] have developed a thermodynamic model for aggregation of binary ionic amphiphile/water [195] and of ternary ionic amphi-
phosphate/alcohol/water systems \cite{196} which includes the interaggregate interactions. In the most recent form, which considers the relative stability of lamellae, and of normal and reversed cylindrical and spherical geometries, their model contains the following contributions to the free energy:

(a) A global electrostatic term, calculated using the Poisson-Boltzmann equation, by dividing the system into a set of identical cells.
(b) An interfacial tension term arising from the hydrophobic effect.
(c) Four entropy terms, arising from the mixing of the components in the aggregates and in the solution, from the mixing of the aggregates themselves, and for inverse phases, from the mixing of overlapping chains.
(d) Molecular packing constraints as proposed by Tanford.
(e) A Marčelja-type hydration force, taken to have an inverse exponential dependence on water content, as found experimentally for various lipid systems \cite{71}.

This model is very successful at describing the ternary phase equilibria in the test system of potassium decanoate/octanol/water, although the calculations are least reliable for the $H_n$ phase.

IV-C Interfacial curvature

There are two fundamental types of curvature which characterize each point of a surface: the mean curvature, $H$, and the Gaussian curvature, $K$. They are related to the principal curvatures $c_1$ and $c_2$ at a point $P$ on the surface by \cite{197}:

$$H = \frac{1}{2} (c_1 + c_2) \quad (21)$$
$$K = c_1 c_2 \quad (22)$$

where the curvatures are related to the local principal radii of curvature by $c_1 = 1/R_1$ and $c_2 = 1/R_2$, as shown in Fig. 20.

When a patch of surface can be expressed in the parametric form $z = f(x,y)$, i.e., $r = (x, y, f(x,y))$, the curvature can be expressed in the differential forms:

$$H = \frac{(1 + f_y^2)f_{xx} - 2f_x f_y f_{xy} + (1 + f_x^2)f_{yy}}{2(1 + f_x^2 + f_y^2)^{3/2}} \quad (23)$$
$$K = \frac{f_{xx} f_{yy} - f_{xy}^2}{(1 + f_x^2 + f_y^2)^2} \quad (24)$$

where $f_x = \delta f / \delta x$, $f_y = \delta f / \delta y$, $f_{xx} = \delta^2 f / \delta x^2$, $f_{xy} = \delta^2 f / \delta x \delta y$, $f_{yy} = \delta^2 f / \delta y^2$ and $f_{xy} = \delta^2 f / \delta x \delta y$.

A compact way of writing the mean curvature at a point $P$ is in terms of the unit normal vector $n$ to the surface at $P$:

$$H = -\frac{1}{2} \text{div} n \quad (25)$$

where $\text{div} n = \delta n_x / \delta x + \delta n_y / \delta y + \delta n_z / \delta z$ and the unit vector $n$ is given by

$$n = -f_x i - f_y j + k \quad \left(1 + f_x^2 + f_y^2\right)^{1/2} \quad (26)$$

Note that there are two equivalent unit normal vectors to the surface at any point $P$, either $n$ or $-n$, and the choice is arbitrary. In Fig. 20, we have taken $n$ to point upwards, and this means that the mean curvature is arbitrarily taken to be negative if the surface curves downwards around $P$ (i.e., $R_1$ and $R_2$ are negative), and positive if it curves upwards (i.e., $R_1$ and $R_2$ are positive). We will adopt the convention that for a lipid monolayer, $H > 0$ when the layer curves towards the hydrocarbon chain region, and $H < 0$ when the layer curvature is towards the aqueous region. This is equivalent to setting the orientation of the monolayer such that the hydrocarbon chains point upwards, as shown in Fig. 21.

The surface area of the patch is given by

$$A = \iint (1 + f_x^2 + f_y^2)^{1/2} \, dx \, dy \quad (27)$$

A parallel small surface patch, a distance $+d$ along the normal $n$ from the surface, has an area given by

$$A(d) = A_0 [1 - (c_1 + c_2) \, d + c_1 c_2 \, d^2]$$
$$= A_0 [1 - 2Hd + Kd^2] \quad (28)$$

where $A_0$ is the area, and $c_1$ and $c_2$ the principal curvatures, of a small patch on the original surface.

Thus for a lipid monolayer, the available area per molecule decreases, or increases, on moving towards the
chain methyl endgroups, depending on whether the lipid/water interface has a mean curvature towards the hydrocarbon region (type I), or towards the aqueous region (inverse, type II).

The mean curvature of this parallel surface is related to the principal curvatures of the original surface by

$$H(d) = \frac{1}{2} \left[ c_1 \left( 1 + c_1 \ d \right) + c_2 \left( 1 + c_2 \ d \right) \right]$$

$$= -K \ d \ \text{when} \ c_1 = -c_2, \ \text{i.e.}, \ H = 0$$

The qualitative nature of the surface at point $P$ is determined by the sign of the Gaussian curvature $K$

<table>
<thead>
<tr>
<th>$K$</th>
<th>$H$</th>
<th>Form of surface</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&gt; 0$</td>
<td>$&gt; 0$</td>
<td>elliptic sphere</td>
<td>sphere</td>
</tr>
<tr>
<td>$0$</td>
<td>$&gt; 0$</td>
<td>parabolic cylinder</td>
<td>cylinder</td>
</tr>
<tr>
<td>$0$</td>
<td>$0$</td>
<td>parabolic plane</td>
<td>plane</td>
</tr>
</tbody>
</table>
| $< 0$   | $< 0$   | hyperbolic saddle surface | saddle surface

A sphere, for which $H = 1/R$ and $K = 1/R^2$ at all points on the surface, is the simplest example of a surface of positive Gaussian curvature. Surfaces for which one of the principal curvatures is zero, for example cylinders or planes, have zero Gaussian curvature. Surfaces of negative Gaussian curvature, where the curvatures $c_1$ and $c_2$ have different signs, are more difficult to visualize. An example is the saddle surface, which is the type of surface which results if a thin fluid film is draped onto a tetrahedral wire frame as shown in Fig 22.

For this surface, the Gaussian curvature, $K$, is negative, because the two principal curvatures, $c_1$ and $c_2$, are of opposite sign. The Gaussian curvature is most negative at the saddle point, and moving along the surface towards the apex points, increases towards a value of zero.

This particular example of a saddle surface is called a minimal surface, because it has zero mean curvature ($H = 0$) at all points (it also minimizes the surface area of the film, but only under the particular boundary conditions imposed by the wire frame in this example). Such minimal surfaces are attracting a great deal of attention at present, since they appear to form the basis of bicontinuous liquid-crystalline cubic phases in lipid/water systems [12,13,90,111,112,198–206].

Parallel surfaces a distance $±d$ from a minimal surface no longer have zero mean curvature, but have curvatures $±H$. If the minimal surface corresponds to the centre of a lipid bilayer, then this corresponds to both monolayers having a negative mean curvature, according to the convention adopted in Fig 21. Hence the available area per lipid molecule decreases on moving from the bilayer centre towards either headgroup region. Thus bicontinuous cubic phases allow a tighter headgroup packing relative to the chains than that of the lamellar phase.
It is important to appreciate that a rigid (inextensible) yet flexible surface (e.g., a sheet of paper) can be easily deformed into a shape of arbitrary mean curvature $H$, but only if the Gaussian curvature $K$ remains zero. This is easily seen for example if one compares 'papering' a cylinder with the process of trying to paper a sphere: the latter cannot be done without wrinkling or tearing the paper. For a fluid surface such as a membrane (above its chain melting transition), Gaussian deformations become permissible, although with an associated elastic energy cost.

For example, a pore in a bilayer membrane represents a monolayer deformation of negative Gaussian curvature, as shown in Fig 23. Similarly, a channel between two apposed bilayers represents a bilayer deformation of negative Gaussian curvature, as shown in Fig 24. Note that the formation of such structures inevitably involves membrane fusion to bring about the changes in topology.

Many of the current ideas relating to the role of molecular structure, packing, interactions and interfacial curvature were already recognized some thirty or more years ago [37,184]. However, it is only quite recently that more quantitative approaches have been attempted.

All of the lyotropic phase behaviour can be categorized in terms of the mean and Gaussian interfacial curvatures, as shown in Table II.

### Table II

<table>
<thead>
<tr>
<th>Phase/structure</th>
<th>Mean curvature $H = 1/2(c_1 + c_2)$</th>
<th>Gaussian curvature $K = c_1 c_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spherical micelle or vesicle (outer half)</td>
<td>$+1/R$</td>
<td>$+1/R^2$</td>
</tr>
<tr>
<td>Oblate/prolate micelles or micellar cubic Q\textsubscript{l} phase</td>
<td>$1/R_1$</td>
<td>$1/R_1^2$</td>
</tr>
<tr>
<td>Cylindrical micelles or H\textsubscript{l} phase</td>
<td>$1/2(1/R_1 + 1/R_2)$</td>
<td>$1/R_1 R_2$</td>
</tr>
<tr>
<td>Ribbon phases (e.g., M\textsubscript{4})</td>
<td>$1/2 R$</td>
<td>0</td>
</tr>
<tr>
<td>Bicontinuous cubic Q\textsubscript{1} and intermediate (R\textsubscript{l}, T\textsubscript{l} phases)</td>
<td>0 to $1/2 R$</td>
<td>$-1/R^2$ to 0</td>
</tr>
<tr>
<td>Planar bilayers</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inverse bicontinuous cubic Q\textsubscript{II}</td>
<td>$-1/2 R$ to 0</td>
<td>$-1/R^2$ to 0</td>
</tr>
<tr>
<td>Inverse cylindrical micelles or H\textsubscript{II} phase</td>
<td>$-1/2 R$</td>
<td>0</td>
</tr>
<tr>
<td>Inverse spherical micelle or inner half of spherical bilayer vesicle</td>
<td>$-1/R$</td>
<td>$1/R^2$</td>
</tr>
<tr>
<td>F-minimal surface</td>
<td>0</td>
<td>$-1/R^2$ (saddle point) to 0 (apex)</td>
</tr>
</tbody>
</table>
curvatures of the aggregates making up the phases and this is summarized in Table II.

For spherical, circular cylindrical and planar aggregates, and for minimal surfaces, the mean interfacial curvature $H$ is uniform at all points of the structure, for all other shapes of aggregate, $H$ varies on moving along the interface in any direction. The Gaussian curvature $K$, on the other hand, is uniform for spheres, cylinders (of any cross-sectional shape), and planes, but is non-uniform for all other shapes, including minimal surfaces. For the minimal F-surface, for example, $K$ varies from a value of $-1/R^2$ ($-11.9$ for unit lattice parameter) at the saddle point, to zero at the apex (umbilical point).

It is instructive to illustrate the relative positions of various membrane geometries as a function of mean and Gaussian interfacial curvatures, as shown in Fig. 25.

**IV-D Lateral expansion and curvature elasticity**

A valuable approach to understanding the stability of different membrane configurations and of lipid mesophases has been to describe the free energy of a lipid layer in terms of various elastic energy contributions [48,207-209]. The various types of elastic deformations of a thin film are illustrated in Fig. 26.

The elastic energy per unit area of a thin fluid membrane is given by the sum of lateral expansion and curvature terms

$$g = (Gd/A) = g_A + g_{cur}.$$  \(31\)

Fig. 26 Elastic deformations of a thin film: (a) lateral expansion, (b) cylindrical bending (zero Gaussian curvature), and (c) elliptic and hyperbolic bending.

where $G_{el}$ is the elastic energy per molecule, $d$ is the interfacial area per molecule. The energy of lateral expansion is [48]

$$\sigma = k_2 H_2,$$  \(32\)

where $A_{ij}$ is the optimum area per molecule, and the isothermal lateral compression modulus is defined by

$$\kappa_A = 4(\delta \sigma / \delta A),$$  \(33\)

where $\sigma$ is the (isotropic) lateral tension (force per unit length), given by

$$\sigma = (\delta G_{el} / \delta A),$$  \(34\)

$G_{el}$ can be equated with the free energy per molecule $(f_h + f_c)$ discussed in subsection IV A, and hence, if estimates for the form of the free energy are known, then $K_A$ can be obtained from its second derivative with respect to area.

Typical values of the lateral compression modulus are [48] $K_A \approx 70$ mN m$^{-1}$ (dyn/cm) for a fluid lipid monolayer, and $K_A \approx 140$ mN m$^{-1}$ (dyn/cm) for a fluid lipid bilayer.

The lateral pressure $\pi$ is given by

$$\pi = \gamma - \sigma,$$  \(35\)

where $\gamma$ is the effective interfacial tension of the hydrocarbon/water interface, which has a value [49,55] in the region of 35-50 mN m$^{-1}$ (dyn/cm). The lateral pressure arises from the lateral repulsions between the chains and the headgroups.

At equilibrium, $\sigma = 0$, since otherwise the bilayer will simply expand or contract its area.

It is usually assumed that the lateral expansion term $g_A$ is much larger than the curvature term $g_{cur}$, i.e., deformations involving stretching are more costly energetically than those involving bending, and so in considering the equilibrium shapes of membranes it is simply ignored.

The curvature elastic energy per unit area, for small deformations, contains the sum of mean and Gaussian curvature contributions, and is given approximately by [207 and 208]

$$g_{cur} = g_M + g_C + 1/2 K_M (\epsilon_1 + \epsilon_2 - \epsilon_0)^2 + K_C (\epsilon_1 - \epsilon_2)^2,$$  \(36\)

where $\epsilon_1$ and $\epsilon_2$ are the principal curvatures, and $\epsilon_0$ is the spontaneous curvature, i.e., twice the equilibrium mean curvature for the case of zero Gaussian curvature, e.g., $\epsilon_0 = \epsilon_1 = \epsilon_2 = 0$.

$K_M$ and $K_C$ are the elastic moduli for mean and Gaussian curvature, respectively. If the variation of $G_{el}$ with curvature is known, then estimates for $K_M$ and $K_C$ can be obtained by differentiating $G_{el}$ with respect to the curvatures $\epsilon_1$ and $\epsilon_2$, for different deformations.
For the case of spherical deformations (where $K \neq 0$), the equilibrium mean curvature is given by

$$H_{eq} = \left( \frac{K_M}{2K_M + K_G} \right) c_0$$  \hspace{1cm} (37)

The case of a planar bilayer corresponds to $c_0 = 0$. This situation must obtain for any symmetrical bilayer with identical conditions (e.g., pressure, pH, etc.) on each side, even though the individual monolayers may have non-zero values of $c_0$.

Thus for a cylindrical deformation ($i.e., c_2 = 0$), the curvature energy increases quadratically upon deforming a bilayer (or a monolayer) away from some preferred value of spontaneous curvature $c_0$, the modulus $K_M$ determining the energy cost of such a deformation $K_M$ is expected to always be positive, although as yet very few experimental values are available. A typical value for fluid phospholipid bilayers is likely to be that determined for egg PC bilayers [208]

$$K_M = 2 \times 10^{-19} J \ (2 \times 10^{-12} \text{erg})$$

$K_M$ decreases strongly with increasing equilibrium area per molecule, with decreasing chainlength, or when mixed lipid chainlengths are present in the bilayer [210]

$$N_B \ K_M^{\text{bilayer}} = 2 K_M^{\text{monolayer}}$$  \hspace{1cm} (38)

The variation of the term $g_M$ with mean curvature is shown schematically in Fig. 27. Note that although for a monolayer we have unambiguously defined positive and negative mean curvatures as curvature of the layer towards the hydrocarbon chain, and polar regions, respectively, no such convention is possible for a bilayer. We may arbitrarily take it to be positive ($i.e., c > 0$).

Each individual monolayer half of a bilayer does however have a well-defined direction of mean curvature, whose signs can either be the same (positive or negative) or opposite to each other ($i.e.,$ as in a spherical bilayer vesicle)

![Image](image_url)

**Fig. 27** Variation of elastic energy with mean curvature $H$, for lipid monolayers and bilayers. (For the case of bilayers only positive mean curvatures are relevant)

The second curvature elastic modulus $K_G$ controls the tendency for the membrane to form structures of non-zero Gaussian curvature. $K_G > 0$ favours saddle-like surfaces (negative Gaussian curvature), whereas $K_G < 0$ favours elliptic surfaces ($i.e.,$ spheres). No reliable estimates of $K_G$ for phospholipid bilayers are as yet available.

The variation of the term $g_G$ with Gaussian curvature is shown schematically in Fig. 28.

![Image](image_url)

**Fig. 28** Variation of elastic energy with Gaussian curvature $K$

The form of these curvature elastic energy contributions for different shapes of thin membranes are listed in Table III.

![Image](image_url)

**Table III**

<table>
<thead>
<tr>
<th>Shape</th>
<th>$g_G$</th>
<th>$g_M$ ($c_0 = 0$)</th>
<th>$g_M$ ($c_0 \neq 0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>$K_G/R^2$</td>
<td>2$K_M/R^2$</td>
<td>1/2 $K_M (2/R - c_0)^2$</td>
</tr>
<tr>
<td>Cylinder</td>
<td>0</td>
<td>1/2 $K_M/R^2$</td>
<td>1/2 $K_M (1/R - c_0)^2$</td>
</tr>
<tr>
<td>Minimal</td>
<td>$-K_G/R^2$</td>
<td>0</td>
<td>1/2 $K_M c_0^2$</td>
</tr>
<tr>
<td>Inverse</td>
<td>0</td>
<td>1/2 $K_M/R^2$</td>
<td>1/2 $K_M (-1/R - c_0)^2$</td>
</tr>
<tr>
<td>Inverse</td>
<td>$K_G/R^2$</td>
<td>2$K_M/R^2$</td>
<td>1/2 $K_M (-2/R - c_0)^2$</td>
</tr>
</tbody>
</table>

The total curvature energy of a spherical vesicle, for a symmetrical bilayer ($c_0 = 0$), is given by integrating the expression for $g_m$ over the surface of the vesicle

$$G_m = 4\pi(2K_M + K_G)$$  \hspace{1cm} (39)

An important result from differential geometry is the Gauss-Bonnet theorem, which states that the integral of Gaussian curvature over a closed surface is a topological invariant, $i.e.,$ it does not depend on the detailed shape or surface area of the surface

$$\int K dA = 4\pi (n_c - n_h)$$  \hspace{1cm} (40)

where $n_c$ is the number of disconnected components.
membranes), and \( n_h \) is the number of 'passages' in the membrane.

For example, a spherical vesicle has \( n_e = 1 \) and \( n_h = 0 \), and \( \int K \, dA = 4 \pi \), whereas for a torus, i.e., a closed bilayer containing a single passage, \( n_e = 1 \) and \( n_h = 1 \), hence \( \int K \, dA = 0 \).

For a periodic minimal surface, for which the mean curvature \( H \) is zero, the total curvature energy is simply

\[
G = -n_h \cdot 4\pi \cdot K_G \tag{41}
\]

where the number of passages \( n_h \) is of the order of one per unit cell. For a lipid/water bicontinuous inverse cubic phase, built by draping a bilayer onto a periodic minimal surface, the unit cell dimension is typically 100 Å, and hence the Gaussian contribution to the total curvature energy can be quite large. Hence, positive values of \( K_G \) will tend to favour the formation of inverse bicontinuous phases.

Thus the energy of creating a passage between two bilayer membranes (see Fig. 24), if this is done in such a way as to maintain approximately zero mean curvature, may be equated to

\[
G_{\text{Gaussian}} = -4\pi \cdot K_G \tag{42}
\]

and has been termed the 'energy of fusion' [202], since the topology of the membrane can only be changed if fusion processes occur.

Kleman has recently analyzed the stability of mesophases adopted by amphiphiles, such as polyoxyethylene surfactants, which have characteristically low values of \( K_M \) (two orders of magnitude smaller than for phospholipids). He finds that for such systems the lamellar phase is expected to have a large range of stability, and that any regions of cubic phase should be of small extent [203].

Attempts have been made to estimate values of \( K_M \) and \( K_G \) from specific microscopic models based upon considerations of the distribution of lateral stress \( t(z) \) (force per unit area) across the bilayer [202, 207–209, 211–213].

NB: \( t(z) \) in the chain region is the negative of the lateral pressure profile \( \pi(z) \) discussed in section IVA (Eqn 20).

Consider first a fluid lipid monolayer, with its associated water of hydration, where we set the origin \( z = 0 \) at the mid-plane. We expect the stress profile to have the qualitative form shown in Fig. 29. In the chain region, the positive lateral chain pressure \( \pi_c \) (corresponding to a negative stress) is expected to have a maximum somewhere near the middle of the chain [174, 175, 213]. The interfacial tension, \( \gamma \), creates a large positive stress centred at the water/hydrocarbon interface. This peak will be somewhat broadened, because the water/hydrocarbon interface is not completely sharp. In addition, any lateral pressure between the lipid headgroups will give rise to a negative stress in the polar region (which will in general extend into the aqueous region).

For a flat monolayer at equilibrium, the net lateral tension, given by summing the stress profile across the layer, must equal zero

\[
\sigma = \int t(z) \, dz = 0 \tag{43}
\]

In other words, the total area under the curve of \( t(z) \) versus \( z \) (see Fig. 29) must equal zero.

In addition, if the positive and negative stresses are not symmetrical about the mid-plane, there will be a spontaneous tendency for the layer to curve away from a planar geometry, either towards a cylindrical shape (zero Gaussian curvature), a spherical shape (positive Gaussian curvature), or a saddle surface (negative Gaussian curvature). These respective deformations will tend to be favoured when the Gaussian curvature modulus \( K_G \) is zero, negative, or positive, respectively. Thus a further condition for a flat monolayer to be stable is that there should be zero net torque (bending moment) exerted around the midplane. The lateral torque tension \( \tau \) in a flat layer with \( \sigma = 0 \), given by the first moment of \( \pi(z) \) with respect to the principal curvatures, may be equated (by differentiating \( \pi(z) \), with respect to the principal curvatures) with [208]

\[
\tau = \int z \cdot t(z) \, dz = -K_M \cdot \epsilon_0 \tag{44}
\]

and thus for a layer to stay flat

\[
\tau = -K_M \cdot \epsilon_0 = 0 \tag{45}
\]

i.e., for a positive \( K_M \), the spontaneous curvature \( \epsilon_0 \) must be equal to zero. If the stress profile can be determined or estimated, we see that \( K_M \) and \( \epsilon_0 \) can be related to each other.

If the monolayer is 'rolled up' into a cylinder with one of the principal curvatures equal to the spontaneous...
curvature \( c_0 \) (i.e., \( c_1 = c_0 \), \( c_2 = 0 \)), it can be shown that the lateral tensions \( \sigma_1 \) and \( \sigma_2 \), parallel and perpendicular to the cylinder axis, both stay equal to zero. Furthermore, the torque tension \( \tau_1 \) clearly must also remain at zero. The expression for the torque tension \( \tau_2 \) around the cylinder axis is now defined by the modified form

\[
\tau_2 = \int z (1 + z c_0) t(z) \, dz
\]  

which, since \( \int z t(z) \, dz = \tau_1 = 0 \), reduces to

\[
\tau_2 = c_0 \int z^2 t(z) \, dz
\]

But \( \tau_2 \) is also given (by differentiating \( g_{\text{curv}} \)) by:

\[
\tau_2 = -K_G c_0
\]

Hence the Gaussian curvature modulus is given by the second moment of the stress profile [208]

\[
K_G = \int z^2 t(z) \, dz
\]

In principle, this equation gives a way of estimating values of \( K_G \) for real lipid monolayers and bilayers, although this approach has yet to be successfully applied.

**Bilayers**

For a bilayer, a positive Gaussian curvature modulus, \( K_G > 0 \), is to be expected when the water/hydrocarbon interfacial tension, \( \gamma \), is balanced primarily by the hydrocarbon chain lateral pressure \( \pi_c \). In this case there will be some tendency for the bilayers to deform from planar into saddle-surfaces, with negative Gaussian curvature. Conversely, when the interfacial tension is balanced primarily by the lateral pressure in the polar region \( \pi_h \), a negative value, \( K_G < 0 \), may result. This situation can occur if the lipid headgroup is very large, is strongly hydrated, or is highly charged; these conditions induce large values of interfacial area per molecule, and hence low values of lateral chain pressure \( \pi_c \).

It has been suggested [208,209] that a negative value of Gaussian modulus leads to spontaneous vesiculation, since \( K_G < 0 \) favours elliptical curvatures, from Eqn 36. However, I argue that there is an alternative type of deformation which will also be favoured by a negative value of \( K_G \), namely a bicontinuous oil-in-water lattice of oil passages, such as are found in the normal type I cubic phases between \( L_a \) and \( H_1 \). The argument for this is as follows: in a multilamellar stack of bilayers, if each single bilayer, plus associated water half-layers, has a negative Gaussian curvature modulus \( (K_G < 0) \) with respect to the bilayer mid-plane, then one can equally define a Gaussian curvature modulus \( K'_G \), with respect to the mid-plane of the aqueous region, and this will be positive (i.e., \( K'_G > 0 \)). Thus such systems will also tend to form saddle surfaces, but ones for which the centre of the water layer is the surface of inextensibility. These correspond to the normal (type I) bicontinuous cubic phases, as found in region \( c \) of the hypothetical phase diagram shown in Fig 7.

We can illustrate these points by considering highly simplified models of the stress profiles expected to be found across lipid bilayers under different conditions. We will ignore the fact that in reality the chain pressure is not uniform across the bilayer interior, that it actually goes through a minimum value at the centre of the bilayer, and that significant amounts of chain interdigitation occur [213].

For example, consider an idealized model bilayer where the interfacial tension, \( \gamma \), is balanced entirely by a uniform chain lateral pressure as shown in Fig 30.

The stress profile is thus of the form:

\[
t(z) = \frac{\gamma}{h} \quad \text{for} \quad |c| \leq z \leq |c + h|
\]

and

\[
t(z) = -\frac{\gamma}{c} \quad \text{for} \quad 0 \leq z \leq |c|
\]
We can then evaluate $K_G$ using Eqn 49, giving

$$K_G = \frac{2}{3} \gamma [2c^2 + 3hc + h^2]$$

(50)

and for such a model, $K_G$ will in general be strongly positive, favouring negative Gaussian curvature ($K < 0$), and such a membrane will tend to deform towards a saddle-surface shape.

For an infinitely sharp water/hydrocarbon interface (i.e., $h \to 0$), Eqn 50 reduces to the previously derived result [214]

$$K_G = \frac{4}{3} \gamma c^2$$

(51)

Conversely, if the interfacial tension were entirely balanced by a lateral pressure in the headgroup region, the stress profile, shown in Fig. 31, would be of the form:

$$\tau(z) = \frac{\gamma}{h} \text{ for } |c| \leq z \leq |c + h|$$

$$\tau(z) = -\frac{\gamma}{p} \text{ for } |c + h| \leq z \leq |c + h + p|$$

and the Gaussian curvature modulus will be given by

$$K_G = -\frac{2}{3} \gamma [3hc + 2h^2 + 3cp + 3hp + p^2]$$

(52)

Fig. 31 Schematic bilayer stress profile and its first and second moments for the case of the lateral repulsion being localized entirely in the headgroup region. $K_G$ is in general negative for such a model.

and for such a model, $K_G$ will in general be negative, favouring elliptic ($K > 0$) curvature of the bilayer. For an infinitely sharp water/hydrocarbon interface (i.e., $h \to 0$), this equation reduces to

$$K_G = -\frac{2}{3} \gamma [3cp + p^2]$$

(53)

Alternatively, when $p = h \neq 0$, the expression reduces to

$$K_G = -\frac{4}{3} \gamma [h^2 + h^2]$$

(54)

Note that if we calculate the alternative Gaussian curvature modulus $K_G'$, by performing the integral of Eqn 49 with the origin set at the mid-plane of the water region (instead of the mid-plane of the bilayer), taken to be at $z = c + h + p$ in Fig. 31, then it has the value

$$K_G' = \frac{2}{3} \gamma [2p^2 + 3hp + h^2]$$

(55)

and is thus in general positive for this model. This is illustrated in Fig. 32. We thus see that such systems may also favour the deformation of membranes towards saddle-surfaces, but with the bilayer "inside-out."
the saddle-surface being centred in the aqueous region between two monolayers. This situation corresponds to the formation of normal type I (oil-in-water) bicontinuous cubic phases.

Similarly, the alternative Gaussian curvature modulus $K_G^c$ for the case considered in Fig 30, of the interfacial tension being balanced primarily by a lateral pressure in the chain region, is in general negative, as shown in Fig 33. Thus an elliptic curvature of the 'inside-out' bilayer would tend to be favoured. This would correspond to the formation of inverse bilayer vesicles, which could occur in the presence of a non-polar solvent.

A more realistic model of a bilayer than those considered above would be that shown in Fig 34, where the interfacial tension is balanced by lateral repulsions in both the hydrocarbon chain and the headgroup regions. In this case, if the repulsion is divided equally between the two regions, the value of $K_G$ will tend to be positive, unless the headgroups extend a long way out from the interface.

We thus expect that the equilibrium 'bilayer' configurations of lipid membranes, with zero spontaneous bilayer curvature $c_0 = 0$, as a function of the mean and Gaussian curvature moduli, to be as shown in Fig. 35. Note that although Eqn. 37 predicts that the equilibrium curvature $H_{eq}$ should be zero for $c_0 = 0$, when account is taken of the hydrophobic free energy cost of exposing bare edges to the water, the bilayers can nonetheless curve to form sealed vesicles when $K_G < 0$, so long as the mean curvature modulus $K_M$ is not too large.

This scheme does not contain the hexagonal $H_I$ or $H_{II}$ phases or micelles, since these aggregate structures are based essentially on curved monolayers. One can in principle compare the relative stability of different aggregate geometries as a function of the elastic moduli by calculating the elastic energy per molecule, which is proportional to the total elastic energy, for each geometry. This requires assumptions to be made about the particular packing constraints to be satisfied (e.g., lipid volume incompressibility, location of surface of inextension, etc). Such calculations have been performed to compare the relative stability of bilayers, cylinders and spheres for microemulsion systems, where the area per surfactant was taken to be constant [215].

**IV-E Spontaneous curvature, chain packing and frustration**

In principle, a fluid lipid monolayer can respond to a non-zero value of spontaneous curvature $c_0$ by defor-
mting away from planar. However, for a bilayer, which consists of two oppositely oriented monolayers placed back-to-back, any tendency for spontaneous curvature is frustrated[53,200] In a planar bilayer, the cross-sectional area per molecule is obviously constant at all depths across the bilayer, \( A_h = A_c \). However, as a parameter such as temperature or water concentration is varied, then the optimal areas of the two regions will tend to become different, in general. For example, increasing temperature will tend to expand the chain region more than the headgroups, due to the increased conformational disorder of the chains. Conversely, increasing the hydration will tend to expand the headgroup lateral packing relative to that of the chains. The individual monolayers would like to become curved to optimise both the headgroup and the chain packingHowever, they cannot, as this would create free volume in the bilayer interior, as shown in Fig. 36.

To remain planar, \( A_h \) and \( A_c \) have to remain equal, and thus one area is reduced and the other increased away from their optimal values. This state of compromise is called frustration, and means that the system is internally stressed, the planar configuration arising from a balance between two opposing stresses (For further discussion of frustration effects in liquid/crystalline systems, see references 216–219).

The build up of frustration is energetically unfavourable, and ultimately the system must find some way of reducing it. Sadoc and Charvolin [200] have identified four different topological ways of relieving this stress:

1. Lateral expansion, with the bilayer remaining flat. This solution leaves the topology unchanged, but costs stretching elastic energy \( \gamma_A \). This is the situation which corresponds to the lamellar \( L_\alpha \) phase below a transition to a non-lamellar phase.

2. Formation of a single, continuous bilayer of negative Gaussian curvature. Such a structure inevitably contains multiple passages, and corresponds to the reverse bicontinuous cubic phases.

3. Formation of an infinite number of infinite disconnected aggregates. This corresponds to the inverted hexagonal \( H_{II} \) phase.

4. Formation of an infinite number of finite disconnected aggregates. This corresponds either to inverted micellar solutions or to inverted mesophases.

In this geometric approach, the non-lamellar phases arise through introduction of defects of rotation, and may thus be described as structures of disclinations (see Appendix A).

### Structure of the \( H_{II} \) phase and interstitial packing

In the case of the \( H_{II} \) phase, although the tendency of the layers to achieve a non-zero (negative) mean curvature can be satisfied, a problem arises due to the necessity to fill all of the hydrocarbon interior of the phase with the lipid chains [90–92]. As shown in Fig. 37, there is an interstitial region (shaded) around the 3-fold axis of the phase (see Appendix A) in which the chains may be stressed, due to their having to stretch from their preferred conformational state towards a more extended conformation. The hydrophobic centre

---

**Fig. 35** Expected equilibrium configurations of fully-hydrated lipid membranes for the case of zero spontaneous bilayer curvature \( (\epsilon_0 = 0) \), as a function of the mean and Gaussian curvature moduli (Adapted from Ref 202).

**Fig. 36** Frustration in bilayer packing with varying temperature or water content (Adapted from Ref 200).
Fig 37 Schematic drawing of the H\textsubscript{II} phase, illustrating the problem of filling the entire hydrocarbon volume of the phase with the lipid chains

point of the H\textsubscript{II} phase is a distance

\[ l_{\text{max}} = \left( \frac{a}{\sqrt{3}} \right) - r_w \]  

(56)

from the lipid/water interface, where \( a \) is the lattice parameter (distance between cylinder axes) and \( r_w \) is the water cylinder radius. This is larger than the 'preferred' monolayer thickness, which may be approximated by:

\[ l_{\text{min}} = \left( \frac{a}{2} \right) - r_w \]  

(57)

The smaller the radius of the water rods, \( r_w \), the smaller are the hydrophobic interstices and the easier it is for the lipid chains to elongate slightly to fill the volume. This, along with the reduction in the spontaneous radius of curvature caused by the lower lateral pressure in the headgroup region, is the reason why the H\textsubscript{II} phase tends to be favoured with decreasing water concentration. Conversely, for a given \( r_w \), the effect of increasing the chainlength should similarly be to favour the H\textsubscript{II} phase, an effect which is abundantly borne out in the phase diagrams of a large variety of amphiphile/water systems.

By adding small amounts of alkanes to phospholipid/water systems, Gruner and co-workers [92] found an ingenious way to try to isolate effects due to relieving packing stress from those due to changes in the preferred spontaneous curvature of the lipid layers. Hydrated DOPE has an L\textsubscript{a}-H\textsubscript{II} phase transition temperature of 5–10°C, with an H\textsubscript{II} phase lattice parameter of 80 Å. On the other hand, a 3:1 DOPE/DOPC mixture was found to have a hexagonal transition temperature 45°C higher, due to the PC component tending to increase the spontaneous radius of curvature of the layers. Addition of 5% dodecane to the pure DOPE system had little effect, lowering the transition temperature by 10°C, and increasing the lattice parameter of the H\textsubscript{II} phase by approx. 4 Å. However, when the experiment was repeated on the 3:1 DOPE/DOPC mixture, the L\textsubscript{a}-H\textsubscript{II} transition temperature decreased by 55°C, and the lattice parameter just above the transition to the H\textsubscript{II} phase had the large value of 92 Å. The radius \( r_w \) of the water cylinder was of the order of 30 Å, far larger than that of the pure DOPE system, estimated to be about 18 Å. The interpretation of these results was that the alkane was allowing the mixed lipid system to adopt its preferred value of spontaneous mean curvature, by partitioning into the hydrophobic region, and hence relieving the packing stress in the H\textsubscript{II} phase. The larger radius of the water cores was taken to be a direct consequence of a larger preferred radius of curvature of the DOPE/DOPC mixture, compared to pure DOPE.

In a further study of PE/PC mixtures, the effect of varying the chainlength of the PC component on the structure of the H\textsubscript{II} phase was investigated [220]. The H\textsubscript{II} lattice dimension systematically increased with increasing chainlength, implying that the spontaneous radius of curvature of the interface increases with chainlength. The fact that the L\textsubscript{a}-H\textsubscript{II} transition temperature was decreased was taken as further evidence that relief of packing constraints must also be taken into account.

It has been demonstrated in a recent study of PEs with various branched and alicyclic chain structures, but with the same effective chainlength (C\textsubscript{18}), that the repeat spacings of the L\textsubscript{a} and the H\textsubscript{II} phases are constant for the different homologues at the transition points [221]. This was taken to imply that there is a critical chainlength at which the lamellar phase becomes unstable with respect to non-lamellar phases.

We may illustrate the effect of varying the chainlength for a pure PE system by considering the experimental data for the C\textsubscript{18} and C\textsubscript{12} dialkyl phospholipids DAPE (28 wt% H\textsubscript{2}O) and DDPE (27 wt% H\textsubscript{2}O) [108]. The radius of the water cores, extrapolated to the L\textsubscript{a}-H\textsubscript{II} transition points, are 22.0 and 12.6 Å, respectively, which shows that for the pure PE system, the preferred radius of curvature of the H\textsubscript{II} phase does indeed systematically increase with increasing chainlength. The values for \( l_{\text{max}} \) and \( l_{\text{min}} \) in the H\textsubscript{II} phase, and for the lipid bilayer half-thickness \( d_{L/2} \) in the L\textsubscript{a} phase, are as follows:

<table>
<thead>
<tr>
<th>Lipid</th>
<th>( T ) (°C)</th>
<th>( L\textsubscript{a} ) phase</th>
<th>( H\textsubscript{II} ) phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( d_{L/2} ) (Å)</td>
<td>( l_{\text{max}} ) (Å)</td>
<td>( l_{\text{min}} ) (Å)</td>
</tr>
<tr>
<td>DAPE</td>
<td>96</td>
<td>23 3</td>
<td>23 5</td>
</tr>
<tr>
<td>DDPE</td>
<td>112</td>
<td>14 2</td>
<td>14 1</td>
</tr>
<tr>
<td>DDPE</td>
<td>(135)</td>
<td>--</td>
<td>(14 2)</td>
</tr>
</tbody>
</table>

The last set of values (in brackets) are those for DDPE at its maximal hydration of 34 wt%, at the point of formation of the H\textsubscript{II} phase; for hydrations above 30 wt%, bicontinuous cubic phases appear between L\textsubscript{a} and H\textsubscript{II}.  

\[ \]
The conclusion that emerges in both cases is that at the transition, the bilayer half-thickness is equal to the distance of the centrepoint of the $H_{II}$ phase from the interface, and so in fact essentially none of the chains stretch to fill the hydrophobic volume. The average chain shortening at the transition is of the order of 10-20%.

The appearance of bicontinuous cubic phases between $L_{\alpha}$ and $H_{II}$ implies that for these phospholipids, the necessity for chain stretching which would occur for the short chainlength lipid to adopt the $H_{II}$ phase at full hydration is energetically too expensive, and the system prefers to adopt new intermediate phases having less curved interfaces than $H_{II}$, but where the packing problem may be less severe. The fact that even when fully-hydrated, DDPE will eventually adopt the $H_{II}$ phase (at 135°C) indicates that the increasing tendency for interfacial curvature, due to increasing chain disorder, eventually outweighs any effects due to interstitial packing problems.

**Frustration and bicontinuous cubic phases**

This result is fully in accord with the suggestion of Sadoc and Charvolin [200], that intermediate inverse bicontinuous cubic phases arise due to a competition between the curvature energy of the lipid monolayer, and the stretching energy of the hydrocarbon chains. This idea was developed by a purely geometric approach, which led to the conclusion that bicontinuous phases could arise, based upon periodic minimal surfaces [222,223]. Such a possibility had previously been suggested by a number of authors [90,111,112,198,199,224].

Gruner and co-workers have further pursued this idea by attempting to quantify the amount by which formation of a bicontinuous cubic phase can relieve the interstitial packing problem of the $H_{II}$ phase, whilst still allowing a partial expression of the monolayer spontaneous curvature [205]. By considering the cubic phase to be formed by 'draping' a lipid bilayer onto the F minimal periodic surface (see Table II and Fig 6a), they examined theoretically the effects of either constraining the bilayer to have a constant thickness, or to have a uniform curvature at the water/lipid interfaces. The results showed that the degree of frustration can be less for the cubic phase than for $H_{II}$. A review of this approach will appear shortly [225]. Charvolin and Sadoc have also considered this problem, but employing a purely geometric approach, with inclusion of packing constraints [223]. They find that this leads to a situation where only within certain composition and/or temperature ranges of the phase diagrams can frustration be relieved by topological transformations (i.e., by transitions to non-lamellar configurations). In those regions of the phase diagrams where global optimization of curvature and chain packing is not possible, the frustration can nonetheless be optimized locally by fluctuations within the structures (see subsection IV-F).

In terms of the stresses existing in the lipid bilayer just below the $L_{\alpha}$-$H_{II}$ transition, it is instructive to examine the values of headgroup area per molecule (at the lipid/water interface) for DDPE (27 wt% H₂O) and DAPE (28 wt% H₂O) at the transition point [108]. The $C_{10}$ lipid DAPE has a relatively low value of 59.6 Å² in $L_{\alpha}$, which drops to 49.5 Å² in the $H_{II}$ phase. The shorter chainlength $C_{12}$ lipid DDPE, on the other hand, has a much larger value of 67.9 Å² in $L_{\alpha}$, which drops to 56.6 Å² in $H_{II}$. In other words, the hydrocarbon chains of the shorter lipid are relatively more disordered than those of the longer lipid in the $L_{\alpha}$ phase at the transition. This difference is not accounted for by the difference in transition temperatures of the two systems, but may be explained in terms of the stress profiles discussed in subsection IV-D because the total chain pressure exerted by a DAPE molecule extends over 20 CH₂ groups per monolayer, as opposed to 12 for DDPE, a smaller degree of chain conformational disorder per methylene group – and hence a smaller area per molecule – is sufficient to overcome the headgroup pressure which is tending to keep the layer flat (see Fig 29). Although DDPE has a greater headgroup area in $H_{II}$ than DAPE, it actually has a smaller hydration: the water cylinder radius of $r_w = 12.7$ Å at 27 wt% water corresponds to 12 waters/lipid (at the limiting hydration of 33.8 wt% at 135°C), $r_w = 16.3$ Å, corresponding to 15.5 waters/lipid. For DAPE at 28 wt% water, $r_w = 21.9$ Å, and there are 17.5 waters/lipid. It should be noted that these hydration numbers are not only considerably larger than the values of 6 and 9 waters/lipid for the limiting hydrations of the gel phases of DDPE and DAPE, but are also larger than the values of 12 and 10 waters/lipid for the limiting hydrations of the $L_{\alpha}$ phase.

It is interesting to compare the ratio of the headgroup area in $L_{\alpha}$ to that in $H_{II}$ at the transition point, since Siegel has suggested (see subsection IV-H) that formation of intermediate phases is to be expected when this ratio falls below a value of approx $1.2$ [135]. For both DDPE at 27 wt% water and DAPE at its limiting hydration of 28 wt% water, the areas quoted above give ratios of 1.2. For DDPE, increasing the water content to the limiting hydration of the $H_{II}$ phase of 33.8 wt% would tend to decrease this ratio below 1.2, and in fact this is precisely the region of the phase diagram where bicontinuous cubic phases appear, as shown in Fig 11.

The temperature dependences of the structural parameters of the lamellar and $H_{II}$ phases of saturated [108] and unsaturated [226] phosphatidylethanolamines have been investigated. The temperature variation, relative to the gel-fluid transition temperature, of the cross-sectional area per molecule at various distances away from the water/lipid interface for fully hydrated DAPE...
is shown in Fig. 38. This emphasizes that the decrease in cross-sectional area per molecule occurring at the water/lipid interface on the lamellar-hexagonal transition can be as great in magnitude as the increase which occurs at the gel-fluid bilayer transition upon chain fluidization. On moving away from the interface into the hydrophobic interior of the $H_{II}$ phase, the area per molecule increases. Close to the polar/apolar interface, a neutral surface is reached, where the area is equal to that in the $L_{a}$ phase. On moving further into the interior, the area increases progressively, reaching quite large values near the midpoint, where the terminal methyl groups of the chains are primarily localized.

Gruner and co-workers [89,91] have developed a thermodynamic model of the $L_{a}$-$H_{II}$ transition in which they specifically include contributions from interstitial packing and from curvature in the free energy of the system. In their model they view the lipid monolayer as having some preferred spontaneous radius of curvature $r_{0}$, determined by the properties of the lipid, and independent of geometry. If the value of $r_{0}$ is infinite, then the $L_{a}$ phase is elastically relaxed. However, if $r_{0}$ has some finite value, then the monolayers would like to curve, either into cylinders or into spheres. In the former case, when the curvature is towards the aqueous region, the system would like to adopt the $H_{II}$ phase, with the radius of curvature, as given by the radius of the water cylinders, equal to $r_{0}$. In the latter case, they considered that a cubic phase $C_{II}$, of close packed spheres, would form (it should be noted that a lipid cubic phase having this structure has not as yet been observed). However, all of the hydrocarbon region of the inverse phases must be filled with lipid chains, and this becomes progressively energetically more expensive as the value of $r_{0}$ increases.

They included the following terms in the free energy:

1. All local interactions (those occurring within a single layer) such as the hydrophobic effect, and headgroup–headgroup and chain–chain interactions, are grouped together into a single (curvature) elasticity term $\mu_{c}$, which is taken to have a quadratic dependence on the mean curvature of the layer. An estimated value for the effective curvature elastic modulus was adopted.

2. A packing term $\mu_{p}$, arising from the filling of the interstices between the lipid cylinders or spheres. This term was estimated from the energy required to stretch a fluid chain to its fully extended state. A surface tension term was added to allow for the formation of free volume at the centre of the hydrocarbon region when its distance from the water/lipid interface exceeded the all-trans length of a lipid molecule.

3. For the case of charged lipid systems, a screened electrostatic term $\mu_{e}$ was calculated by solving the linearized Poisson-Boltzmann equation in the different geometries, at fixed surface charge density.

4. A hydration term arising from the polarization of water molecules by the lipid headgroups was calculated from the model of Marčelja [227] by a similar procedure to the electrostatic term, making the appropriate correspondence between the two sets of parameters.

The predictions of their model for the dependence of the various free energy contributions on water concentration for an uncharged dicyl lipid system are shown in Fig. 39. The picture that emerges is that the $H_{II}$ phase should be the stable phase at low hydration, due to the favourable curvature elastic energy, and a relatively low hydration free energy. The $L_{a}$ phase has a constant curvature term, because its curvature is constant at zero, and a packing term which is constant at zero. With increasing water concentration, the hydration free energy of the $L_{a}$ phase drops steeply, and the packing free energy contribution of the non-lamellar phases rises, so that beyond a certain water content, the $H_{a}$ phase is lowest in total free energy. No region is found where the $C_{II}$ phase should be stable. This model has a number of unsatisfactory features, and yet, by focussing attention on the importance of spontaneous curvature, and of the packing problems arising from the desire of the hydrocarbon chains to maintain a constant conformational state, it serves as a very useful starting point for the development of more realistic theories of non-lamellar phase formation.

The osmotic stress method of Parsegian and Rand has been used to measure the energy required to dehydrate phospholipid $H_{II}$ phases and hence to test some of the predictions of the Gruner model [228,229]. At low stress (large hydrations), the work was found to vary quadratically with the curvature of the water/lipid interface of the cylinders, arising mainly from the elas-
tectic energy associated with changing the curvature. This was the case for pure DOPE with or without 5% added alkane, and for a 3/1 DOPE/DOPC system containing 20% alkane, which forms an H_{II} phase with much larger water cylinder dimensions. The curvature elastic modulus was independent of the stress-free radius of curvature. At high stress, on the other hand, the work was found to rise far more steeply than could be accounted for by changes in the hydration force, and was proposed to be mainly associated with the work of dehydrating the lipid headgroups.

Cevc has proposed a phenomenological molecular theory of lamellar-to-nonlamellar transitions, where the transition point is determined by the balance between the repulsive lateral pressure of the chains, \( \pi_c \), and the corresponding lateral pressure from the heads, \( \pi_h \) (Cevc, G., unpublished data). Landau theory was used to evaluate the chain pressure, with the degree of chain rotational isomerization as the order parameter. The headgroup lateral pressure was approximated by a term due to surface hydration, evaluated from previous theory of the author. The results that emerge are that the chain pressure jumps steeply at the chain-melting transition, by an amount which increases with chainlength \( n_c \), and then rises steadily with temperature due to increasing chain disorder. The headgroup pressure, on the other hand, is rather insensitive to either temperature or chainlength, but is very sensitive to the effective hydrophilicity of the lipid headgroup. As shown in Fig 40, the transition to the non-lamellar phase is predicted to occur with increasing temperature at the point where the chain pressure curve crosses the headgroup pressure curve (which is taken to fall slightly with increasing temperature). This model explains in a rather clear way a number of the observed [157] facts: the inverse chain-length-dependence of the L_α-H_{II} phase transition temperature, the fact that direct gel-H_{II} transitions occur when the chains are sufficiently long, and the lowering of the L_α-H_{II} transition temperature, either by addition of salt, or by exchange of the phospholipid ester chain linkages for ether linkages. The latter two effects are due to a reduction of the lateral hydration pressure. However, by neglecting factors such as interstitial packing, this model is unable to make any structural predictions, nor any thermodynamic ones, under conditions where such effects become important.
IV-F Fluctuations, defects and intermediate phases

The assumption that the lipid/water interface of the cylindrical aggregates of the H_{II} phase has a circular cross-section is made purely as a convenient first approximation to the true structure [3]. In principle, the cross-section could, for example, become somewhat hexagonal in shape and still be compatible with a hexagonal packing, as shown in Fig. 41. It has been pointed out that a deformation of the interface towards a hexagonal cross-section, although costing curvature elastic energy, would partially relieve the chain packing stress of the H_{II} phase, by bringing the interface closer to the centre points (i.e., the 3-fold axes) of the hydrophobic interior [92,109]. However, a Fourier synthesis of diffraction data from the H_{II} phase of DOPE indicates that the cylinders are in fact close to circular [230]

Deformation towards an elliptical cross-section has been detected in the normal hexagonal H_{I} phase of a phosphatidylcholine/cholate/water ternary system [231], and in sodium dodecyl sulphate (SDS)/decanol/water ternary mixtures on increasing the decanol content [232]. The explanation for this effect in the latter case is that the SDS and the decanol have different tendencies for interfacial curvature, and as the decanol concentration increases, a partial lateral phase separation of the surfactant molecules occurs, leading to an inhomogeneous interfacial curvature. Beyond a critical shape anisotropy of the aggregates, hexagonal packing is no longer possible and a transition to a less symmetrical rectangular phase occurs. In fact, the decanol component is not strictly necessary to bring about such deformations (although it may be to stabilize them) the purely binary system SDS/water adopts a series of intermediate phases between the H_{I} and the L_{a} phases [107,233]. As the water content is reduced, the cylinder radius of the H_{I} phase grows until it reaches a value of 18.3 Å, equal to the all-trans length of an SDS molecule. Beyond this point, the water layer thickness between cylinders would have to decrease rapidly if the cylinder cross-section remained circular. However, this is resisted by repulsive electrostatic and hydrational forces between the aggregates. Instead, it costs less free energy for the cylinders to distort towards an elliptical shape, since in this way the opposing surfaces can maximise their separation. However, this then induces a phase transition to a less symmetrical oblique (two-dimensional monoclinic) phase.

Although this system is nominally a binary one, the fact that ionic amphiphiles can either have a counterion bound, or be dissociated means that in effect there are three components present (including water).

Inhomogeneous interfacial curvature can then arise from a partial lateral phase separation of the charged and uncharged forms of the amphiphile. Conversely, deviations from homogeneous curvature could affect the degree of dissociation of an ionic lipid system.

All of these considerations should apply equally to phospholipid inverted phases, although there will of course be important differences due to the opposite topology. For example, it is frequently observed that cubic phases form in binary mixtures of phospholipids,

![Fig 41 Possible deviations of cylindrical aggregates from circular to hexagonal cross-section in the H_{II} phase](image-url)
even though the individual components only form Lα and H1 phases (see subsection V-D). It is likely that a requirement for inhomogeneous interfacial curvature in the cubic phases is more easily met by a binary mixture of lipids.

**Defects**

Defects have been observed to occur in the Lα phase of hydrated non-ionic polyoxyethylene surfactants, studied either by ESR spectroscopy [234], electron microscopy [235], or by X-ray scattering [236]. The properties of such defects, and their relationship to the low value of curvature (splay) elastic modulus characteristic of these systems, have been discussed [237, 238]. They probably consist of coupled pores connecting a number of adjacent bilayers (dislocation loops), which increase in density with increasing temperature, and which are involved in the mechanism of the transition to the isotropic phase. At low temperatures, conical (trumpet)-shaped structures are observed, which are thought to reflect a tendency for the bilayers to adopt a negative Gaussian curvature (see subsection IV-C). It has been speculated that trapped defects are responsible for the formation of cubic phases in certain lipid systems [110].

Diffuse scattering, indicative of defects, has in fact been observed in X-ray and neutron experiments from a number of lamellar phases of binary or ternary surfactant systems. In some cases, the structure of the individual layers is thought to consist of arrays of small oblate (disc-like) aggregates, rather than bilayers [238, 239]. In other systems, the defects appear to consist of regions of correlated rod-like aggregates, either elliptical [240] or circular in cross section [236]. In the lamellar phase of the ternary system sodium dodecyl sulphate/1-decanol/water, increasing the ratio of soap to decanol (which increases the tendency for positive monolayer interfacial curvature) firstly introduces pores into the bilayers, then these pores become correlated as a phase boundary with a (type I) non-lamellar phase is approached [241]. In the polyoxyethylene system discussed in the previous paragraph, diffuse scattering is seen in the Lα phase, but only for those compositions where there is a cubic phase at lower temperatures [236]. On approaching the cubic phase, the diffuse scattering exhibits a hexagonal symmetry, which indicates that the cubic phase grows from small domains of hexagonal phase embedded in the lamellar phase. The cubic phase itself exhibits considerable diffuse scattering, which is anisotropic, and which indicates the presence of displacement disorder in the cubic phase in certain preferred directions [242]. Also the hexagonal H1 phase of this system shows evidence of displacement disorder, of a planar type [236]. Some unknown deformation of the cylinders occurs, which is correlated over large distances (≈ 1000 Å) in directions normal to the cylinder axes, but which is only correlated over short distances (≈ 100 Å) along the cylinders.

In the system sodium dodecyl sulphate/water, on increasing the water content to close to the Lα-Tα phase boundary, defects develop in the bilayers, which are precursors to the tetragonal phase [233]. Regions of the flat bilayers form arrays of pores arranged on a square lattice. When the positions of these square arrays become correlated between the layers, the Tα phase sets in.

Although the fascinating effects I have described in this section relate to type I (oil-in-water) phases, it is likely that analogous phenomena will be observed in the near future in the type II (inverse) phases which are of more direct relevance to biological lipids.

Fromhertz has extended his ‘surfactant-block’ model for micelle structure (in which blocks of essentially parallel molecules are rotated away from the bilayer configuration to produce small aggregates with rather stiff conformations of the hydrocarbon chains) to propose that ‘inverted’ block defects may occur in bilayer membranes, leading to the formation of hydrophilic channels across the bilayer [243–245]. He envisages that the formation of such structures may be facilitated by a variety of chemical agents which would reduce the hydrophobic energy cost of forming the block defect. Furthermore, he proposes that membrane junctions might result from the formation of such defects between two apposed membranes, thus being stabilized by the resulting shielding of water–chain contact at the surfaces, and in some cases leading ultimately to membrane fusion. The surfactant-block model has some attractive features, but it must be considered to be somewhat idiosyncratic in its approach.

**Local biaxiality**

For either a spherical micelle, a spherical bilayer vesicle, or a flat bilayer, the curvature is rotationally symmetric about any normal to the interface (i.e., the principal radii of curvature are equal). This is not the case for the hexagonal phase, and this might cause a tendency for phospholipid molecules (which are non-circular in cross-section) to lie with a preferred average alignment, either along or normal to the cylinder axis. It is unclear what consequences this might have for features such as the strength of the interactions between the headgroups.

**IV-G Geometric and epitaxial relationships**

An important question which has so far been little explored by researchers is that of the geometric relationships between the lattices of lyotropic phases during phase transitions. Such information is clearly liable to be of great relevance to understanding structural transformations in membranes, and from the point of view of physical chemistry, may shed light on the mecha-
nisms underlying such transitions. For example, a $^{31}$P-
NMR study of aligned PE bilayers showed that upon
heating into the $H_{II}$ phase, the lipid/water cylinders
formed parallel to the bilayers [246]. Furthermore, it has
been found [247,248] that in purified rod outer-segment
photoreceptor membranes, and in reconstituted rhodopsin/
phosphatidyethanolamine membranes, domains of inverted hexagonal $H_{II}$ phase and lamellar $L_n$
phase coexist, with a well-defined orientation between
the two lattices. This orientation, with the hexagonal
phase (10) planes parallel to the lamellar phase (001)
planes, corresponds to the rows of inverse cylinders
lying coplanar with the lamellae, as shown in Fig 42.
This simple result is by no means a trivial one, and
indicates that the spatial alignment of a phase may be
dictated by that of another phase from which it is
derived. Furthermore, the repeat spacings of the two
lattices were observed to be frequently commensurate,
in the ratios $d_L/d_{H} = 2:3$ and $1:1$ for the natural and
reconstituted systems, respectively. It was also noted
that the edges of the $H_{II}$ domains are faceted, which
implies that it is energetically favourable for the system
to minimize the area of domain boundary. It was sug-
gested that the diffraction patterns obtained by Fimean
and co-workers from dehydrated natural membranes
[249] actually showed evidence of aligned domains of
$H_{II}$ phase [247].

![Fig 42: Schematic diagram of the epitaxial relationships observed between the lamellar, cubic $Q_{23}^{23}$ (space group Ia3d) and hexagonal $H_{II}$ phases of the polyoxyethylene surfactant $C_{12}EO_6$ [250]. (Reproduced with permission from the J Phys Chem (1988) 92, 2646–2651. Copyright (1988) American Chemical Society.)](https://example.com/fig42)

Such epitaxial relationships between lyotropic liquid-crystalline mesophases have recently been ob-
served in a polyoxyethylene surfactant system forming
type I (oil-in-water) hexagonal and cubic (spacegroup
Ia3d) mesophases [250]. A previous NMR study of this
system [251] had shown that the $H_{II}$ phase sponta-
neously formed domains which were aligned along four
unique directions arranged tetrahedrally with respect to
each other. An aligned sample of hexaethylene glycol
mono-$n$-dodecyl ether ($C_{12}EO_6$) with a water content
of 62 wt% gave the phase sequence $H_{II}-Q_{23}^{23}-L_n$
upon heating from below 20 to 35°C. It was found [250] that the (10)
planes of the hexagonal phase were parallel to the (211)
planes of the cubic phase, which in turn were found to
be parallel to the (001) planes of the lamellar phase.
These are in fact the planes of highest density in each of
the three phases. Also, the lattices were observed to be
commensurate, in the sense that the repeat spacings of
these planes were identical

$$d_{10}(H_{II}) = d_{211}(Q_{23}) = d_{001}(L_n) = 48\,\text{Å}$$

These epitaxial relationships are illustrated in Fig
43.

Furthermore, the hexagonal phase cylinders were
found to be aligned along the four equivalent 3-fold
(111) (body-diagonal) axes of the cubic phase, and also,
the cylinder spacing of 55 Å in the $H_{II}$ phase was
identical to the spacing between the 3-fold axes of the
cubic phase.

Similar epitaxial relationships have been demon-
strated in the type I system sodium dodecyl sulphate
(SDS)/water, which has the rich phase sequence with
increasing water at 55°C of $L_n-L_{c}-T_{c}-Q_{c}-R_{c}-M_{c}-H_{II}$
[233]. In this case the (001) planes of the $L_n$ phase
were found to be aligned with the (002) planes of the tetra-
gonal phase, which in turn were aligned with the (222)
planes of the cubic phase, the repeat spacing of these
planes of 33 Å being found to be equal for each phase.

These extremely important results demonstrate that
very precise, yet subtle epitaxial relationships exist in
general at transitions between lyotropic mesophases having different topologies. It is highly likely that analogous behaviour will be discovered in future for transitions between inverse phases of biological lipids, for example for \( L_{\alpha}-Q_{11} \), \( Q_{11}^{*}-Q_{11}^{*} \) and \( Q_{11}^{*}-H_{11} \).

The large body of structural data on thermally induced \( L_{\alpha}-H_{11} \) transitions in hydrated lipid systems shows that an equivalence in the principal \( d \)-spacings at the transition point is not in general observed. However, by varying the water content, it may well become possible to find a composition where the \( d \)-spacings do (accidentally) become equal at the transition.

The situation at other non-lamellar transitions is less clear. In the case of a lamellar-cubicle transition observed in hydrated 1-monostearin, it was claimed that the \( d(001) \) Bragg spacing of the \( L_{\alpha} \) phase was continuous with the \( d(222) \) spacing of a body-centred cubic phase [252]. It was further claimed that the cubic (222) spacing of an unsaturated acylglycerol system was also continuous with that of the \( H_{11} \) phase [253]. However, such equivalences were not observed at various non-lamellar transitions of hydrated monoelaidin and monoolen [254].

**IV-H Kinetics, reversibility and transition mechanisms**

The use of intense synchrotron radiation permits time-resolved X-ray diffraction study of lipid phase transitions to be carried out with a time resolution of 100 ms or better [255,256]. A number of authors have used this approach to investigate the kinetics of the \( L_{\alpha}-H_{11} \) and \( L_{\beta}-H_{11} \) phase transitions of phosphatidylethanolamines [257,258] and phosphatidylcholine/fatty acid mixtures [259]. The use of time-resolved diffraction to study biological systems has been reviewed [260]. Fig. 44 shows some of the diffraction patterns observed during the first 13 s of a temperature-jump experiment, where a fully hydrated sample of DHPE (dihexadecyl-PE) was rapidly heated from 37 to 93°C. The conclusions that emerge from such studies are:

(a) The transitions are fast, requiring of the order of 1-3 s for completion, irrespective of whether the lamellar phase is fluid or solid-like (gel or crystalline).

(b) The transitions are two-state processes, with no intermediate phases (additional sharp diffraction lines) or structures/defects (diffuse scatter) being observed, at least at the sensitivity of the experiments so far performed.

(c) The behaviour on heating and cooling is fully reversible.

(d) The diffraction lines of the different phases remain sharp throughout the transitions, indicating a high degree of long range order within the coexisting phase domains.

An important point which has been emphasized [257] is that the rate of the \( L_{\alpha}-H_{11} \) transition is probably in general limited by the speed at which water re-equilibrates between the two phases, rather than being con-

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**Fig. 44** Kinetics of the \( L_{\alpha}-L_{\beta} \) and \( L_{\alpha}-H_{11} \) phase transitions of fully hydrated dihexadecylphosphatidylethanolamine, as studied by time-resolved X-ray diffraction [257] (Reproduced with permission from Biochemistry (1985) 24, 4826-4844. Copyright (1985) American Chemical Society).
trolled by the time required for the topological re-
arrangement of the lipid molecules occurring at the
transition

Very different results were reported for the reversibil-
ity of the \(L_{\alpha}-H_{II}\) transition, studied by \(^{31}\)P-NMR [261], in mixtures of phosphatidylglycerol with phosphatidyl-
ethanolamine, monogalactosyldiacylglycerol, and mono-
glucoosyldiacylglycerol, in the presence of Ca\(^{2+}\) or Mg\(^{2+}\). It was found that the reverse transition to the lamellar
phase was slow upon cooling, taking from hours to
days. It is likely that this behaviour is related to the
strength of binding of the divalent ions to the charged
lipid component in the \(H_{II}\) phase.

Much useful work on lipid mixtures is still to be
done, and it remains to be established whether dialkyl
lipid mixtures undergo fully reversible, fast transitions
to and from the \(H_{II}\) phase.

The situation with regard to reversibility can be very
different with other non-lamellar phase transitions. For example, a fully-hydrated polar lipid extract from the
extreme thermoacidophile \(S\) \(sulfataricus\) was found to
undergo a nearly irreversible transition from \(L_{\alpha}\) to an
inverse cubic phase (space group \(Pn3n\), No 224) upon
heating to \(85^\circ\)C (which is the optimal growth tempera-
ture of the organism) [262]. Although this behaviour
might be attributed to the fact that the bacterial lipids
have very unusual chemical structures, containing single,
long (C\(_{40}\)), branched hydrocarbon chains ether-linked
to polar headgroups at each end, there is some evidence
that it might be a more general effect. In a time-re-
solved diffraction study of transitions between lamellar,
cubic and inverse hexagonal phases in hydrated mono-
acylglycerol, it was found that, although most of the
transitions occurred within 1-3 s both on heating and
on cooling, one particular cubic-cubic transition (tenta-
ively assigned as \(1m3m-Pn3m\)) required tens of minutes
for completion, upon cooling [254].

In certain phospholipid systems, metastable cubic
phases have been observed. These may be very difficult
to induce to form, yet strongly metastable once formed.
For example, a cubic phase (spacegroup \(Pn3m\)) was
observed in fully hydrated DOPE after rapidly cycling
the temperature hundreds of times between \(-5\) and
\(15^\circ\)C [110]. Once formed, it was necessary to cool the
sample a number of times to \(-30^\circ\)C to return it to the
\(L_{\alpha}/H_{II}\) phases; heating to above room temperature did
not completely transform the sample to the \(H_{II}\) phase.
Similarly, a sample of fully hydrated \(N\)-methyl-DOPE,
which has an \(L_{\alpha}-H_{II}\) transition at \(65-75^\circ\)C, did not
return to \(L_{\alpha}\) upon very slow cooling, but instead it
transformed to two co-existing cubic phases [109]. Such
metastable effects were not observed with cubic phases
observed in saturated phosphatidylethanolamine sys-
tems [108], but do appear to be present in the methyl-
ated saturated PE derivatives (Hogan, J L. and Seddon,
J M., unpublished data)

**Mechanisms of non-lamellar transitions**

The possible role of non-lamellar structures such as
inverted micelles as intermediates in the \(L_{\alpha}-H_{II}\) transition has been discussed a number of times by Culhs, De
Krujff, Verkleij and co-workers [4,14,15,17–19,124]
They suggested that the transition proceeds via forma-
tion of lipidic particles, or intermembrane inverted
micelles [15,19,131,263]. Formation of such point de-
fects may not invariably happen, however, as for some
systems, no lipidic particles are detected [264]. These
authors suggested that the primary mechanism of the
transition involves the formation of fused linear contact
regions, which are line defects, between apposed bi-
layers. A morphological study of egg PC/cardiolipin in
the presence of calcium appeared to support the latter
mechanism, and led to the suggestion that a complex
hexagonal phase, composed of cylindrical bilayer tubes,
forms as an intermediate stage in the transition [136]. A
similar mechanism was also proposed by Caffrey [257].

Siegel has developed a model for lamellar to non-
lamellar transitions, which attempts to analyze the role
of intermediate structures in the mechanisms of the
transitions, and to obtain information on the kinetics
[85,86,133–135]. The details of this model are too exten-
sive to review adequately here, and only a few of the
findings will be discussed. The model takes the primary
initial event in the transition to be the formation of
‘inverted micellar intermediates’ (IMI) between pairs
of apposed bilayers, as shown in Fig. 45 (it is calculated
that the formation of intrabilayer inverted micelles
should be very unlikely). After formation, so long as
their density is sufficiently high, and the rate of rever-
sion is sufficiently slow, the IMI can either fuse into
‘rod micellar intermediates’ (RMI), or form ‘line defects’
(LD), which then assemble into the \(H_{II}\) phase, as shown
in Fig. 46.

The IMI are estimated to occur within the lamellar
phase only when close to the \(L_{\alpha}-H_{II}\) transition (within
approx. \(10^\circ\)C). He estimates that \(10^{9}–10^{12}\) IMI per cm\(^2\)
of apposed bilayers will form during the transition. The
lifetime of an IMI close to the transition is estimated to
be \(\leq 1\) ms, and this is predicted to increase upon
dilution with ‘\(L_{\alpha}\)-forming’ lipids. This might explain
why PE/PC mixtures tend to show lipidic particles in
freeze-fracture micrographs, but pure PE systems often
do not. For systems such as PE, where the optimal value
of headgroup area is relatively small, the transition
tends to be directly from \(L_{\alpha}\) to the \(H_{II}\) phase. However,
for lipids where the optimal area is larger, intermediate
phases, such as bicontinuous cubic phases, often ap-
ppear. Siegel attempts to explain this by considering
another possible outcome of IMI formation. As shown
in Fig. 45, instead of of fusing laterally to form RMI,
the IMI can fuse with the outer monolayers to form an
‘interlamellar attachment’ (ILA), a channel through the
Formation of non-bilayer structures in membranes [134] (a) two bilayers become closely apposed over small areas, (b) a fluctuation away from a planar configuration occurs, (c) formation of an inverted micellar intermediate (IMI) between the membranes, (d) reversion to original bilayers, or formation of an interlamellar attachment (ILA), which is a fusion step which may lead to formation of bicontinuous cubic phases, such as Im3m (Q229) (Reproduced with permission from Biophys J (1986) 49, 1155-1170).

Pair of bilayers This results in the two membranes fusing together, and can lead to formation of bicontinuous inverse cubic phases such as Im3m (Q229). Siegel takes the ratio of the headgroup areas in the Lα and HII phases as a measure of the tendency to form ILA. When this ratio exceeds 1.2, ILA should form only rarely. For example, for egg PE, where the area ratio is considerably larger than 1.2, the Lα→HII transition is complete within <1 s [153]. On the other hand, for systems where the ratio is less than 1.2, ILA are estimated to form at a rate of approx. 0.1–1 per IMI per s, and they should be relatively long-lived, allowing observation of extensive arrays of lipidic particles by electron microscopy, isotropic signals by NMR, and coalescence of the ILA to form intermediate cubic phases. This area ratio used by Siegel is in fact roughly equivalent to the intrinsic curvature parameter of Gruner and co-workers. The Siegel model invokes metastable cubic phases; this has been further discussed by Gruner and co-workers, who found that cubic phases could be formed in DOPE and N-methyl-DOPE only with great difficulty [109, 110]. In fact, in some cases, cubic phases may form rapidly, but then exhibit metastability [13,262].

Formation of IMI can be a mechanism for intervesicular lipid exchange (of the outer monolayers). If there is a strong tendency for HII phase formation, then the vesicles will rupture and release their contents. Alternatively, if ILA formation is favoured, then mixing of vesicle contents without leakage can occur.

IV-1 Relationship to micellar solutions and microemulsions

Microemulsions are stable, isotropic solutions typically containing surfactant, oil and water, and frequently also alcohol and/or salt. Unlike the geometri-
cally ordered liquid-crystalline phases, they possess only topological order, associated with the surfactant-rich interface between the aqueous and hydrocarbon regions. Unlike the translationally ordered mesophases, it is possible to vary the interfacial curvature continuously from strongly positive (oil-in-water) to strongly negative (water-in-oil) without any apparent structural discontinuities. At one extreme of low oil content, normal micellar solutions are formed, consisting of aggregates of surfactant molecules dispersed in aqueous solution (see Ref 35 for a comprehensive review). In the oil-rich region of the phase diagram, conversely, inverted micelles are found. Evidence is accumulating that on moving from one extreme to the other, by varying the ratio of water to oil, bicontinuous structures are formed, having dynamic structures which are locally, and at a given moment similar to the bicontinuous phases of lipids, yet which constantly break and reform through fusion events between the interfaces. These properties are a consequence of the curvature elastic moduli having low values, permitting thermally excited fluctuations of the interfaces, which, in the absence of strong long-range interactions, breaks up the lattice and hence the long-range order. It was proposed that these bicontinuous microemulsions might be based upon periodic minimal surfaces [112,198], and this idea has stimulated a great deal of interest.

Statistical models of phase equilibria in microemulsion systems have been presented by a number of authors [265–267]. By inclusion of an additional free energy term arising from interfacial curvature, to those arising from the entropy of mixing, the interfacial tension, and the adsorption of surfactant at the water-oil interface, quite good agreement with some features of the experimentally determined phase diagrams is obtained. An important aspect which has been discussed by De Gennes and Taupin [268] is the flexibility of the interface, given by its curvature elasticity. The effect of co-surfactants (small amphiphilic molecules which adsorb at the polar/non-polar interface) is to strongly increase the flexibility of the layers. This clearly also has important implications for biological membranes, which in the absence of co-surfactants are invariably far less flexible than the interfaces of microemulsions, but which in the presence of molecules such as short-chain fatty alcohols should become much more deformable. The role of interfacial curvature in determining the microstructure of microemulsions has been further described by Evans and co-workers [269,270].

V. Factors affecting phase behaviour

The factors affecting lipid polymorphic phase behaviour have been extensively reviewed elsewhere [4,13–20,56], and I will here only include selected data to illustrate the principal effects I wish to discuss.

In general terms, any factors which expand the chain region lateral packing relative to that of the headgroup region will tend to favour the formation of inverse non-lamellar phases, as discussed extensively in section IV. For lipid systems such as PE, where a tight headgroup packing is favourable, the transitions tend to occur directly from \( L_\alpha \) to the \( H_{II} \) phase. On the other hand, for lipids which require a somewhat more expanded headgroup packing, intermediate phases, e.g., bicontinuous cubics, frequently appear. For large, strongly hydrated headgroups, the tendency to adopt a negative interfacial mean curvature is no longer present, and the lamellar phase is stabilised.

V-A Headgroup region

Headgroup type

The single most important property of the headgroup in regulating the lipid-phase behaviour is its effective hydrophilicity, which determines the strength of the interaction between the headgroups and the water [271,272]. This hydrophilicity is primarily a function of the headgroup chemical structure, but can be strongly modified by the presence of solutes (see subsection V-C). If attractive headgroup–headgroup interactions, such as hydrogen-bonding, are present, the hydrophilicity will be reduced (vide infra). If we compare the molecular conformations of PE and PC, as shown in Fig 47, we see no immediate indication that these two classes of phospholipid might have dramatically different phase behaviour. However, for the PEs, which can form direct phosphate-ammonium hydrogen bonds, as shown from the crystal structure in Fig 48, the tendency to hydrate is bound to be less than that of the PCs, for which direct hydrogen-bonding between headgroups is not possible. For the former class of lipid, this leads
to reduced hydration and hence smaller values of interfacial area per molecule. This has dramatic consequences for the phase behaviour, leading both to an enhanced tendency for gel-phase metastability [275–277], and to the appearance of non-lamellar phases, even at high levels of hydration, for the PEs.

For PC gel and fluid lamellar phases, the repulsive hydration force (enhanced by thermal fluctuations [278]), balanced by van der Waal’s attraction, leads to equilibrium separations between the bilayers in the range 20–30 Å [279,280]. For phosphatidylethanolamines, the hydration repulsion is of shorter range, leading to equilibrium bilayer separations in the range 8–20 Å [108,279–281]. Furthermore, the attractive component of the total force is much stronger for PEs, leading to adhesion energies which are one order of magnitude larger than for PC bilayers [282,283]. Force measurements on methylated PEs have led to the suggestion that there is an additional attractive interaction between PE headgroups, caused by charge/dipole correlations in headgroups facing each other across the aqueous region [281].

The strongly hydrophobic nature of the PC headgroup means that this class of phospholipid, whether saturated or unsaturated, does not in general (unless bulky substituent groups are attached to the chains, see subsection V-B) form any non-lamellar phases, except at low hydration levels below approx 5 wt% [3].

Phosphatidylethanolamines, on the other hand, have a strong tendency to adopt the HII phase [15,19,34]. Almost every member of this class of lipid so far examined will adopt the HII phase in excess water above some temperature (which may be quite high for saturated species), and we may surmise that this also applies to all lower levels of hydration, even down to the anhydrous lipid.

For charged phospholipids, the situation is somewhat more complicated.

Phosphatidylserines can form the HII phase in the anhydrous state [24,284], or in excess aqueous buffer, when the charge is neutralized by low pH values in the region of 3 [285], or in the presence of lithium ions [286].

Phosphatidylglycerols tend to form lamellar phases, and in fact have strikingly similar phase transitions to the corresponding phosphatidylcholines. However, the C14 ether compound DTPG has been reported to form the HII phase at 90°C in the presence of 1 M CaCl2 solution [287].

Phosphatidic acids, which are doubly-ionizable, can adopt the HII phase at low pH, whether saturated [155], or unsaturated [288]. At higher pH values, in the region of 5.5–6, divalent ions induce formation of the HII phase in various phosphatidic acids [288–291].

Cardiolipin, which is a doubly-ionizable tetra-acyl class of phospholipid found in high concentration in the inner mitochondrial membrane, forms the HII phase at low hydration [292], in excess solution in the presence of divalent ions [292–295], or at pH values below 3 or at NaCl concentrations above 1.5 M [296]. Interestingly, a cardiolipin analogue with an additional oleoyl chain attached to the 2-position of the glycerol headgroup bridge was found to adopt the HII phase in the presence of excess water, without any added salt to screen the headgroup charges [297]. Various other tetra-acyl phospholipids can form the HII phase. For example, phosphatidlydiacylglycerols with either tetra-myristoyl or tetra-lauroyl chains undergo direct gel-HII transitions upon heating [164].

Sphingomyelins do not in general form any non-lamellar phases, whether saturated [298], or unsaturated [299], and in fact they stabilize the lamellar phase in mixed systems [300]. A hexagonal phase had been reported in an NMR study of hydrated bovine brain sphingomyelin at 46°C [165], but this was subsequently refuted [301].

Hydrated gangliosides and charged cerebrosides may form hexagonal mesophases, but normally of the oil-in-water HII type. For example, bovine brain mixed gangliosides adopt the HII phase at 50°C for water contents of 18–50 wt% [302]. Similarly, the glycosphingolipid psychosine hydrochloride forms the HII phase between approx 47 and 66 wt% water [303].

Various other unusual hydrated phospholipids can form the HII phase. For example, aminoethylphosphonolipid, an analogue of PE extracted from tetra-hymena membranes, forms an HII phase at 40°C [304], and dipalmitoyl phosphatidylcholine forms an HII phase at 54°C [164].

Glyceroglycolipids are found as major constituents of the membranes of plants, bacteria, and mycoplasma. Unsaturated monogalactosyl diacylglycerols (MGDG) extracted from plants adopt the HII phase in the presence of excess water, whereas digalactosyl diacyl-
glycerols (DGDG) form only lamellar phases [305]. The effect of hydrogenation of natural MGDG, to produce saturated chains, was found to be to suppress the H\textsubscript{II} phase [306]. However, it was subsequently found that aqueous dispersions of the saturated ether-linked (C14) glycolipid, ditetradecyl glucopyranosylglycerol, showed a small enthalpy calorimetric transition 6°C above the main transition [307], which has been identified as a transition to the H\textsubscript{II} phase [178]. It was pointed out that this temperature is strikingly lower than that of the corresponding C14 ether-linked PE (96°C, see Fig 50), and this was suggested to be a reflection of stronger hydrogen-bonding in the glycolipid system. A study of a homologous series of saturated glucopyranosylglycerols has shown that this is a general effect [308].

The phase behaviour of glucosyl diacylglycerols extracted from mycoplasma has been found to be strikingly similar to that of the corresponding galactosyl lipids from plants. For example, monoglucosyl diacylglycerol (MGDG) forms an H\textsubscript{II} phase in water at 40°C, whereas the diglucosyl compound DGDG is lamellar [309].

Finally, Murakami and co-workers have synthesized a number of artificial peptide lipids which can form stable cubic and H\textsubscript{II} phases [310,311].

Modification to headgroup structure

Methylation of PE has dramatic effects on the molecular conformation in the crystalline lamellar phase. The bent headgroup conformation of phosphatidylethanolamine [274,312] changes for the N,N-dimethyl derivative to a conformation where the headgroup is essentially normal to the bilayer, and interdigitated with the headgroups of the apposed bilayer [313]. This is illustrated in Fig 49. For the trimethylated derivative phosphatidylcholine (PC), as shown in Fig 47, the conformation reverts towards being nearly parallel to the bilayer surface, and is non-interdigitated [273].

Step-wise methylation of the ammonium group of either phosphatidylethanolamine [314] or the headgroup-lengthened analogue phosphatidylpropanolamine [315] has the effect of progressively reducing the chain-melting transition temperature.

The effect of increasing the headgroup length and the degree of N-methylation on the L\textsubscript{α}-D\textsubscript{II} transition of fully-hydrated ditetradecyl PE (C\textsubscript{14} ether-linked) was studied by DSC and X-ray diffraction [157]. Lengthening the headgroup by a single methylene group increased the hexagonal transition temperature by 18°C. Addition of a single terminal methyl group had much more dramatic effects, increasing the H\textsubscript{II} transition temperature by 52°C, and inducing the formation of additional non-lamellar phases between L\textsubscript{α} and H\textsubscript{II}. The additional phases were suppressed by high salt concentrations. Lengthening the headgroup by two methylene groups also led to the appearance of additional non-lamellar phases, which again could be suppressed by high salt concentrations. For the dimethyl compound, no H\textsubscript{II} phase could be detected, although now addition of high salt induced the appearance of additional non-lamellar phases at high temperatures. Some, if not all, of these additional phases are in fact cubic. These results illustrate the interplay between the effect of increasing the headgroup hydrophilicity by chemical modification, and reducing the effective headgroup hydration by addition of salt. The overall effect is that increasing hydrophilicity first raises the L\textsubscript{α}-H\textsubscript{II} transition temperature, then induces new intermediate non-lamellar phases to form. This behaviour is strikingly similar to that caused by reducing the chain-length (see subsection V-A), and emphasizes the interplay between the lateral interactions in the headgroup region, and the hydrocarbon chain region, in determining the phase behaviour.

The effect of N-methylation of unsaturated PE is rather similar [109,316,317]. It was found that fully hydrated N-methyl DOPE has a transition to the H\textsubscript{II} phase at 70°C, some 60°C higher than the L\textsubscript{α}-H\textsubscript{II} transition of DOPE [316]. However, between 20 and 70°C, an isotropic \textsuperscript{31}P-NMR resonance was observed, which has now been identified as arising from two coexisting cubic phases, of probable spacegroups Pn3m (Q\textsuperscript{224}) and Im3m (Q\textsuperscript{229}) [109]. In this latter study, it was inferred that methylation increases the lipid monolayer spontaneous radius of curvature r\textsubscript{0}, which, since periodic minimal surfaces represent geometries in which intermediate values of r\textsubscript{0} can be obtained, along with...
near constant monolayer thickness, leads to cubic phases becoming favoured.

Trinitrophenylation of the amino group of unsaturated PE systems was found to greatly reduce the headgroup hydration [318]. For the pure systems, even in the presence of excess buffer, no headgroup rotation occurs, even though the lipid chains are fluid. When mixed with either PE or PC, strong destabilization of the bilayer phase occurs, with promotion of H_{II} and/or isotropic phases. Since on purely steric grounds, the opposite effect might be expected due to the bulk of the extra group, these results implied that it was a dehydrating effect of the trinitrophenylated derivative which was dominant.

A similar effect was that due to addition of alkyl substituents to the C-2 position of the PE headgroup, which was found to destabilize the lamellar phase relative to H_{II} [317]. Since this perturbation neither (directly) affects the ability of the amine group to form hydrogen bonds, nor alters the phosphate-amine distance, this was taken as evidence that the H_{II} phase was favoured due to a reduction in the repulsive hydration force for the (C-2)-alkylated derivatives.

The effect of acylation of the terminal ammonium group of DPPE and DOPE has been studied by 31P-NMR and DSC [319]. The main transition of DPPE was depressed by N-acylation with short chainlengths, and raised for longer chainlengths. Although N-acyl-DPPE and N-palmitoyl-DOPE form lamellar phases, the N-oleoyl-DOPE was observed to form an H_{II} phase at room temperature.

The pH-sensitive derivative N-succinyl-DOPE was found to remain in the lamellar phase, either upon lowering the pH to 4.0, or in the presence of calcium [320]. Incorporation into DOPE led to stabilization of the lamellar phase for amounts greater than 10 mol%, and addition of calcium then induced a transition to the H_{II} phase.

The effect of lipid peroxidation was found to be to stabilize the L_{α} phase of egg PE, inhibiting the H_{II} phase up to at least 70°C [321]. The mechanism proposed involved cross-bridging by formation of Schiff’s base linkages between the PE headgroup and bifunctional malondialdehyde, formed as a product of the peroxidation.

Hydrogen bonding

The crystal structure of DLPE shows clear evidence for an extensive network of direct intermolecular headgroup-headgroup hydrogen bonds between ammonium and phosphate groups [22,274,312]. Two bonds are formed laterally to neighbouring molecules within the same bilayer, as shown in Fig. 48. In addition, a further bond is formed transversely to a headgroup in the adjacent bilayer (via an acetate acid of crystallization in this particular case).

This gives a strong cohesion to the crystalline multilayer, and reduces the effective hydrophilicity of the PE headgroup, since its neighbouring headgroups can compete with water for binding to it. It is likely that the low hydration of PE systems, with the concomitant dramatic consequences for the polymorphic phase behaviour, can largely be traced to this effect. The possibility that residual hydrogen-bonding could affect the interactions within and between bilayers of such systems has been suggested [322–324]. A recent analysis of the variation of the repulsive forces between hydrated phospholipid bilayers as a function of the degree of headgroup methylation has in fact led to the suggestion that, in addition to the Marčelja and Radic hydration repulsion [325], an attractive hydration force may also exist [281]. It was suggested that this force could be mediated by interbilayer hydrogen-bonded water bridges across the aqueous layer. A study of a number of surfactant systems in the aprotic yet polar solvent 3-methylsydnone found no evidence for monomer aggregation, which was taken to imply that hydrogen bonding in the solvent is a prerequisite for amphiphilic self-assembly [326].

The role of hydrogen-bonding in lipid polymorphism has been reviewed, and a case was argued for the persistence, at least partially, of hydrogen-bonding in the liquid-crystalline phases of lipids such as PE and MGDG [74–76].

A number of NMR studies support this assertion. The activation energy for the motion of the headgroup terminal C-N bond of DPPE was found from 15N-NMR to be 53 kJ mol⁻¹, compared to 32 kJ mol⁻¹ for DPPC, reflecting a higher barrier to rotation of the amine headgroup [327]. Similar results were obtained by 2H-NMR [328]. Furthermore, it was found in a study by 31P- and 2H-NMR that the phosphate group rotational correlation time of 0.6 ns for fully hydrated DOPC in the L_{α} phase at 20°C, was increased to 1.65 ns for bilayers of E. coli PE [329].

Further support for hydrogen-bonding is lent by a number of infrared studies. Infrared absorption showed that the phosphate absorption bands of anhydrous DPPE or egg PE multilayers were shifted to lower frequency compared to those from various PC films [330]. Infrared dichroism found that even in the fully hydrated gel phase, the PO₃⁻ symmetric stretching vibration of DPPE was shifted by 12 cm⁻¹ to lower frequency compared with DPPC, implying that hydrogen-bonding is present in the gel phase of PE [331,332]. Fourier transform (FT) infrared studies of hydrated unsaturated PE systems indicated that the degree of hydrogen-bonding is similar in the gel, L_{α} and H_{II} phases [181,182].

It was further argued by Boggs that lipids tend to transform to the H_{II} phase at pH values where their hydrogen-bonding interactions are strongest. However,
the fact that $H_{II}$ phase formation in DPPE (C$_{12}$ ether PE) is promoted at low pH (see Fig. 52), shows that this suggestion is not necessarily correct in general, and other factors, such as the intrinsic hydrophilicity of the phosphate group, may predominate.

An osmotic swelling study of unilamellar vesicles found that PE systems (at pH 8.6) had unusually large values of elastic modulus, of the order of 10–80-times as large as those found for other lipid systems [7]. This was ascribed to residual hydrogen-bonding between the headgroups.

V-B Hydrocarbon chain region

Type of chain linkage

Ether-linked lipids appear to form non-lamellar phases more easily than their ester-linked counterparts, presumably because they are less hydrophilic, owing to the absence of the carbonyl groups. For example, it was found that fully hydrated ether-linked saturated phosphatidylethanolamines formed the $H_{II}$ phase at temperatures well below 100°C [154–156], whereas the ester compounds did not. Subsequent work showed that exchange of ester for ether chain linkages has the effect of reducing the lamellar to non-lamellar transition temperature by as much as 50°C, the difference between esters and ethers being found to decrease progressively with increasing chainlength [157].

These differences are not just confined to phospholipids, but are also observed for glycolipids. Ether-linked ditetradecyl glycosyloxyglycerol adopts the $H_{II}$ phase from myelin was found to adopt the $H_{II}$ phase at a few degrees above the chain-melting temperature [156]. On comparing PE systems having diacyl, alkylacyl, and alkenylacyl (plasmalogens) linkages, a progressive reduction in the temperature of $H_{II}$ phase formation was seen, showing that the vinyl ether linkage is even more effective than a normal ether linkage [333].

Fig. 50 shows the chainlength-dependence of the transition temperatures for fully hydrated diacyl (a, c, e) and dialkyl (b, d, f) phosphatidylethanolamines, dispersed in water (a, b), 2.4 M NaCl (c, d), and saturated NaCl (e, f) [157]. The upper transition in each case is to an $H_{II}$ phase, except for the C$_{12}$ dialkyl DDPE, which transforms first to a cubic phase, the $H_{II}$ phase transition occurring at 135°C (Reproduced with permission from Biochemistry (1983) 22, 1280–1289 Copyright (1983) American Chemical Society).

Fig. 50 shows the chainlength-dependence of the transition temperatures for fully hydrated diacyl and dialkyl phosphatidylethanolamines.

Chain unsaturation and branching

The effect of chain unsaturation on membrane structure, with regard to the effects on enzyme kinetics, has been reviewed [335].

It is well documented that insertion of cis-double bonds in the hydrocarbon chains of saturated phospholipids lowers the gel-fluid transition temperature by approx. 60°C (depending on the position and number of the double bonds), due to the stiff kink in the chain at the double bond being sterically incompatible with the nearly all-trans configuration of the rotating chains in the gel phase. Surprisingly, the effect on the bilayer-$H_{II}$ transition is similar. For example, the saturated di-C$_{18}$ DSPE in excess water has a $T_{c}$ of 74°C and a $T_{h}$ of 101°C [157], the corresponding trans-unsaturated DEPE has a $T_{c}$ of 35°C [152] and a $T_{h}$ of approx. 61°C [336], whereas the cis-unsaturated DOPE has a $T_{c}$ of approx. –16°C [152] and a $T_{h}$ of approx. 10°C [336]. It has in fact been shown in a recent study of PEs with
various branched and alicyclic chain structures, but with the same effective chainlength \( (C_{18}) \), that the difference in temperature between the two transitions has a constant value of 25–30 °C \[221\]. Thus, although one would expect that the presence of double bonds would have a much smaller perturbing effect on the structure of the fluid phases than on the gel, they nonetheless are able to reduce the bilayer-hexagonal transition temperature by approximately the same amount as the gel-fluid transition. This effect is probably in part a reflection of the relatively low entropy of the former transition (see subsection IV-A, above), but suggests that the particular distribution of chain conformational states has a profound importance for the phase stability. Furthermore, it is conceivable that the more severe packing constraints imposed by the kink in the chains could actually lead to the formation of new phases (e.g., cubic) intermediate between the lamellar and hexagonal \( H_{II} \) phases. In other words, the necessity to fill all of the hydrocarbon volume may become too severe a constraint (at a particular temperature and chainlength) in the \( H_{II} \) phase when the chains contain a kink. Thus, in a certain sense, the effect of unsaturation could be analogous to that of reducing the chainlength, which promotes the appearance of intermediate cubic phases (Ref 108, and Seddon, J.M., unpublished data). A recent finding which seems to bear out this suggestion is the formation of a bicontinuous cubic phase by fully hydrated DOPE \[110\], whereas the corresponding saturated lipid DSPE above its chain-melting transition adopts only \( L_{n} \) and \( H_{II} \) phases \[155,157\]. Furthermore, the effect of branched chains may also be to induce cubic-phase formation between the \( L_{n} \) and \( H_{II} \) phases. A study of PE, extracted from *Bacillus megaterium*, containing roughly 90% branched acyl chains, was found to adopt a bicontinuous cubic phase at high temperatures, for water contents up to 14 mol/mol lipid \[337\]. On the other hand, replacement of the unsaturated bond in the oleoyl chain of POPE with a cyclopropane ring has the effect of reducing the \( L_{n}-H_{II} \) transition by 15–20 °C \[170\], apparently without inducing cubic phases to appear. A recent study shows that the presence of bulky substituent groups attached to the hydrocarbon chains can cause the appearance of \( H_{II} \) and cubic phases in hydrated diacyl phosphatidylcholines \[338\].

**Lyso phospholipids**

Hydrated lyposphospholipids can form either normal \( H_{I} \) or inverse \( H_{II} \) phases, depending on the lipid headgroup structure, and the chainlength and degree of unsaturation.

Lyso phosphatidylcholines do not form inverse non-lamellar phases owing to the reduced hydrophobicity of the hydrocarbon chain region relative to the diacyl compounds, they form micellar solutions at water contents greater than 60–70 wt% \[100,101\]. With reducing water content, they tend to form firstly the normal \( H_{I} \) hexagonal phase, then a bicontinuous cubic phase, followed by the fluid bilayer \( L_{n} \) phase, in the case of 1-palmitoyl PC. A further type I cubic phase is observed between the micellar solution and \( H_{I} \) \[101\]. The ordered mesophases can be induced to form in the presence of an excess solution, if a large molecular weight polymer is added to reduce the water activity, thereby effectively dehydrating the system \[339\]. Lyso phosphatidylethanolamines have been found to adopt either \( L_{n} \) or \( H_{II} \) phases, depending on the degree of unsaturation. For 1-oleoyl-PE, having a monounsaturated, \( C_{18} \) chain, the \( L_{n} \) phase was formed in the presence of excess water. For the di- and tri-unsaturated linolenoyl and linolenoyl lyso compounds, on the other hand, an \( H_{II} \) phase was observed at 0 °C \[340\].

Removal of a single one of the four acyl chains from cardiolipin is sufficient to prevent formation of the \( H_{II} \) phase \[297\], observed for cardiolipin at high salt concentrations \[296\]. Removal of a second chain induces formation of a micellar solution, which then transforms to a lamellar phase with increasing salt.

**V-C Solvent and solutes**

**Hydration**

In general, low hydration tends to favour the formation of inverted non-lamellar phases provided that a fluid-ordered chain transition does not intervene \[3,25,108,157\]. Note that this does not imply that the \( H_{II} \) phase is always less hydrated than the lamellar phase. The limiting hydrations of the \( H_{II} \) phase for saturated phosphatidylethanolamines are in general greater than those for the \( L_{n} \) phase formed at lower temperatures \[108\].

Dehydration can be brought about either by vapour pressure (drying), by osmotic or hydrostatic pressure, or by freezing the water \[254\]. It was suggested that lipids of dried biological membranes might adopt the \( H_{II} \) phase \[341\], and this has now been observed in certain systems upon drying. For example, muscle microsomes were found to undergo lateral phase separation of the lipid and protein components upon dehydration, and domains of \( H_{II} \) phase were observed in the lipidic regions \[342\]. Similarly, \( L_{n}-H_{II} \) transitions were induced in the plasma membrane of protoplasts by freeze-induced dehydration \[343\]. Interestingly, osmotic dehydration of total lipids from rye-leaf plasma membranes induced \( H_{II} \) phase formation in non-cold-acclimatised, but not in cold-acclimatised, leaves \[344\]. It is known that anhydrobiotic organisms (i.e., those capable of surviving dehydration), such as brine shrimp cysts, produce carbohydrates such as trehalose and glycerol in response to dehydration, and it was suggested that this might be in order to protect against lipid phase transi-
tions which could destroy membrane integrity [342]. It has now been demonstrated that trehalose preserves membrane integrity on dehydration [345], and has been reported to inhibit (but see section on polar solutes below) the L_α-H_II transition in egg PE and DOPE [346].

**Polar, non-polar and amphiphilic solutes**

The effects of solutes on non-lamellar phase transitions will depend in general on where they partition. Solutes which are located in the aqueous region will change the solution properties, and can thereby exert indirect effects by modifying the hydration of the headgroups, or the strength of hydrogen-bonding in the headgroup region. Ionic solutes will in addition exert effects due to screening and/or binding to the lipid headgroups. Osmotic dehydration effects can in general be expected, and these should scale logarithmically with the change in the water activity coefficient due to the presence of the solute [347]. Another factor is a possible modification of the water structure by the solute. The presence of ‘structure-breaking’ or ‘structure-making’ solutes, by modifying the hydrophobic interactions with the lipid, will tend to disfavour, or favour, ‘inverted’ phases, respectively. Thus for hydrated soybean PE, the presence of structure-breaking solutes such as guanidine hydrochloride, urea or sodium thiocyanate (NaSCN) in the aqueous solution was found to stabilize the lamellar phase [348]. Because non-lamellar transitions tend to be of low-entropy, such effects can be quite large. For example, 1 M NaSCN increased the L_α-H_II transition temperature by more than 40 °C [348].

Solute which bind to the interface may be expected to make a negative contribution to the interfacial tension, and hence disfavour ‘inverted’ phases such as H_II, however, in some cases precisely the opposite effect may occur, if the solute binds to the lipid headgroups in such a way as to dehydrate them, and if this positive contribution to the interfacial tension outweighs the negative one arising from the solute adsorption.

Non-polar solutes which partition strongly into the hydrophobic region of the lipid phase will tend to increase the lateral pressure within the hydrocarbon interior, and hence will favour the formation of inverted non-lamellar phases. Another effect of non-polar solutes is to relieve possible packing stresses due to the requirement to entirely fill the hydrocarbon volume of the phase [91]. The different mechanisms by which alkanes and diacylglycerols promote the formation of inverted phases have been investigated by a combination of NMR and diffraction [349].

**Monovalent and divalent cations and pH**

Monovalent ions can induce lamellar-inverted hexagonal transitions in charged phospholipid systems, presumably mainly by screening the electrostatic interactions. For example, cardiolipin (diphosphatidylglycerol) buffered to pH 7 forms bilayers at low NaCl concentrations, but transforms to the H_II phase when the salt concentration is increased above 1.6 M [296].

However, monovalent ions also strongly reduce the L_α-H_II transition temperatures of fully hydrated zwitterionic phospholipid systems, such as the phosphatidylethanolamines, where screening cannot be invoked [155,157]. For example, Fig 5.1 shows the salt dependence of the chain-melting (T_c) and lamellar-hexagonal (T_h) transition temperatures for the C-14 ester- and ether-linked compounds DMPE and DTPE. Both systems show a strong dependence of T_h on concentration, the effect being larger for the ester-linked compound DMPE, than for the ether-linked DTPE. The plot shows that the reason why no H_II phase has been reported for DMPE in excess water is simply that T_h is inaccessibly high. For this lipid, T_h is reduced by approx. 7 °C per mol of NaCl. At first sight, one would tend to interpret these effects as due to ‘salting-out’, whereby the salt effectively dehydrates the phospholipid headgroups by reducing the water activity. However, the phenomenon is clearly more complicated, since the aqueous layer thickness of the L_α phase of PE actually increases with increasing salt. The addition of salt also modifies the phase behaviour of non-ionic lipids such as monoacylglycerols, causing the appearance of an H_II phase for monoelaidin, which in the absence of salt forms only lamellar and cubic phases [254].

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*Fig 5.1 Dependence of the gel-fluid (T_c) and lamellar-hexagonal (T_h) transition temperatures on NaCl concentration for fully hydrated DMPE and DTPE [157]. Reproduced with permission from Biochemistry (1983) 22, 1280–1289 Copyright (1983) American Chemical Society*
As we have seen in subsection V-A., lowering the pH induces L\textsubscript{a}-H\textsubscript{II} transitions in a variety of charged, fully hydrated phospholipid systems, such as phosphatidylserine, phosphatidylglycerol, phosphatidic acid and cardiolipin. However, varying the pH can also have striking effects on the phase behaviour of zwitterionic phospholipids. For phosphatidylethanolamines, it is not possible to produce unilamellar vesicles unless the ammonium group is (partially) deprotonated by increasing the pH above 8-9 [350,351]. The effect on a dispersion of such vesicles of imposing a pH-jump from 9.2 to 7 was to induce aggregation [324]. Furthermore, it is well documented that increasing the pH has the effect of reducing the chain-melting transition temperature \( T_c \), by as much as 25 °C for full deprotonation [352,353]. The effect on lamellar-nonlamellar transitions is the opposite [153,157,168]. For example, Fig. 52 shows the effect of pH on the C\textsubscript{12} ether-linked compound DDPE. Above pH 8.5, as the headgroup develops a negative charge, \( T_h \) increases extremely steeply [157]. This could in principle be accounted for by a combination of electrostatic effects, and weakened hydrogen-bonding. However, a striking finding was that \( T_h \) decreases at low pH, notwithstanding the fact that a net positive charge develops upon phosphate protonation, and that the hydrogen-bonding is also expected to become weaker in this case. The conclusion reached was that the dominant effect was a reduced hydration of the phosphate group upon protonation, favouring the formation of the non-lamellar phases.

The effects of divalent ions on the polymorphism of charged phospholipids are quite complicated, and often differ between calcium and magnesium. Such ions tend to bind stoichiometrically to the lipid headgroups, even at low concentrations, because the binding constants are quite high. For cardiolipin [292–295] and phosphatidic acid [288–291], both of which can have up to two negative phosphate group charges per molecule, calcium and other divalent ions tend to induce the H\textsubscript{II} phase to form, above the chain-melting transition temperature of the lipid (which tends to be strongly increased by binding of the ions). For phosphatidylserine, on the other hand, calcium stabilizes a highly ordered lamellar phase [285,354,355]. However, when the PS is mixed with other lipids, the H\textsubscript{II} phase can form in the presence of calcium. This tends to occur by phase separation of a crystalline lamellar calcium-phospholipid complex from the mixture [356,357], but in the presence of cholesterol, phase separation is inhibited, and the entire mixture adopts the H\textsubscript{II} phase [358,359].

The situation with regard to phosphatidylglycerol is less clear. One group reporting formation of an H\textsubscript{II} phase in the presence of calcium [287], and another group finding only lamellar phases unless the PG was mixed with PE, in which case an H\textsubscript{II} phase was induced [360].

For phosphatidylinositol, H\textsubscript{II} phase formation does not occur, and in mixtures with PE, it progressively stabilizes the bilayer phase until, beyond 20 mol%, no H\textsubscript{II} phase is observed, even in the presence of high concentrations of calcium [361].

**Polar solutes**

The presence in the aqueous solution of 50% (wt/vol) of the fusogen poly(ethylene glycol) has been reported to dehydrate various phospholipid mesophases, yet to increase the L\textsubscript{a}-H\textsubscript{II} transition temperature of soybean PE [362].

Cryoprotectants have been reported to inhibit the formation of the H\textsubscript{II} phase. The presence of trehalose at a concentration of 4 mol/mol lipid was found to increase the bilayer-hexagonal transition of hydrated dioleoyl PE by 20–30°C [346,363], the amphiphilic cryoprotectants trethoxycholesterol and trethoxycholesterol galactose were found to have similar effects at much lower ratios of 1.4 mol/mol [364]. However, these results have been disputed by subsequent work, which found that disaccharides such as sucrose, trehalose, lactose and maltose, and to a lesser extent sugar alcohols such as sorbitol and myo-inositol, actually decrease the L\textsubscript{a}-H\textsubscript{II} transition temperature of DEPE [365]. For example, 15 M solutions of sucrose and trehalose were found to decrease the transition temperature by 18°C. The reason for these diametrically opposed findings is not clear. The mechanism of action of the sugars probably involves binding by hydrogen-bonding to the non-esterified oxygens on the phosphate group of the lipid headgroup, resulting in an effective dehydration.

![Fig 52 pH Dependence of the gel-fluid (○) and lamellar-hexagonal (□) transition temperatures of DDPE in 2.4 M NaCl [157]. The dotted line indicates the appearance of additional non-lamellar (cubic) phases in the region of the transition.](image-url)
Non-polar solutes

The effect of addition of non-polar solutes on the polymorphism of hydrated phospholipids has only been studied relatively recently [92–94,220,366–368], although a vast literature exists on ternary phase equilibria in amphiphile/water/non-polar solute systems [37]. The most recent studies on phospholipids have been carried out primarily to test the model of Gruner and co-workers for the L$_\alpha$-H$_{II}$ phase transition (see subsection IV-E). The basic idea is that non-polar solutes can both increase the tendency for a negative mean curvature of the lipid monolayer, and, by partitioning into the interstices between the water/lipid cylinders, can also relieve stress in the H$_{II}$ phase caused by the necessity that all of the hydrophobic volume be filled. Addition of alkane is not entirely equivalent to increasing the phospholipid chainlength without adding alkane, since the alkane can diffuse freely within the hydrophobic region, whereas the phospholipid chains are partly constrained by being pinned at one end to the interfacial regions by the polar head groups.

In general, incorporation of C$_6$–C$_{20}$ n-alkanes promotes the formation of non-lamellar phases such as H$_{II}$. Thus, for example, incorporation of approx. 12 mol% dodecane (C$_{12}$ n-alkane) lowers the L$_\alpha$-H$_{II}$ transition temperature of fully hydrated egg PE by 18°C [366]. For a given total number of added carbon atoms, addition of alkane is more effective than increasing the phospholipid chainlength in reducing the L$_\alpha$-H$_{II}$ transition temperature [220]. The effectiveness of added alkane decreases with increasing chainlength of the alkane, at constant added carbon atoms [94,368]. For pure PE systems with a strong tendency to form H$_{II}$ phases, the effects are relatively small. However, for mixtures of PE with added PC, which has the effect of strongly stabilizing the lamellar phase, dramatic effects are observed. For example, addition of 5% dodecane to a 3:1 DOPE/DOPC mixture reduced the L$_\alpha$-H$_{II}$ transition temperature by 55°C [92].

Alkanes can even induce cubic and H$_{II}$ phases in pure PC systems, when the amount of incorporated alkane is of the order of 8 wt% or greater, depending on temperature, water content, and chainlength of the alkane [93,94,368]. At a fixed water content and alkane chainlength, an increasing amount of alkane is required to induce non-lamellar phases in PCs which are of increasing saturation. An intriguing finding of these workers is that in these systems, a phase sequence H$_{II}$–L$_\alpha$-H$_{II}$ can apparently be induced by dilution with water, at constant temperature and constant alkane/PC molar ratio. It is not yet clear whether these two H$_{II}$ regions are in fact connected towards the pure alkane corner of the ternary phase diagram.

Amphiphilic solutes

Although fully protonated free fatty acids do not form mesophases in the presence of water, partially deprotonated systems do [369–371]. Such acid-soaps may be formed by pH titration of micellar solutions of soaps. Although the pK of the carboxyl group of a fatty acid monomer has a value close to 4.6, the value in aggregated structures is much larger, being in the region of 7.4, both for pure fatty acids [371] and for fatty acid/PC 2:1 mixtures [372]. Acid-soaps may form mesophases in the presence of a large excess aqueous phase, and can also adopt inverse non-lamellar phases. For example, potassium hydrogen dioleate forms an L$_\alpha$ phase at high hydration, and a pure H$_{II}$ phase below about 40 wt% water [371]. This emphasizes the point that many single-chained lipids can form inverse phases, under certain conditions.

Unsaturated fatty acids are known to be fusogenic agents, and so it was of great interest when it was found [263,373] that their incorporation in phospholipid bilayers, or in natural (erythrocyte) membranes, tends to induce the formation of an H$_{II}$ phase, as well as promoting fusion. Retinol and monoolein were found to have similar effects on erythrocyte membranes. The effect of alcoholysis on hydrated egg PE membranes was found to be to stabilize the L$_\alpha$ phase for short chains (ethanol, butanol), but for longer chains to tend to promote H$_{II}$ phase formation [366].

It has been shown for some time that incorporation into PC bilayers of long-chain fatty alcohols, acids or amines, all of which have the capacity for hydrogen-bonding to the PC phosphate group, has the effect of raising the gel-fluid transition temperature [374–378]. It has been shown that this effect also occurs with PG, but not with PE [379]. For the case of hydrated fatty acid/phosphatidylcholine mixtures, it was found that they form stoichiometric 2:1 mol/mol complexes, with sharp calorimetric transitions at temperatures some 20°C greater than that of the pure phosphatidylcholine component [377]. The pseudo-binary phase diagrams (in excess water) for a number of DPPC/fatty acid systems were subsequently reported [380]. However, it was subsequently found that addition of stoichiometric 2:1 mol/mol amounts of palmitic acid to DPPC actually completely suppresses the fluid bilayer phase, and leads to the formation of an H$_{II}$ phase, in equilibrium with excess buffer, for pH values less than approx 6 [381]. An apparent discrepancy exists between these findings and carboxyfluorescein trapping experiments, which indicated no effect on membrane structure at fatty acid/PC ratios of up to 4:1 [382]. However, the formation of the H$_{II}$ phase by 2:1 fatty acid/PC mixtures has now been fully confirmed [259,372,383], and has
been shown to be a general property of all fatty acid/PC mixtures, for chainlengths greater than C₁₄ (Seddon, J. M., Cevc, G. and Marsh, D., unpublished data) An interesting finding is that for the C₁₂-chamlength mixtures, a bicontinuous cubic phase is formed (Seddon, J. M., unpublished data)

Incorporation of the fusogen monoleen into bilayers tends to promote formation of non-lamellar phases such as H₁₁ [373,384,385] and bicontinuous cubic phases [386] This behaviour is not entirely unexpected, since pure monoacylglycerols themselves have a strong tendency to form inverse phases such as H₁₁ and bicontinuous cubic phases, in the excess water region [111,386, 387]

The effects of diacylglycerols on lipid membranes is of particular interest because they are produced during the turnover of phosphatidylinositol, and act as second messengers, evoking a number of cellular responses, such as the specific activation of protein kinase C [388] An important finding is that diacylglycerol promotes the activity of the intracellular phospholipases A₂ and C, apparently by its perturbing effect on the membrane structure [389,390] A similar effect was induced by cardiolipin or MGDG [391] This result appears to be correlated with the finding that diacylglycerols are potent promoters of non-lamellar phases in various phospholipid systems, such as PE, PC and PS [390,392, 393] Upon incorporation of as little as 1 mol% diolein or dilinoleen, the L₁-H₁₁ transition temperature of fully hydrated palmitoyl-oleoyl PE is lowered by approx 9°C [367,394] Interestingly, these structural effects do not seem to be related to changes in interbilayer interactions the addition of 12.5 wt% diacylglycerol to PC, or 4 wt% to PE, does not appear to affect the bilayer thickness, interbilayer force, or the interbilayer separation [393]

When the amount of diacylglycerol incorporated into PC is very large (approx 70 mol%), the H₁₁ phase is transformed into a cubic phase, identified as Pn₃m [393] However, this identification has been disputed, it being claimed that the probable space group is Fd₃m (O²⁻) [Seddon, J. M., manuscript in preparation]

The very long-chain (= C₉₀) polyisoprenoid lipids dolichols, and their phosphorylated derivatives, are potent in promoting the H₁₁ phase [91,395–397] In the case of fully hydrated 3:1 DOPE/DOPC mixtures, incorporation of 2 wt% dolichol reduced the L₁-H₁₁ transition temperature by 50°C [91] Both dolichol and dolichyl phosphate were found to enhance DOPE/DOPC vesicle fusion, in a concentration- and temperature-dependent way, and for 2:1 DOPE/DOPC mixtures, incorporation of 1–5 mol% dolichyl phosphate induced H₁₁ phase formation at 20°C [398] For these latter samples, heating to 70°C led to the appearance of an isotropic ³¹P-NMR signal, which remained on cool-
Cholesterol was found to promote $\text{H}_{11}$ phase formation in glucolipid mixtures extracted from A. laudanum [410]. For this system, the ability of various sterols to affect cell growth and lipid headgroup composition was correlated with the lipid phase behaviour [411]. Under certain circumstances, changes to the biosynthetic ratio of MGDG/DGDG did appear to be correlated with the appearance of an $\text{H}_{11}$ phase in the extracted mixtures, and certain sterols induced non-lamellar phases in vivo mixtures.

Cholesterol has been found to enhance the activity of sarcoplasmic reticulum Ca-ATPase, reconstituted into PE/PC vesicles [10]. It was suggested that the enhancement was due to the tendency for bilayer destabilization, notwithstanding the fact that non-bilayer structures were not actually induced in the proteoliposomes by the cholesterol.

**Anaesthetics and drugs**

Local anaesthetics can have a variety of effects on lipid polymorphism. Dibucaine and chlorpromazine were found to induce an $\text{L}_{\alpha}$-$\text{H}_{11}$ phase transition in cardiolipin hydrated at close to neutral pH, probably by charge neutralization [293]. For phosphatidic acid systems, chlorpromazine was effective in inducing $\text{H}_{11}$ phase formation at pH 6, but not pH 4 [290]. On the other hand, dibucaine was found to reverse the calcium-induced $\text{L}_{\alpha}$-$\text{H}_{11}$ transition in PE/PS mixtures [412], and various such charged anaesthetics stabilize the bilayer phase of PE systems [366].

The general anaesthetic etomoxir lowers the $T_{c}$ of DPPC by 10 °C at a lipid drug molar ratio of 5·1, and induces the formation of the $\text{H}_{11}$ phase, apparently by dissolving in the hydrocarbon chain region [413].

Lipidosis in rats, induced by the cationic amphiphilic drug chlorpromazine, was found to cause the appearance of cytoplasmic inclusions of $\text{H}_{11}$ phase in the retinal pigment epithelium (see subsection VI-A) [414].

The cationic surfactants $N$-alkyl trimethylammonium iodide have a variety of effects on biological membranes, which depend strongly on the chainlength of the surfactant, it has been found that this correlates with the appearance of non-lamellar phases in model surfactants/phospholipid systems [415].

Anisodamine, a herbal drug which is used to treat toxic shock, induces the formation of $\text{H}_{11}$ phase in cardiolipin liposomes [416].

The antineoplastic drug adriamycin inhibits the formation of non-lamellar phases in cardiolipin/PC (1·1) model membranes [417], but induces $\text{H}_{11}$ phase formation in cardiolipin/PE (1·2) and PE/PS (1·1) mixtures [418].

The mechanisms by which these anaesthetics and drugs exert their effects on lipid polymorphism probably span all of the effects discussed at the beginning of this section.

**Peptides and proteins**

Most of the work on the effects of proteins on lipid polymorphism has been carried out by Gulik-Krzywicki and co-workers [12,122,419] and by De Kruijff and co-workers (reviewed in Ref. 18 and 420). It has been suggested that protein insertion and transport across membranes may involve non-lamellar lipid structures [18,421].

The effects of proteins and peptides will obviously be strongly dependent upon whether they are water-soluble, and bind to the lipid headgroups (extrinsic), or whether they become integrated within the lipid region of the phase (intrinsic). Certain proteins such as cytochrome $c$ bind electrostatically, specifically to negatively charged lipids, and then become partially inserted into the lipid hydrocarbon chain region. In general, it is by no means straightforward to predict what the effects on the polymorphism will be. Incorporation of peptides and proteins sometimes stabilizes the lamellar phase, but sometimes induces the $\text{H}_{11}$ phase, or other non-lamellar structures, to form.

An example of a polypeptide which binds electrostatically to the lipid headgroups is poly(L-lysine), which stabilizes the lamellar phase of cardiolipin in the presence of calcium, but induces $\text{H}_{11}$ phase formation in cardiolipin/PE mixtures [422,423]. The latter effect is due to a phase separation of the lipid components $\text{H}_{11}$ phase formation was induced in dispersions of phosphatidic acid by myelin basic protein [424].

Small amphiphilic peptides such as cardiotolins and melittin interact both electrostatically and hydrophobically with membranes, penetrating the hydrocarbon interior. The effect of snake venom cardiotoxin on axonal lipids was to induce the appearance of an $\text{H}_{11}$ phase [425]. A study of cardiotoxin/cardiolipin found $\text{H}_{11}$ phase formation under certain conditions [426]. Similarly, melittin induces $\text{H}_{11}$ phase formation in cardiolipin membrane models [427]. Virus replication inhibitory peptides were found to raise the $\text{L}_{\alpha}$-$\text{H}_{11}$ transition temperature of DEPE (up to 10°C at 0.1 mol%), the magnitudes of the shifts being correlated with the potency of the anti-viral action [428]. However, the effects of other anti-viral agents such as cyclosporin A were more complex [429].

The mitochondrial protein cytochrome $c$ binds electrostatically to charged lipids, then inserts into the hydrophobic region. It was found to induce $\text{H}_{11}$ phase formation in extracted mitochondrial lipids [419], and in cardiolipin itself, but not in PC, PE, PG or PS [430]. On the other hand, the precursor molecule apocytochrome $c$ does not induce the $\text{H}_{11}$ phase to form in either cardiolipin or PS [431].

Very interesting behaviour was found by Gulik-Krzywicki in the ternary system cytochrome $c$/monoaoylglycerol/water, and a structural analysis of this system has been performed [12]. For hydrated mono-
OLEIN, incorporation of increasing amounts of cytochrome c was found to convert the Pn3m (Q2 \textsuperscript{24}) cubic phase to the Im3m (Q2 \textsuperscript{29}) cubic phase. On reducing the water content, whereas the pure lipid/water system undergoes a transition to an Ia3d (Q2 \textsuperscript{20}) cubic phase, the system with incorporated cytochrome c transforms to a P4 \textsubscript{1}22 (Q2 \textsuperscript{12}) cubic phase. This is a fascinating result, because this space group is chiral, and this is the first example of a non-centrosymmetric lyotropic cubic phase.

In terms of the effects of more hydrophobic peptides or proteins on lipid polymorphism, one of the most extensively studied systems has been that of the pore-former gramicidin [420,432–441]. Addition of gramicidin to various fully hydrated synthetic lipid bilayers, for example DOPC, induces the formation of hexagonal H \textsubscript{11} phase when the gramicidin/phospholipid molar ratio exceeds 1:10. The tryptophan residues seem to play an important role [438]. The presence of gramicidin has little effect on the hydrocarbon chain order in the H \textsubscript{11} phase (although it does at lower hydrations, where the lamellar phase is adopted), but it restricts the fast chain motions [439].

Incorporation of the intrinsic membrane glycoprotein, glycoporphin, into either PE [442] or cardiolipin [443], has the effect of preventing the L \textsubscript{α}-H \textsubscript{11} transition. Similarly, cytochrome oxidase [444] and chlorophyllase [445] have the effect of stabilizing the lamellar phase (but it has been reported that chlorophylls a and b tend to induce formation of the H \textsubscript{11} phase in lipid mixtures [446]).

V-D Lipid mixtures

Biological membranes of course contain complex mixtures of lipids, and so it is of great importance to understand the polymorphic phase behaviour of such mixtures, in well-defined model systems. It should be noted that an additional complexity exists in biomembranes, in that the lipid composition is normally asymmetric across the bilayer.

Generally speaking, for an H \textsubscript{11}-forming system, mixing in a ‘bilayer forming’ lipid has the effect of tending to stabilize the lamellar phase. Thus for unsaturated PE systems, addition of PC has the effect of stabilizing the bilayer structure [92,220,394,402,407–409,447–450]. In many cases, isotropic \textsuperscript{31}P-NMR signals were seen for certain composition ranges, and freeze-fracture electron microscopy showed arrays of lipidic intramembranous particles. It is very likely that these are associated with the appearance of a cubic phase, observed for various unsaturated PE/PC mixtures containing in the region of 5–50 mol% PC [448, 449,451]. It is interesting to note that evidence of an intermediate non-lamellar phase has been found in mixtures of a trans- with a cis-unsaturated PE [336]. For mixtures of PE with charged phospholipids such as PE [356,357,394], PG [360] and PI [361], the picture is more complicated, as effects due to charge come into play, rendering these mixtures very sensitive to pH, and univalent and divalent ions. In general, Ca\textsuperscript{2+} ions (but usually not Mg\textsuperscript{2+}) tend to induce the appearance of an H \textsubscript{11} phase in such mixtures, sometimes with phase separation of the lipid species, but sometimes without. The presence of cholesterol tends to inhibit this phase separation [359]. For PE/PS mixtures, lowering the pH can induce an L \textsubscript{α}-H \textsubscript{11} phase transition [356].

For equimolar mixtures of PC with cardiolipin, a cubic phase can be induced by low levels of calcium [452]. For glucolipids extracted from A. laevis, the bilayer configuration is stabilized by addition of DGDG to MGDG, the latter forming the H \textsubscript{11} phase on its own [410].

A total lipid extract from maize chloroplasts, containing a mixture of phospholipids and glycolipids, was found to form an H \textsubscript{11} phase at low water contents [453]. The extracted galactolipid mixture, containing predominantly MGDG, was found to form an H \textsubscript{11} phase between −20 and +100 °C at low water contents, and a cubic phase (Q2 \textsuperscript{20}, spacegroup Ia3d) at larger hydration, at temperatures above 60 °C. The ternary phase equilibria of MGDG/DGDG/D\textsubscript{2}O for galactolipids extracted from chloroplasts were studied by \textsuperscript{2}H-NMR, and it was found that for MGDG/DGDG mole ratios of 1.2 and 2.1, an H \textsubscript{11} phase formed at very low hydration, which transformed to an Ia3d cubic phase at water contents in the region of 3–5 mol/mol lipid [454].

VI. Implications for biomembrane structure and function

VI-A Non-lamellar structures in membranes

Many natural membranes are rich in lipids which have strong tendencies to form H \textsubscript{11} phases, and considerable effort has been spent on trying to detect such structures in biological systems.

Chemical fusogens were found to induce the formation of an H \textsubscript{11} phase in erythrocyte ghosts [263,373]. Isotopic \textsuperscript{31}P-NMR signals were observed from microsomes (endoplasmic reticulum membranes) isolated from liver cells, implying that some kind of non-lamellar structures were present [455–457]. It has been found that partial dehydration of microsomal membranes leads to the appearance of an H \textsubscript{11} phase [342].

Mitochondrial inner membranes are particularly rich in cardiolipin which forms the H \textsubscript{11} phase in the presence of low levels of divalent ions [292]. It was suggested that lamellar fusions induced in the inner mitochondrial membrane by Mg\textsuperscript{2+} ions have a structure consisting of inverse lipid cylinders embedded between two monolayers [458]. This idea was supported by subsequent
work, which found evidence for non-lamellar structures in a total mitochondrial lipid extract [459]. Furthermore, it was found that addition of millimolar amounts of Mn\(^{2+}\) to intact mitochondria causes the appearance of regions of H\(_{II}\) phase [460].

Paracrystalline inclusions of H\(_{II}\) phase lipid domains were observed in frog retinal rod outer segments (ROS) by freeze-fracture electron microscopy [127] (see Fig. 13). A previous study had found H\(_{II}\) phase cytoplasmic inclusions in rat retinal pigment epithelium under conditions of drug-induced lipidosis [414]. Non-lamellar structures were detected by \(^{31}\)P-NMR in the extracted lipids of bovine ROS, but not in the intact photoreceptor membrane, and this was suggested to imply a role of rhodopsin in stabilizing the bilayer structure [461]. However, a later study found no evidence for non-lamellar structures in the extracted ROS lipids [462]. The discrepancy may be due to a difference in the concentration of divalent ions present in the buffers, since millimolar amounts of Ca\(^{2+}\) were found to induce L\(_{a}\)-H\(_{II}\) transitions in extracted ROS lipids [463].

It has been proposed that the structure of tight junction strands consists of pairs of lipid cylinders formed within linear fusion regions between adjacent cell membranes [464-466]. Similarly, contact sites between the inner and outer membrane of gram-negative bacteria may also involve non-lamellar lipid structures [467].

Detailed arguments have been presented which suggest that the plasma membrane of the archaeabacterium Sulfolobus solfataricus is based upon the bicontinuous cubic phase Q\(^{224}\) (Pn\(_{3m}\)) [81].

It is interesting to note that human hybridoma lupus anticoagulants, which are a class of anti-phospholipid antibody, appear able to distinguish between lamellar and hexagonal phospholipid phases [468].

A role has been suggested for the involvement of non-lamellar phases in artificial pulmonary surfactant systems [469].

**Lipidic particles**

Electron micrographs of membrane systems containing lipid components with a tendency for non-lamellar phase formation frequently exhibit so-called 'lipidic particles' (reviewed in Ref. 124), globular objects embedded within the lamellae, whose diameters are of the order of 60-130 Å [129-132,264,290,293,447,470,471]. These particles were initially thought to represent inverted micelles within a single bilayer, however, it is now believed that they represent inverted micelles formed between two apposed bilayers. A third possibility, that they represent fused cusps between apposed bilayers, has been suggested [472,473], but this is now considered to be energetically unlikely [134].

In some cases, the lipidic particles seen by electron microscopy may actually correspond to cubic phases, whose structures have only been partially preserved by the freezing procedure [451,474].

It was suggested that lipidic particles are involved in membrane fusion [130], the mechanism of the L\(_{a}\)-H\(_{II}\) transition [131] and transbilayer transport [14] It was found that the formation of lipidic particles correlates with a loss of permeability of cardiolipin/PC bilayers, upon addition of calcium [475]. The model of Siegel (see subsection IV-H) attempts to assess and quantify these ideas.

**VI-B Membrane fusion**

**Molecular mechanism of membrane fusion**

It was first suggested by Lucy that the mechanism of fusion might involve a phase change in the lipid, although he envisaged the formation of normal micelles at the fusion sites [476]. Subsequently, it was proposed that the formation of inverted micelles might drive the fusion process [263,477-480]. The implications of non-lamellar structures for membrane fusion have been discussed by a number of authors [14,17-19,68,71,82-85,87,88,124,133,373,481-484]. It has been reported that fusion provides a mechanism for the transbilayer redistribution of PE in vesicles [485]. It is now recognized that there are at least three distinct steps involved in the molecular mechanism of fusion: membrane aggregation, bilayer destabilization, then merging of the membrane (and aqueous) components [486,487]. Non-bilayer lipid phases may be indirectly involved in each of these steps.

Before fusion can occur, it is obvious that the membranes must be brought into close apposition. However, to do this, even for neutral systems, strong repulsive hydration forces [488] must be overcome. For example, the work required to bring two 300 Å unilamellar vesicles to within a separation of 13 Å has been estimated (from hydration force measurements on phospholipids) to be of the order of 10 \(kT\) (25 kJ·mol\(^{-1}\) at 37°C), corresponding to a statistical weighting (exp\([-E/kT]\)) of \(10^{-4}\) against this mutual approach [279,489]. It is very likely that this hydration force must be reduced in some way before fusion can occur, and we must consider what mechanism might effect this reduction. One possibility is that partial phase separation of the lipid component may occur, with accumulation of more weakly hydrated lipids (such as PE) in the fusion region [71]. Furthermore, in addition to promoting aggregation by lowering the hydration repulsion, this could also have the effect of destabilizing the bilayer, since many of these lipids either tend to form non-lamellar phases themselves, or promote their formation when present above certain concentrations. Lucy and Akhong have proposed a model for fusion, where osmotic pressure is viewed as the crucial step driving...
the bilayer destabilization, possibly in conjunction with non-lamellar structures [490].

In the bilayer destabilization step, it is likely that the primary event is the formation of inverted micelles (IMI) between apposed bilayers (see subsection IV-H and Figs 45 and 46). In order for fusion to occur, the IMI must then fuse with the outer monolayers, transiently forming interlamellar attachments (ILA), i.e., channels between the bilayers. These channels then expand, allowing a merging of the aqueous contents on either side of the original bilayers, without mixing with the aqueous layer which was initially between the membranes.

There is now strong evidence that non-lamellar structures are indeed involved in the molecular mechanism of membrane fusion [491].

Freeze-fracture electron microscopy of fused vesicles showed the appearance of "lipidic particles" localized in the fusion regions, supporting the idea that inverted micelles do form transiently during fusion [130,131]. A recent time-resolved electron microscopy study claims to have visualized interlamellar attachments (ILA) formed during membrane fusion [492].

The external monolayer of synaptic vesicles — a naturally fusing system — was shown to be enriched in polyunsaturated PE and PS, and this was proposed to be relevant to the mechanism of fusion, possibly involving non-lamellar structures [493].

Creutz has shown that oleic acid induces the fusion of chromaffin granules, aggregated by synexin, at 37°C [494]. It was shown that incubation of granules with vesicles containing 'HII-phase forming' phospholipids led to fusion and release of granule contents upon addition of calcium [495].

It was found [263,373] that addition of fusogenic agents such as unsaturated fatty acids to phospholipid bilayers, or to natural (erythrocyte) membranes tends to induce the formation of an HII phase as well as promoting fusion. However, as discussed below, unsaturated fatty acids can actually stabilize the bilayer structure of PE systems, at physiological pH values. It has been demonstrated that physiological levels of diacylglycerol can promote the formation of inverted phases, and also induce membrane fusion [496].

Fusogenic liposomes

A number of authors have investigated the effects of pH on aggregation and fusion of phospholipid vesicles. This idea of pH-sensitive liposomes is of interest for liposomal drug delivery, since pathological tissues have lowered local pH environments [497].

It was shown that PE vesicles buffered at pH 9.2 rapidly aggregate when the pH is dropped to 7 [324].

PS and PS/PE vesicles were found to fuse when the pH was lowered to below 3.5 [498,499], or in the presence of calcium [499–502].

Although PI vesicles aggregate in the presence of calcium, it was shown that the presence of PE was necessary for fusion to occur [503]. However, it was suggested that the fusion was facilitated by a dehydrating effect of the PE component, rather than by the tendency of the PE to form the HII phase.

DOPE vesicles, stabilized by incorporation of the charged PE analogue N-succinyl-PE, were induced to fuse by lowering the pH from 7 to 4 [504].

Dolchyl phosphate induces fusion in DOPE/DOPC vesicles, and this correlates with the appearance of lipidic particles, and with HII phase formation [398].

For PE vesicles stabilized by incorporation of oleic acid (7.3 mol ratio), rapid aggregation and fusion were induced by lowering the pH to below 6.5 [505]. This effect was not observed for PC/oleic acid (7.3 mol ratio) vesicles, indicating that the presence of the PE component was necessary.

Similarly, palmitoylhomocysteine/PE vesicles fuse when the pH is lowered below 7 [506].

Cytochrome-c oxidase proteoliposomes containing 30 mol% cardiolipin and varying proportions of PC and PE were found to undergo calcium-induced fusion, but only for PE contents greater than 10% [507]. A similar effect was found for the protein-free system, the implication being that the fusion required the presence of sufficient amounts of 'HII phase-forming' lipids.

The role of non-lamellar structures in promoting destabilization and fusion of liposomes has been studied in detail by Ellens, Bentz and co-workers in systems consisting of PE [483], PE/cholesterol succinate [481,482,508], and N-methyl-DOPE [484,491] PE liposomes stabilized by incorporation of the charged cholesterol ester cholesteryl hemisuccinate were found to undergo fusion with release of their contents, on lowering the pH to 4.0. Analysis of the kinetics of release showed that bilayer destabilization occurred subsequent to aggregation. The conclusions that emerge are that aggregation, destabilization, and leakage can occur well below the Lc–HII transition temperature. Increasing the temperature to the transition caused a massive increase in the rate of leakage. However, the mixing of the vesicle contents was diminished above the transition temperature, due to a tendency for the system to form domains of HII phase. For the N-methyl-DOPE system, the rate of fusion (defined in terms of mixing of the aqueous contents) is maximal [484] in the temperature range where isotropic NMR signals are observed [316], then decreases at higher temperatures, where the HII phase is formed. Within this intermediate temperature range, it is now known that the bicontinuous inverse cubic phases, Im3m (Q^22) and Pn3m (Q^24), are formed [109].

Although calcium ions have been shown to induce aggregation and fusion of didodecyl phosphate [509,510], and didodecylmethylammonium bromide vesicles...
[511,512], only in the former case was \( H_{II} \) phase formation observed, and then only after fusion of small vesicles into larger ones had occurred.

It is known that incorporation of small amounts of saturated fatty acids (2–4%) induces fusion in PC vesicles [512]. When the molar ratio of fatty acid to PC is increased to 2.1, direct gel-\( H_{II} \) transitions are observed [381] (see subsection V-C) The phase and colloidal behaviour of stoichiometric 2.1 fatty acid/PC mixtures have been studied [372]. It was shown that such liposomes undergo rapid aggregation and fusion when subjected to a fast pH jump from 10 to \(< 4\), when the temperature was close to or above the gel-\( H_{II} \) transition temperature.

The technique of electrofusion, whereby membranes are induced to fuse by application of short-lived electric field pulses has been developed by Zimmermann and co-workers [514–518], and is described briefly in subsection VI-H.

**VI-C Membrane protein activity**

There is evidence which suggests that the activity of membrane proteins is in some way dependent upon the phase preference of the lipid component.

The \( \text{P}^{31} \)-labelled ATP exchange activity of mitochondrial proteins reconstituted into lipid vesicles was found to be enhanced by the presence of a large proportion of PE in the vesicle lipids [8]. Similarly, it has been found that the presence of lipids which tend to form non-lamellar phases, such as PE and monogalactosyldiacylglycerol, enhances the calcium transport of reconstituted Ca-ATPase vesicles [9]. A similar enhancement was observed in the presence of unsaturated, but not saturated, monoacylglycerols [519]. Furthermore, the transport is stimulated by the presence of cholesterol [10], which tends to induce \( H_{II} \) phase formation in the pure lipid mixtures. For mannosyltransferase II reconstituted in the presence of phospholipids, optimal activity was also found in the presence of PE [395,520]. The activity was reduced by dilution with PC, but could then be restored by incorporation of dolichol, which destabilizes the bilayer.

In reconstitution experiments with erythrocyte membrane proteins, it was found that incorporation of the protein into PC/PE vesicles was maximal at a lipid ratio of 1 3 [521]. It was pointed out that this ratio is where the highest density of lipidic particles is seen by electron microscopy, and is in the region where cubic phases are observed.

Spin-label studies of reconstituted lipid/cytochrome-c oxidase complexes [522] have shown that there is a specific, preferential interaction with cardiolipin, compared with other phospholipids. Cardiolipin has a strong tendency to form the \( H_{II} \) phase, either in the presence of millimolar concentrations of divalent cations [292], or in molar concentrations of univalent ions or at low pH [296].

In reconstituted rhodopsin systems, it has been found that optimal photochemical activity occurs for an equimolar mixture of PE and PC [523], and this was suggested to be because the lipid is close to a hexagonal or cubic phase.

**VI-D Bioregulation of membrane lipid phase properties**

There is ample evidence that cells respond to changes in their physicochemical environment (temperature, pressure, pH, salt concentration, etc.) by adjusting the lipid composition of their membranes in such a way as to maintain optimal stability and function. Most membranes have highly asymmetric lipid compositions [524,525], and this has profound implications for the possible role of lipid polymorphism in modulating the membrane structure [15,19]. It has been suggested that cells exhibit ‘homeoviscous adaptation’, whereby the property they control is the membrane fluidity [526]. However, more recent work suggests that the critical parameter is the ratio of ‘bilayer-forming’ to ‘non-bilayer-forming’ lipids, and that cells seek to homeostatically control the net intrinsic curvature of the individual bilayer leaflets of their membranes [91].

The first evidence for this concept was the finding that the organism *Pseudomonas fluorescens* responds to a lowering of its growth temperature by increasing the proportion of unsaturated chains in its phospholipids, and that this lowers the \( L_{\alpha}-H_{II} \) transition temperature [527]. It was suggested that a biological mechanism exists for regulating the lipid phase behaviour. This idea has been considerably extended and developed by the elegant work of Wieslander, Lindblom, Rolfors and co-workers, using the mycoplasma *Acholeplasma laidlawii* (see Ref. 528 for a review of the plasma membrane structure and function) as a test system [410,411,529–533]. They have produced a large body of data on the effects of varying temperature, and incorporation of fatty acids, sterols, anaesthetics, alkanes, alcohols and detergents into *A. laidlawii* membranes, providing strong evidence for the importance of bioregulation of lipid phase behaviour. Related studies employing the system *Clostridium butyricum* lend further support to this concept [534–537].

**VI-E Photosynthetic membranes**

The molecular organization of photosynthetic membranes, and the possible roles of non-lamellar structures, have been reviewed a number of times [538–542].

Plant photosynthetic membranes contain 35 wt% acyl lipid, of which 70% consists of the polyunsaturated (predominantly dihlinolenyl, 18 3\(_n\)) glycolipids monogalactosyldiacylglycerol (MGDG) and dигалактосидиацилглицерол (MGDG).
acylglycerol (DGDG) [540]. The former lipid, MGDG, which is in fact the most abundant polar lipid species in Nature [543], forms the inverted hexagonal \( H_{II} \) phase at all temperatures between 0 and \( 80 \degree C \) and at all compositions between 90 wt\% lipid and excess water [305]. DGDG and the charged glycolipid sulphoquinovosyldiacylglycerol, on the other hand, form only lamellar phases over this range of conditions. A hydrated galactolipid extract from maize chloroplasts was found to form the inverted hexagonal phase over a wide range of temperature, but only at water concentrations less than 15 wt\% [453]. At slightly higher water contents, an \( \text{Ia}3\text{d} \) cubic phase was observed at high temperatures. A more recent study of mixtures of MGDG and DGDG extracted from wheat found that a bicontinuous \( \text{Ia}3\text{d} \) cubic phase was formed between the \( L_{\alpha} \) and \( H_{II} \) phases, with varying water content [454].

The effect of chlorophyll on the lipid phase behaviour is somewhat unclear. Incorporation of more than 20-30 mol\% of chlorophyll \( a \) was found to tend to inhibit \( H_{II} \) phase formation in hydrated MGDG [544]. However, another study found that chlorophyll \( a \) and \( b \) induce formation of the \( L_{\alpha} \) phase in MGDG/PG and MGDG/DGDG/PG mixtures [446].

Non-lamellar structures have been observed by electron microscopy in galactolipid extracts from chloroplasts [470,545,546]. For total lipid extracts, non-lamellar structures were observed to form either by lowering the pH, or by addition of divalent ions [547]. For native thylakoid membranes, non-lamellar structures are not normally observed, but can be induced by heating [546,548,549], or by lowering the pH [550]. The degree of unsaturation of the acyl chains was found to be important in controlling the appearance of the non-lamellar structures [551].

From study of the ternary phase equilibria of wheat chloroplast MGDG/DGDG/\( D_{2}O \), it was suggested that the observation of lipidic particles in freeze-fracture electron micrographs of aqueous dispersions of such mixtures can sometimes be artefactual [454]. In fact, it has been pointed out that many of the published micrographs showing non-lamellar structures, probably represent partially preserved cubic phases, rather than inverted micelles per se [451]. It is interesting to note that the prolamellar body of etiolated chloroplasts has a structure [552,553] which is strikingly similar [454] to that of the \( \text{I}m\text{3}m \) bicontinuous cubic phase (although not identical).

**VI-F Biopolymers**

It is becoming clear that lyotropic liquid crystals and non-lamellar phases may have a far more general biological significance than their role in membranes. Any biological polymer, such as DNA, actin, myosin, or collagen may potentially form lyotropic liquid crystalline mesophases in the presence of an aqueous solvent. DNA may be packaged very tightly in vivo, for example in sperm heads or virus capsids. The concentration may approach 70\% (w/v) (see Ref 554 for references). At these high concentrations, liquid-crystalline phases are formed [554]. In addition to a lyotropic cholesteric phase (the DNA is chiral), at slightly higher concentrations a phase was observed which was interpreted as being smectic. This is almost certainly a two-dimensional hexagonal phase, similar to the normal hexagonal \( H_{I} \) phase formed by many monoacyl lipids. Parsegian and co-workers have in fact performed osmotic stress measurements on such a DNA hexagonal phase [555,556]. The A bands of striated muscle also seem to have a structure which is rather similar to the hexagonal \( H_{I} \) phase [99] (the authors actually compared the structure to the thermotropic \( S_{B} \) phase, but the analogy with the hexagonal phase is more realistic). The myosin filaments within one band are arranged on a two-dimensional hexagonal lattice with a lattice parameter in the region of 630 Å. Each myosin filament being surrounded by 12 actin filaments (1 myosin 6 actin per unit cell). Osmotic pressure measurements gave a value for the isothermal bulk compressibility of \( 1.5 \times 10^{5} \text{ N m}^{-2} \).

Similarly, the molecules in native collagen fibrils appear to pack in quasi-hexagonal arrays with a (pseudo) lattice parameter of 15 Å [557]. This structure contains considerable disorder, believed to be of the "mixed crystal" type. It has been suggested that collagen fibres can form fluid lamellar-like liquid-crystalline phases [99].

**VI-G Membrane permeability, ion transport and channels**

The spontaneous translocation of ions across phospholipid bilayers is an extremely slow process, due to the high free-energy cost of moving an ion into the weakly polarizable ("low-dielectric constant") hydrocarbon interior of the bilayer. Membrane ion transport thus requires either the presence of a hydrophilic channel across the bilayer, or of a suitable molecule within the membrane (protein, ionophore or lipid) which is able to effect the translocation.

Already in the earliest studies of lipid polymorphism, some of the implications for biological membranes were appreciated. It was suggested by Luzzati and Husson that if inverse \( H_{II} \)-phase like structures exist in vivo, they would have remarkable permeability properties [103]. Conversely, Lucy proposed that regions of membranes might contain arrays of normal micelles, with permeability channels between them [558]. This idea now seems physically implausible. The possibility that micelles might be involved in permeability changes in membranes was further discussed by Maas and Colburn, who considered that inverse micelles might form
in each monolayer of the synaptic membrane in the presence of magnesium, copper and iron ions, causing a large permeability increase [559]. It was proposed that the presence of ATP, by binding to the phospholipid headgroup-metal ion complex, would stabilize the purely bilayer configuration. This idea was further developed by Cullis and De Kruijff and co-workers, based on the tendency for some lipids to form non-bilayer phases such as H\(_{II}\) [246]. It is envisaged that the binding of Ca\(^{2+}\) ions (for example) to negatively charged lipids such as cardiolipin might induce formation of inverted micelles, which could be solubilized in the membrane interior, thereby providing a mechanism not only for the translocation of polar solutes across the bilayer, but also for phospholipid flip-flop [560]. It has been pointed out that a bilayer containing a proportion of 'H\(_{II}\)-forming' lipids will tend to have a lower passive permeability than one without such lipids, due to the greater order existing in the hydrocarbon interior of such a bilayer [4].

The bilayer permeability barrier is, not surprisingly, lost at the \(L_{a}-H_{II}\) transition [402]. It was suggested that the effects of fatty acids and monoacylglycerols on enhancing the permeability of phospholipid bilayers might be due to bilayer destabilization induced by the transient formation of complexes, which would have a tendency to prefer a non-lamellar configuration [561]. Similar effects on permeability were seen in cardiolipin/phosphatidylcholine bilayers on addition of calcium [475].

VI-H Electric and magnetic fields, and pressure

Remarkably little research has yet been done on the effects of electric and of magnetic fields, or of pressure, on lipid polymorphism. This neglect is surprising, given that not only are all three fundamental physical variables, but also that they are all of great importance in biological and biotechnology.

Electric and magnetic fields

It has been appreciated for many years that large electric fields exist across many biological membranes: 80 mV across a 40 Å thick bilayer corresponds to a field of 2 \(\times\) 10\(^7\) V \(\cdot\) m\(^{-1}\), dielectric breakdown will occur for membrane voltages less than 1 V. This property has been exploited in the technique of electrofusion [516–518], whereby it is possible to induce cell-cell fusion by the application of non-uniform electric fields to bring about close contact between the cells (by 'dielectrophoresis'), followed by microsecond, high-field (kV \(\cdot\) cm\(^{-1}\) range) electrical pulses. Alternatively, using a different electrical pulse procedure, it is possible to induce transient permeability channels to form in bilayers (electroporation), without causing cell fusion [514].

The electric fields appear to facilitate a number of steps in the fusion process: (1) dipoles generated within the cells generate attractive forces which overcome the repulsive electrostatic and hydration forces between cell membranes, bringing about aggregation, (2) lateral redistribution of protein and lipid components around the fusion region may be facilitated; and (3) non-bilayer (inverted micellar) structures might be formed in response to the field in the fusion region, bringing about destabilization of the bilayers.

No effects of magnetic fields on non-lamellar transitions have as yet been reported. For phospholipids, whose diamagnetic susceptibility anisotropies are normally very small, any such effects will probably be negligible, even at quite large fields. However, for lipids containing substituent groups such as aromatic rings, the coupling to a magnetic field would be much stronger, and such effects might then become detectable.

Pressure

Pressure is one of the principal thermodynamic variables and is of great direct relevance, for example to marine biology. It is known that pressure has profound effects on many membrane-mediated processes such as reversal of anaesthesia [562], and in fact affects a range of functions such as permeability, active transport, excitability, and synaptic transmission [563]. However, very little work has as yet been carried out on its effects on non-lamellar transitions in lipids. Direct effects may be expected from the tendency for the more dense phase to be stabilized with increasing pressure. A scanning densitometry study was unable to detect any volume change at the \(L_{a}-H_{II}\) transition of either egg PE or DOPE [158]. It may be concluded that the expected small volume increase at this transition is more than an order of magnitude smaller than that occurring at the gel-fluid transition, measured to be 2.4% for DMPE. Although this suggests that direct pressure effects on the \(L_{a}-H_{II}\) transition should be smaller than those on the chain-melting transition, the fact that the former transition is of very low enthalpy compensates for this. The reason for this is that, from thermodynamic arguments, one can show that the pressure-induced shifts in transition temperature should be inversely proportional to the transition entropy. Experimentally, it was found that the \(L_{a}-H_{II}\) transition of egg PE increased roughly linearly by 0.05 \(^\circ\)C per atm of pressure up to 320 atm [564, 565]. This is roughly twice as large as the effect on the main transition of DPPC [566, 567]. Similar effects have been observed with DOPE [568]. Furthermore, it was found that the lattice parameter of the H\(_{II}\) phase increased by 0.01 Å per atm of pressure, an effect which was 10-times larger than that observed for the \(L_{a}\) phase [568].

In addition to such direct pressure effects, more indirect effects may also be operative, such as changes...
in the strength of hydrophobic interactions, or in disso-
ciation of charged groups [569] It is very likely that
striking effects of pressure on lipid polymorphism, such
as the induction of intermediate phases (such as cubic
phases) in systems which at atmospheric pressure have
simpler phase sequences (e.g., \( L_n-H_{11} \)), will be found in
future

VII. Appendix

VII-A Geometric and symmetry aspects of hexagonal
phases

A number of authors have discussed the properties of
hexagonal phases from the formal viewpoint of geometry [98,570,571] Such considerations are important for
an understanding of the characteristic (microscopic) textures of the phases (see subsection III-A), moreover
they may give insight into the roles of deformations, dislocations, and defects in the mechanism of non-
lamellar phase transitions (Section IV)

Symmetry

A perfect hexagonal phase, where the optical axis is
represented by a unit vector \( \mathbf{n} \) (the director) along the
direction of the rod axes, contains the following symmetry elements (in addition to inversion symmetry and the
identity operation), as shown in Fig 53 [571]
2-, 3- and 6-fold longitudinal rotation axes \( (L_2, L_3 \text{ and } L_6) \)
2-fold transverse rotation axes \( (T_2, T_3) \)
Mirror planes \( (P, L_6-T_2, \text{ and } L_6-O_2) \)
The translational symmetry operations consist of any
translation along \( \mathbf{n} \), and any translation which is a
multiple of the lattice vectors \( \mathbf{a} \) and \( \mathbf{a}' \)

Defects can be introduced into a lattice without
destroying the overall crystallinity by dislocations, where

the lattice is locally displaced by an integral number of
lattice vectors (i.e., the Burgers vector \( \mathbf{b} \)). These can
either take the form of pure screw, longitudinal edge,
transverse edge, or hybrid (screw and edge) dislocations
(Fig 54)

In addition to these translational dislocations, it is
possible to have rotational ones, or disclinations, where
the lattice vector rotates through a certain angle on
traversing a closed path around the disclination. For a
hexagonal phase, the smallest possible longitudinal (i.e.,
around \( \mathbf{n} \)) rotation angle (and in fact the only probable
one) is \( \pm \pi/3 \). Rotation by \( +60^\circ \) leads to 5-fold coor-
dination around the central site, rotation by \( -60^\circ \)
leads to 7-fold coordination (Fig 55). Note that the
longitudinal edge dislocation of Fig 54 (B) is equivalent
to a pair of coupled disclinations, \( -\pi/3 \) and \( +\pi/3 \)

Although further types are possible, the last we will
mention is the transverse disclination \( +\pi \), where the
director \( \mathbf{n} \) rotates by \( +180^\circ \) around either of the 2-fold
transverse rotation axes \( T_2 \) or \( \theta_2 \), as shown in Fig 56

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Fig 53 Symmetry elements of the hexagonal phase [571] (Re-
produced with permission from the J Phys France (1980) 41,
1297–1306)

Fig 54 Dislocations of a hexagonal phase [571] (A) Screw, (B)
longitudinal edge, (c) transversal edge (Reproduced with permission
from the J Phys France (1980) 41, 1297–1306)
Deformations

Deformation of a 'single crystal' of a liquid-crystalline phase (i.e., a single domain where the director $n$ has a uniform direction throughout the domain) raises the free energy of the system. There are two contributions to this energy [572]; firstly, there is an elastic (strain) energy due to distortion of the optimal packing, secondly, there is a curvature energy. There are three principle terms in the curvature contribution – splay, twist, and bend – and each is characterized by an elastic constant, $K_1$, $K_2$, and $K_3$, respectively. It has been shown that for hexagonal phases, bend deformations are much easier than twist or splay, and in fact can occur with zero elastic strain energy [570]. This is why freeze-fracture micrographs of $H_{II}$ phase domains frequently exhibit regions where the hexagonal bundle of lipid rods is curved. For lyotropic liquid crystals, a further curvature term, the 'saddle-splay' elastic constant, is important. This term arises from deformations of layers towards a Gaussian curvature (see subsections IV-C and IV-D).

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