Chapter 2

Structural Properties of Lipids and Their Functional Roles in Biological Membranes[1]

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Introduction

It is commonly assumed that lipids in biological membranes play rather inert structural roles, serving to maintain a permeability barrier between external and internal environments as well as providing a matrix with which functional membrane proteins are associated. However the sheer diversity of membrane lipids, as indicated in Fig. 1 for phospholipids, suggests that this does not provide a complete picture. A single phospholipid species such as phosphatidylcholine could maintain the required liquid crystalline bilayer envelope, immediately leading to questions concerning the functional roles of the many other lipid species present.

The nature of the problem can be more clearly indicated from the ^{31}P nuclear magnetic resonance (NMR) spectra presented in Fig. 2. As explained in slightly more detail in the next section, the observation of broad asymmetric ^{31}P-NMR signals with a low field shoulder and high field peak from large (diameter > 2000 Å) phospholipid systems indicates the presence of phospholipids in a bilayer arrangement. Thus Fig. 2 shows that phospholipids of the human erythrocyte membrane experience a bilayer organization (reflecting the behavior of over 97% of the endogeneous phospholipids [1]), as do dispersions of the total lipids extracted from the erythrocyte membrane. These results are consistent with the view that the lipid component provides the basic bilayer structure of biomembranes, since

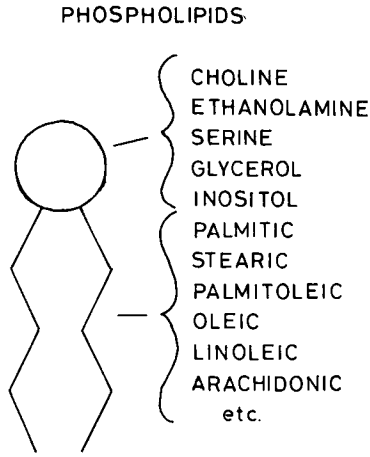

Fig. 1. Chemical diversity in the headgroup and acyl chain regions of phospholipids.

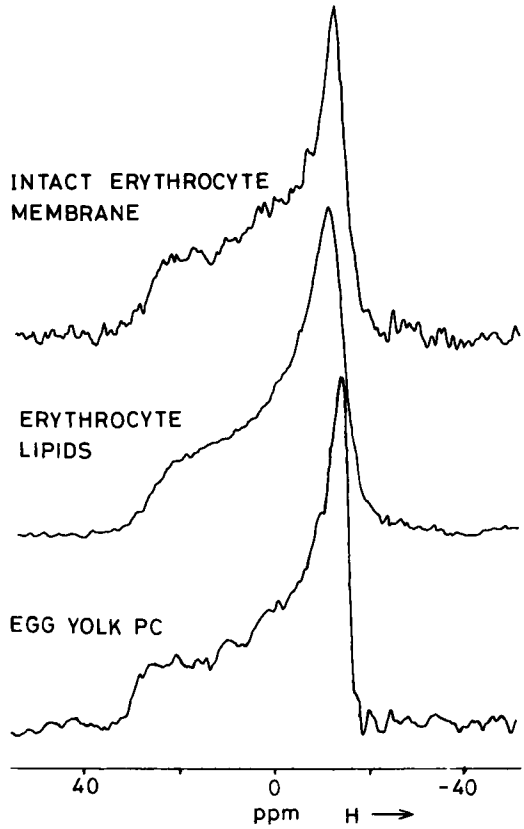

Fig. 2. ^{31}P-NMR spectra (81.0 MHz) at 37°C of human erythrocyte ghost preparations (100 mg dry wt. ghosts), total extracted rehydrated lipids of the erythrocyte (100 mg), and hydrated egg PC (100 mg). All preparations were hydrated in 1 ml of a 100 m*M* NaCl, 10 m*M* Tris-acetic acid (pH = 7.4) buffer containing 10% D_2O by vortex mixing. Spectra were collected from 1000 transients employing a 0.8 sec interpulse time, an 11 μsec 90° r.f. pulse and high power broadband proton decoupling. An exponential filter corresponding to 50 Hz line broadening was applied prior to Fourier transformation.

such structure is maintained in the absence of protein. The problems concerning lipid diversity are implicitly posed by the bilayer ^{31}P-NMR spectrum obtained from egg yolk phosphatidylcholine (PC) (see Fig. 2) suggesting that this PC (as well as PC with a single species of acyl chain substituent—see Fig. 5) could satisfy this structural demand. In the particular case of the erythrocyte membrane, the question is then, What are the functional roles of major phospholipids such as phosphatidylethanolamine (PE), phos-

phatidylserine (PS), and sphingomyelin (SPM) which respectively make up 30, 15, and 25 mol% of the total membrane phospholipid? Indeed, this is a great oversimplification of the problem, which must also include questions about the reasons for acyl chain diversity and location, the roles of cholesterol, the reasons why phospholipids are asymmetrically distributed across the membrane, and the roles of a host of minority lipid species found in this single membrane.

Previous attempts to rationalize lipid diversity have emphasized a hypothetical need for proteins to experience local regions of differing "fluidity" (as provided by local regions of varying lipid composition) for regulation of function, or a need of individual proteins for specific lipids for activity. As we have indicated elsewhere (see introduction of Cullis and de Kruijff [2]), the evidence supporting such a rationale for lipid diversity remains unconvincing. In spite of intensive effort there is little evidence to support the possibility that modulation of fluidity in the region of membrane protein plays an important regulatory role *in vivo*. Reasons for this situation include the fact that gel state lipids, which can modulate (inhibit) the activity of integral proteins in model reconstituted lipid–protein systems (see Warren, *et al.* [3] for example) do not appear to exist in most biological systems. They are notably absent from eukaryotic cell membranes. Further, the ability of physiologically relevant factors (such as pH, ionic strength, divalent cation concentration or even membrane protein) to isothermally modulate membrane fluidity to gain the necessary regulation in appropriate lipid mixtures is far from established.

In this work we review and develop recent work showing that the structural properties of lipids, as indicated by the macroscopic structures they adopt on hydration, are strongly dependent on the type of polar headgroup and acyl chain composition. Further, it is shown that these structural preferences are sensitive to and can be modulated by pH, Ca^{2+} and membrane protein among other factors. This leads to the possibility that some functional roles of lipids in membranes may be dictated by their ability to adopt bilayer or nonbilayer configurations. Evidence suggesting a role of nonbilayer lipid structures in processes such as membrane fusion is reviewed. Subsequently it is noted that the structural preferences of lipids appear to reflect, at least in part, the molecular shape of the lipid molecule, and this shape factor alone may play structural roles related to optimum biomembrane packing and sealing. In turn, this suggests a possible rationale for lipid diversity in terms of the molecular shapes of various components, allowing the formation of bilayer or nonbilayer structures as well as allowing local architectural roles at the lipid–protein interface.

Structural Preferences of Membrane Lipids

Introduction

In the presence of excess water and at concentrations above the "critical micellar concentration" (CMC) lipids form a variety of macromolecular organizations (the most common of these are indicated in Fig. 3). As we shall indicate momentarily, major membrane lipids generally assume the bilayer

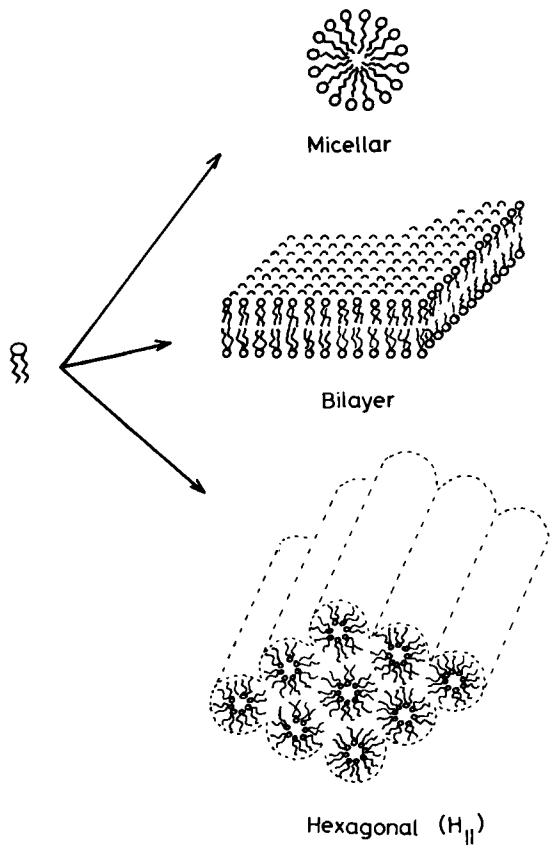

Fig. 3. Structural preferences of lipids at aqueous concentrations above the critical micellar concentration (c.m.c.).

or hexagonal (H_{II}) organization when hydrated. Lipids that assume micellar structures (e.g., lysophospholipids) are normally minority components of biomembranes. This is not the case for lipids preferring the hexagonal (H_{II}) phase in isolation, however, which often constitute up to 30% of the membrane lipid. The structure of this phase as elucidated by X-ray techniques [4] contrasts dramatically with the familiar bilayer organization, and two points must be emphasized. First, the H_{II} phase is basically a liquid crystalline hydrocarbon matrix penetrated by hexagonally packed aqueous channels (of approximately 20 Å diameter [4]) toward which the polar groups of the lipid are oriented (see Fig. 3). Second, lipids in the H_{II} phase cannot provide a permeability barrier between internal and external environments, and it is not clear that they can help to maintain a stable bilayer organization These functions are commonly thought to constitute the major role of membrane lipids. The observation that such lipids are major components of biological membranes immediately raises questions about the other functional properties of membranes they may satisfy, and such considerations have provided the major impetus for many of the studies summarized in this chapter.

Techniques for Visualizing Lipid Organization

There are several techniques available to investigate the structural properties of hydrated lipids, namely X-ray diffraction [4], freeze-fracture [5, 6], and NMR [2, 7]. X-ray diffraction techniques are clearly the classical procedures allowing unambiguous determinations of the structure of hydrated lipid aggregates, provided that these structures are present in some regular array. Freeze-fracture electron microscopy procedures, on the other hand, provide a local visualization of the macromolecular lipid structures present, rather than averaged information arising simultaneously from many sites. Structures that can be detected include not only the ordered lamellar and hexagonal (H_{II}) phases [5], but also local intermediary structures [7] which may not be organized in a regular pattern. Finally, NMR techniques, particularly ^{31}P NMR for phospholipids [2], provide a valuable diagnostic procedure for determining lipid organization in large (diameter $>$ 2000 Å) bilayer systems or cylindrical structures such as the H_{II} phase. However, NMR techniques do not provide unambiguous structural information for phospholipids in smaller systems where isotropic motional averaging (due to tumbling or lateral diffusion) occurs. Some of the advantages and disadvantages of the ^{31}P NMR-technique have been discussed elsewhere [2].

The ^{31}P-NMR and freeze-fracture characteristics of phospholipids in bilayer, H_{II} phase, and alternative structures are summarized in Fig. 4. Briefly, phospholipids in lamellar arrangements give rise to broad, asymmetric ^{31}P NMR spectra with a low field shoulder and high field peak separated by approximately 40 ppm, whereas freeze-fracture micrographs show large sheets which may be occasionally interrupted by ridges which appear to represent jumps of the fracture plane from the interior of one bilayer to an adjacent one. Phospholipids organized in the hexagonal (H_{II}) phase, on the other hand, give rise to asymmetric ^{31}P-NMR spectra which have reversed asymmetry compared to the bilayer spectrum and are narrower by a factor of two. This structure is visualized by freeze-fracture as a regular striated pattern created as the fracture plane fractures at all angles between the hexagonally packed cylinders (see Deamer *et al.* [5], Verkleij and Ververgaert [6], Verkleij and deGier [7], and Cullis *et al.* [8]). Finally, as mentioned previously, lipids in a variety of other structures allowing isotropic motional averaging give rise to narrow, symmetric ^{31}P-NMR spectra. Freeze-fracture representations can give less ambiguous assessments of the structures present, as shown for sonicated vesicles and "lipidic particle" structures (see next section).

Phase Preferences of Lipids

In order to understand the behavior and organization of complex lipid mixtures such as those obtained in biomembranes, it is necessary to understand the structural preferences of the individual lipid species themselves. As indicated in Fig. 2, egg PC assumes a bilayer organization when hydrated, and similar behavior is observed for synthetic $18{:}1_c/18{:}1_c$ (DOPC) PC and bovine brain SPM as shown in Fig. 5. Such bilayer ^{31}P-NMR spectra are also observed in the presence of equimolar cholesterol. These results are consistent with extensive X-ray investigations of long chain PCs [9] and SPM [10], and suggest that a primary functional role of PC and SPM is to maintain the bilayer organization of biological membranes. A similar function may be ascribed to glycolipids such as diglucosyl and digalactosyl diglycerides, which also assume a lamellar organization upon hydration [11, 12]. This contrasts strongly with the behavior of naturally occurring PEs, which adopt either the bilayer or hexagonal (H_{II}) phase depending on the temperature [13, 14]. The ^{31}P-NMR behavior of a variety of PEs is illustrated in Fig. 6, where bilayer to H_{II} transitions are observed as the temperature is increased through ~8°C for erythrocyte PE, ~3°C for rat liver endoplasmic reticulum

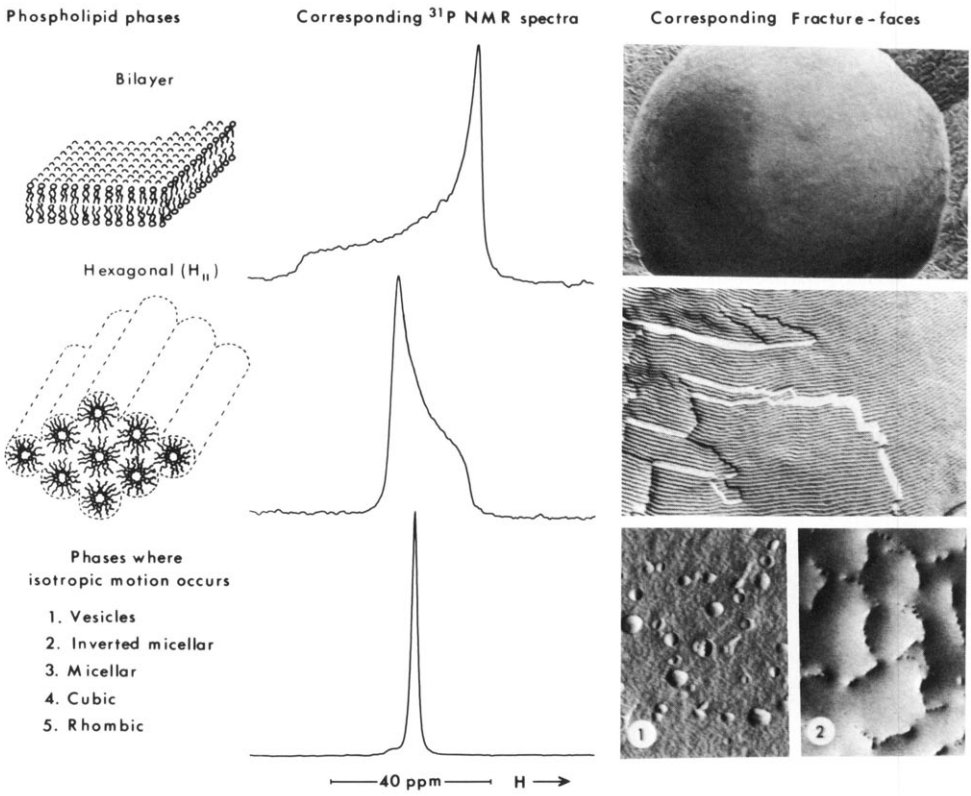

Fig. 4. ^{31}P-NMR and freeze-fracture characteristics of phospholipids in various phases. The bilayer spectrum was obtained from aqueous dispersions of egg yolk phosphatidylcholine, and the hexagonal (H_{II}) phase spectrum from phosphatidylethanolamine prepared from soya bean phosphatidylcholine employing the head group exchange capacity of phospholipase D. The ^{31}P-NMR spectrum representing isotropic motion was obtained from a mixture of 70 mol% soya phosphatidylethanolamine and 30% egg yolk phosphatidylcholine. All preparations were hydrated in 10 m*M* Tris-acetic acid (pH 7.0) containing 100 m*M* NaCl and spectra recorded at 30°C in the presence of proton decoupling. The freeze-fracture micrographs represent typical fracture faces obtained. The bilayer configuration (total erythrocyte lipids) gives rise to a smooth fracture face whereas the hexagonal (H_{II}) configuration is characterized by ridges displaying a periodicity of 6–15 nm. Two common conformations that give rise to isotropic motion are represented in the bottom micrograph (**1**) small bilayer vesicles (sonicated PS–PE [1:4] vesicles) (< 200 nm diam.) and (**2**) large lipid structures containing lipidic particles (PI–PE containing 15 Mol% PI: Ca^{2+}:PI = 1:2).

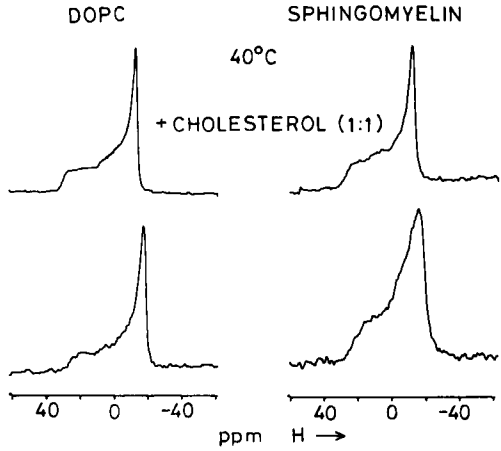

Fig. 5. 81.0 MHz ^{31}P-NMR spectra at 37°C arising from aqueous dispersions of 18:1$_c$/18:1$_c$ PC (DOPC) and bovine brain sphingomyelin (SPM) in the presence and absence of equimolar cholesterol. The cholesterol-containing samples were prepared from appropriate mixtures of DOPC and SPM in chloroform, which was subsequently evaporated under a stream of nitrogen and subsequent high vacuum (1 hr). The lipid film was then hydrated by vortex mixing in the aqueous buffer described in the legend of Fig. 2. All other conditions same as for Fig. 2.

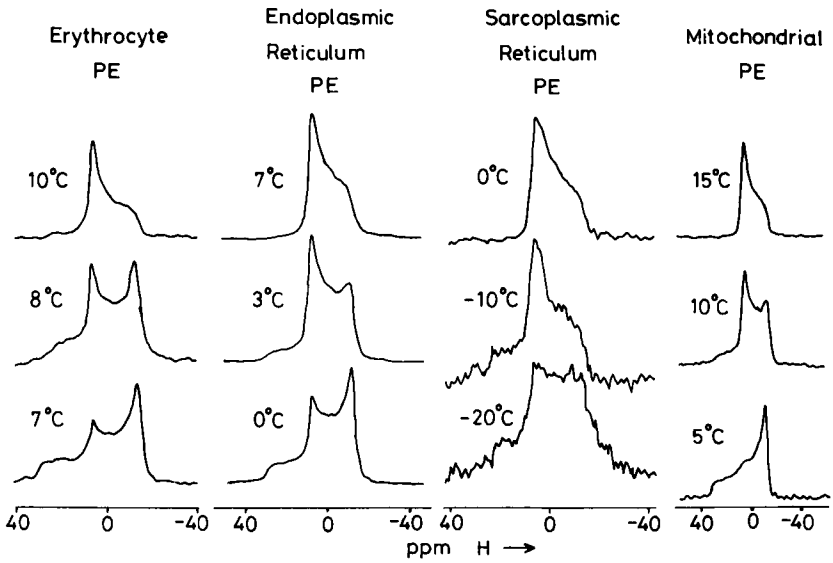

Fig. 6. ^{31}P-NMR spectra at selected temperatures illustrating bilayer to H_{II} transitions for various species of naturally occuring phosphatidylethanolamines hydrated in excess aqueous buffer. For experimental conditions and other details on erythrocyte PE, see Cullis and deKruijff [85] endoplasmic reticulum PE, see deKruijff *et al.* [86]; and mitochondrial PE, see Cullis *et al.* [91]. Sarcoplasmic reticulum PE was isolated from rabbit muscle sarcoplasmic reticulum.

PE, $\sim -10°C$ for rabbit muscle sarcoplasmic reticulum PE, and $\sim 10°C$ for rat liver mitochondrial PE. Thus all these PEs prefer the H_{II} organization at physiological temperatures. Similarly, hydrated monoglucosyl and monogalactosyl diglycerides from different sources prefer the hexagonal phase at growth temperatures [11, 12]. It is intriguing that there appears to be a relationship between the occurrence of these glycolipids and PE in bacterial membranes [15], despite their chemical disparity, suggesting a regulated requirement for lipids favoring the H_{II} organization.

The behavior of acidic (negatively charged) phospholipids is of particular interest because they experience strong interactions with divalent cations, thus the macroscopic structures assumed after hydration may be modulated by the presence or absence of Ca^{2+}, for example. The structural preferences of hydrated beef heart cardiolipin (CL), egg PS, egg phosphatidylglycerol (PG), and beef brain phosphatidylinositol (PI) in the presence and absence of Ca^{2+} are indicated in Fig. 7. Two points are apparent. First, in the absence of Ca^{2+}, all these negatively charged phospholipids adopt the bilayer organization as indicated by ^{31}P NMR. Second, the addition of Ca^{2+} can have a variety of effects depending on the lipid species involved. These cation-dependent effects are discussed in greater detail in the next section dealing with isothermal regulation of lipid structure.

In mixed lipid systems, phospholipids preferring a bilayer organization in isolation (such as PC, SPM, and the acidic phospholipids) would be expected to stabilize a bilayer arrangement when mixed with an H_{II} phase lipid such as an unsaturated PE. This is the case for DOPC, as equimolar mixtures of this lipid with $18{:}1_c/18{:}1_c$ PE (DOPE) (which adopts the H_{II} phase above 15°C [16]) exhibit bilayer ^{31}P-NMR spectra (Fig. 8(a)). It is difficult to predict a priori, however, the influence of a lipid such as cholesterol, which adopts neither the bilayer nor the H_{II} phase when hydrated but remains in a crystalline form. In mixtures with bilayer lipids cholesterol produces a state of intermediate fluidity, consistent with an ability to condense PC bilayers [17], and decrease the permeability of such systems. This may suggest a bilayer-stabilizing role, and the action of cholesterol in PC and SPM bilayers (Fig. 5) in no way contradicts this. However the presence of cholesterol in mixed unsaturated PE–PC systems produces a strong bilayer destabilization effect, inducing the hexagonal H_{II} phase at equimolar cholesterol to phospholipid concentrations (see Fig. 8(d)).

An interesting feature of the cholesterol titration of Fig. 8 is the appearance of a narrow spectral feature indicating the presence of phospholipid in structures allowing isotropic motional averaging. Similar components are observed in unsaturated PE–PC systems at PC concentrations below those required to achieve full bilayer stabilization (see Cullis and de Kruijff [18], Cullis *et al.* [19]). As noted earlier, ^{31}P NMR does not provide an unam-

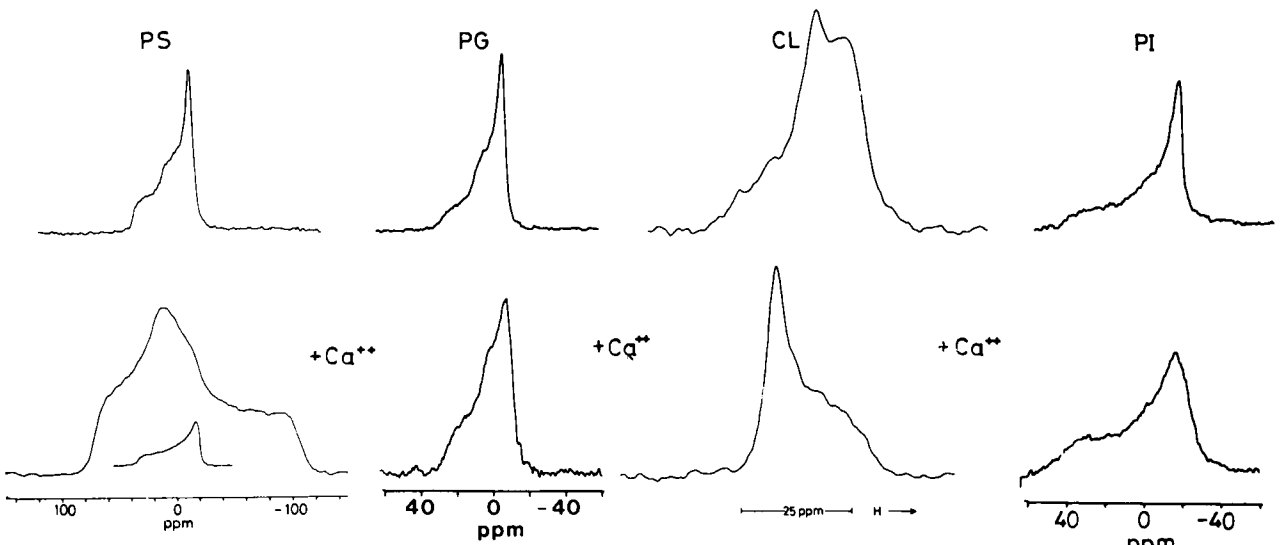

Fig. 7. ^{31}P-NMR spectra obtained for various species of acidic phospholipids (sodium salt) in the absence and presence of equimolar (with respect to charge) Ca^{2+}. For full details of sample preparation and signal accumulation procedures for cardiolipin (CL), see Cullis *et al.* [8], for phosphatidylserine (egg PS), see Hope and Cullis [29], for phosphatidylglycerol (egg PG), see Farren and Cullis [33], and for phosphatidylinositol (soya PI), see Nayer *et al.* [34]. The small insert on the PS + Ca^{2+} spectrum indicates the influence of Mg^{2+}. All previously published spectra reproduced with permission.

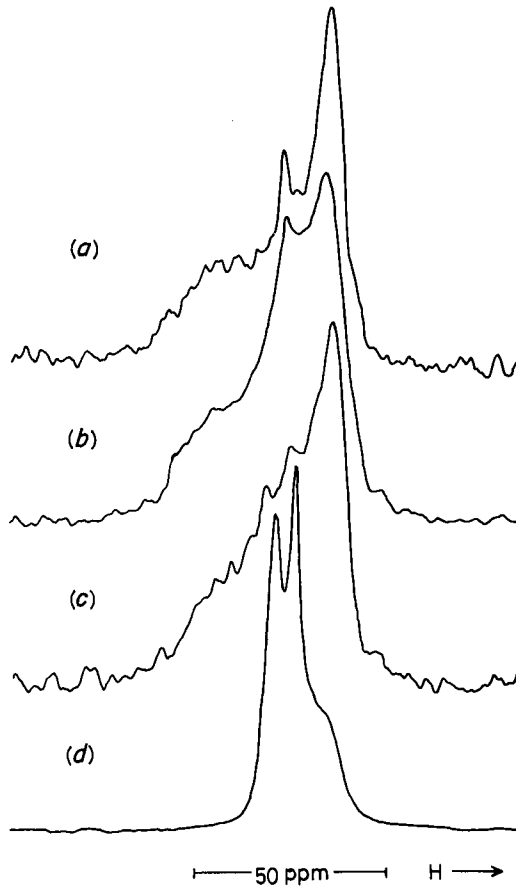

Fig. 8. 36.4 MHz ^{31}P-NMR spectra obtained at 30°C from equimolar mixtures of DOPE with DOPC in the presence of (*a*) 0 mol%; (*b*) 15 mol%; (*c*) 30 mol%; and (*d*) 50 mol% cholesterol. All dispersions contained 10 m*M* Tris-acetic acid (pH = 7.0) and 2 m*M* EDTA. Reproduced with permission from Cullis *et al.* [19].

biguous determination of the structures present in such situations. However the freeze-fracture results obtained from systems composed of mixtures of bilayer and H_{II} preferring lipids are most intriguing because small "lipidic particles" [20] are visible on the fracture face (Fig. 9). Although alternative possibilities have been proposed [23, 24], available evidence strongly suggests that these uniformly sized particles correspond to intrabilayer inverted micelles as illustrated in Fig. 9(b) [21, 22]. These inverted lipid micelles can occur as an intermediate structure between the bilayer and H_{II} organizations [25]. An important aspect of such a structure is that if nonbilayer lipid organizations are present in biomembranes, inverted micelles are more at-

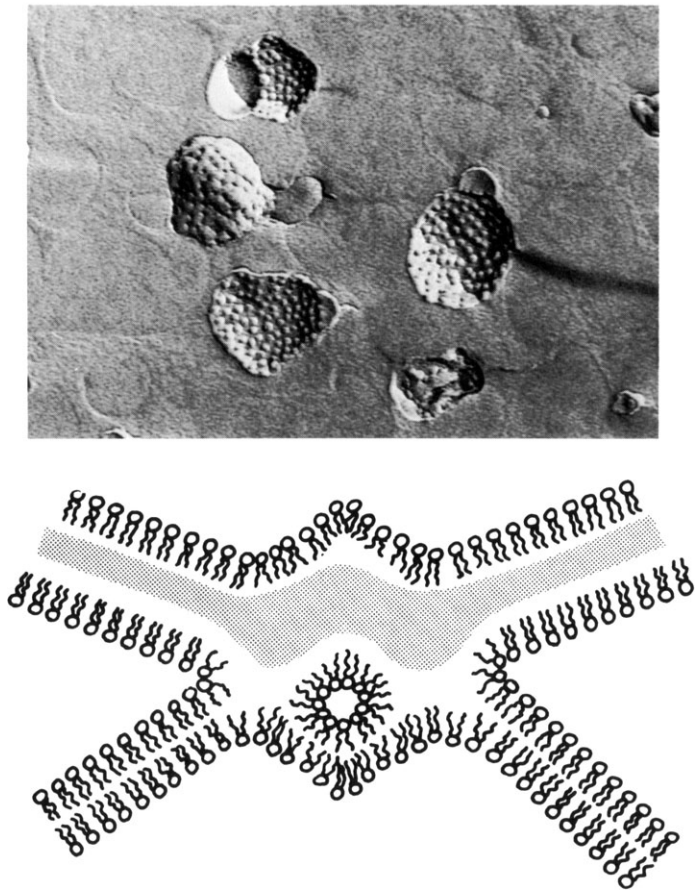

Fig. 9. Freeze-fracture micrograph of lipidic particles induced by Ca^{2+} in a lipid system consisting of cardiolipin and soya phosphatidylethanolamine in the molar ratio of 1:4 (magnification 80,000). A model of the lipidic particle as an inverted micelle is depicted below the micrograph. The shaded area represents the fracture region.

tractive candidates than the long inverted cylinders characteristic of the H_{II} phase. Reasons for this include the obvious fact that biomembranes are mixed lipid systems and thus would be mimicked more closely by the model systems composed of bilayer and H_{II} phase lipids, as well as the fact that a membrane containing an intrabilayer inverted micelle can conceivably maintain a permeability barrier. Further, inverted micelles can be generated at discrete locales as required, in contrast to a long inverted cylinder. However, such cylinders may play roles in "arrested fusion" situations such as tight junctions [92].

Isothermal Modulation of Membrane Lipid Structure

Influence of Divalent Cations and pH

It is clear that in order for nonbilayer lipid structures such as inverted micelles or extended inverted cylinders to be of some functional use in biomembranes mechanisms must exist for their isothermal regulation and control. Logical agents for such control include the local pH, ionic strength, divalent cation concentration, and membrane protein. In this section we point out that the pH and the divalent cation concentration (particularly Ca^{2+}) can strongly influence the structural preferences of membrane phospholipids under conditions that are within physiological bounds.

The ability of Ca^{2+} to modulate the structures formed by acidic (negatively charged) phospholipids is clearly illustrated in Fig. 7. In the case of beef heart CL, Ca^{2+} triggers a bilayer to H_{II} phase transition (see Cullis *et al.*, [8] for background detail) in agreement with previous X-ray results [26]. Similar behavior is observed for PA at pH 6 [28], results supported by freeze-fracture [27] and ^{2}H-NMR [28] techniques. It may be noted that other divalent cations such as Mg^{2+} and Ba^{2+} are able to trigger H_{II} phase formation for CL [26], as can Mg^{2+} and Mn^{2+} for PA at pH 6 [27, 28]. In the case of PS, the addition of Ca^{2+} results in a much broader ^{31}P-NMR spectrum (see Hope and Cullis [29] for details) which corresponds to a "rigid lattice" (no motion) situation commonly observed for anhydrous phospholipids. This appears to be characteristic of the "cochleate" Ca^{2+}–PS structure identified by Papahadjopoulos and co-workers [30]. A specificity of the divalent cation—PS interaction is indicated by the fact that Mg^{2+} is not able to produce similar effects (PS remains in the liquid crystalline lamellar organization in the presence of Mg^{2+}, see Fig. 7). Much different behavior is exhibited by egg PG and soya PI. In common with the other acidic phospholipids they precipitate on addition of Ca^{2+}, but remain in lamellar structures as indicated by ^{31}P NMR.

The ability of Ca^{2+} and other divalent cations to influence the structural preferences of acidic phospholipids (particularly CL, PA, and PS) is clearly of fundamental interest. However it is difficult to extrapolate this behavior to biological membranes where these phospholipids are not majority components. We have therefore examined the influence of Ca^{2+} on systems that may be expected to be both more representative of biomembrane lipid mixtures as well as more sensitive to the presence of divalent cations. Particularly interesting combinations are those containing acidic phospholipids with a PE species which prefers the H_{II} organization in isolation. In such systems situations can be achieved where the acidic phospholipid is barely

stabilizing a bilayer configuration, and the net structure may thus be expected to be very sensitive to factors affecting the distribution and/or net charge on the negatively charged species.

The ability of representative acidic phospholipids to stabilize the bilayer in the presence of a polyunsaturated (soya) H_{II} phase PE at 30 mol% concentrations is illustrated in Fig. 10. Also illustrated is the ability of Ca^{2+} in all these systems to trigger a bilayer to H_{II} phase transition. It is interesting to

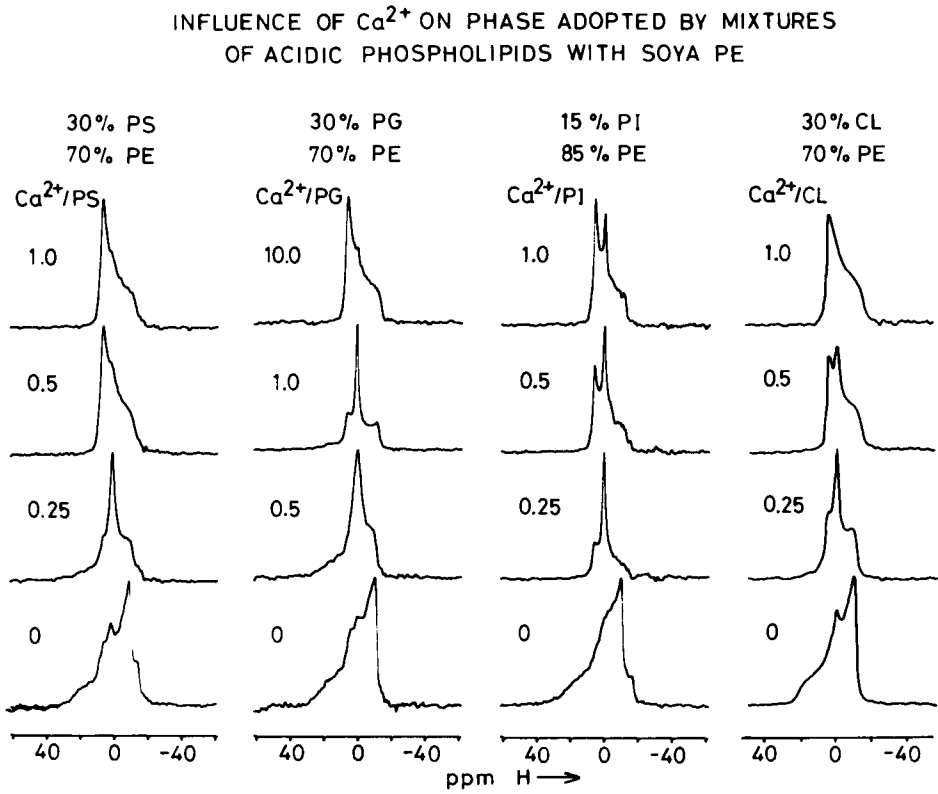

Fig. 10. ^{31}P-NMR spectra arising from mixtures of acidic phospholipids with soya phosphatidylethanolamine (a polyunsaturated PE derived from soya PC employing the base exchange reaction catalyzed by phospholipase D—see Comfurius and Zwaal [88] and Cullis and Hope [89] for further detail on PE preparation) in the presence of the various molar ratios of Ca^{2+}. All samples were prepared from 50 μmol total phospholipid mixed in chloroform, from which the chloroform was removed, and the lipid then hydrated in 1 ml of the buffer employed for Fig. 2 by vortex mixing. The Ca^{2+} was added as aliquots from a 100 m*M* stock solution. For further details for (egg) PS–PE, see Tilcock and Cullis [32], (egg) PG–PE, see Farren and Cullis [33], (soya) PI–PE, see Nayar *et al.* [34], and for (beef heart) CL–PE, see deKruijff [31]. All previously published spectra reproduced with permission.

note that the detailed mechanism involved differs according to the acidic phospholipid species involved. In the case of CL, Ca^{2+} converts the CL to an H_{II}-preferring species, thus allowing the entire mixture to adopt the H_{II} phase (see deKruijff and Cullis [31]). Alternatively, in the case of PS, Ca^{2+} appears to induce a lateral segregation of PS into "cochleate" domains [32], allowing the PE to adopt the H_{II} configuration it prefers in isolation. This contrasts with the behavior of systems stabilized by PG, where the presence of Ca^{2+} appears to reduce the bilayer-stabilizing capacity of PG, and both the PG and PE enter the H_{II} configuration [33]. Conversely a Ca^{2+}-induced lateral segregation of PI into liquid crystalline lamellar domains is possible, although not yet proven, leaving the PE to revert to the H_{II} phase [34]. It may also be noted that the molar ratios of Ca^{2+}:acidic phospholipid required to induce H_{II} phase organization vary significantly, with the CL–PE and PS–PE systems being more sensitive to the presence of Ca^{2+} than the PI–PE and PG–PE systems. Systems stabilized by 30 mol% or more PI will not adopt the H_{II} phase even at very high (50 m*M*) Ca^{2+} concentrations.

The polymorphic phase preferences of acidic phospholipids would be expected to be most sensitive to pH changes in the region of the pK of ionizable groups. This is indeed the case for $18{:}1_c/18{:}1_c$ PS (DOPS) and DOPA, which have a pK in the region of 3.5 and 4 respectively, as illustrated in Fig. 11. Both these lipid species exhibit bilayer to H_{II} transitions as the pH is lowered so that the charge on the head group is neutralized. DOPA undergoes this transition at pH values above 3.5 due to the presence of 100 m*M* NaCl in the buffer, which acts to raise the pH at which the H_{II} phase is preferred. Following the reasoning indicated above, it may be expected that the polymorphism of PE systems stabilized by these acidic phospholipids would be particularly sensitive to a reduction of pH. This is indeed the case for soya PE systems stablized by egg PS, as shown in Fig. 12. It may be noted that systems in which bilayer structure is stabilized by low (15 mol%) PS concentrations exibit the strongest effects on lowering the pH, and components indicative of H_{II} phase structure are visible at pH values as high as 5.5. The mechanism involved would appear to correspond to the Ca^{2+} induced bilayer–H_{II} transitons in CL–PE systems (Fig. 10) because the PS is converted to an H_{II} lipid species as the pH is reduced.

A question that was not addressed for the Ca^{2+}-induced bilayer–H_{II} transitions in acidic phospholipid–PE systems concerns the absolute Ca^{2+} concentrations required. In the case of the PS–PE systems, for example, Ca^{2+} concentrations on the order of 2 m*M* or more (much higher than cytosol Ca^{2+} concentrations) are necessary to trigger such transitions [32]. Given the ability of cholesterol to destabilize PC–PE systems (Fig. 8), as well as the fact that cytosol levels of Mg^{2+} (~2 m*M*) can enhance the ability of Ca^{2+} to effect PE–PS bilayer–H_{II} transitions [35], it may be expected

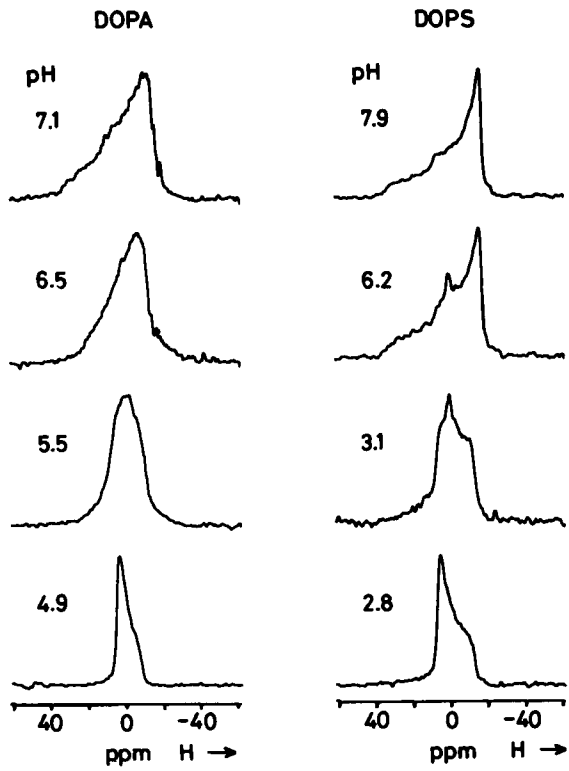

Fig. 11. 81.0 MHz ^{31}P-NMR spectra at 30°C arising from aqueous dispersions of DOPS and DOPA (sodium salt) at various pH values. The samples (50 μmol phospholipid) were hydrated in 1 ml of buffer containing 100 m*M* NaCl, 20 m*M* HEPES and the pH was adjusted by adding aliquots of 0.1*M* HCl. For further details, see Hope and Cullis [29] and Farren and Cullis [28].

that much lower Ca^{2+} concentrations are required to trigger these structural reorganizations in PE–PS–cholesterol systems in the presence of 2 m*M* Mg^{2+}. This is indeed the case as illustrated in Fig. 13, where Ca^{2+} concentrations in the region of 0.2 m*M* are sufficient to induce appreciable effects.

These types of investigations can be extended to lipid mixtures more closely representative of biological membranes. One such system is the inner monolayer of the erythrocyte, which is composed (on a molar basis) of 50% PE, 25% PS and 12.5% each of PC and SPM [36]. The polymorphism of systems composed of erythrocyte lipids reconstituted in these proportions (in the presence of equimolar cholesterol) is indeed sensitive to the presence of Ca^{2+} (see Fig. 14), where a Ca^{2+}:PS molar ratio of 0.5 induces an

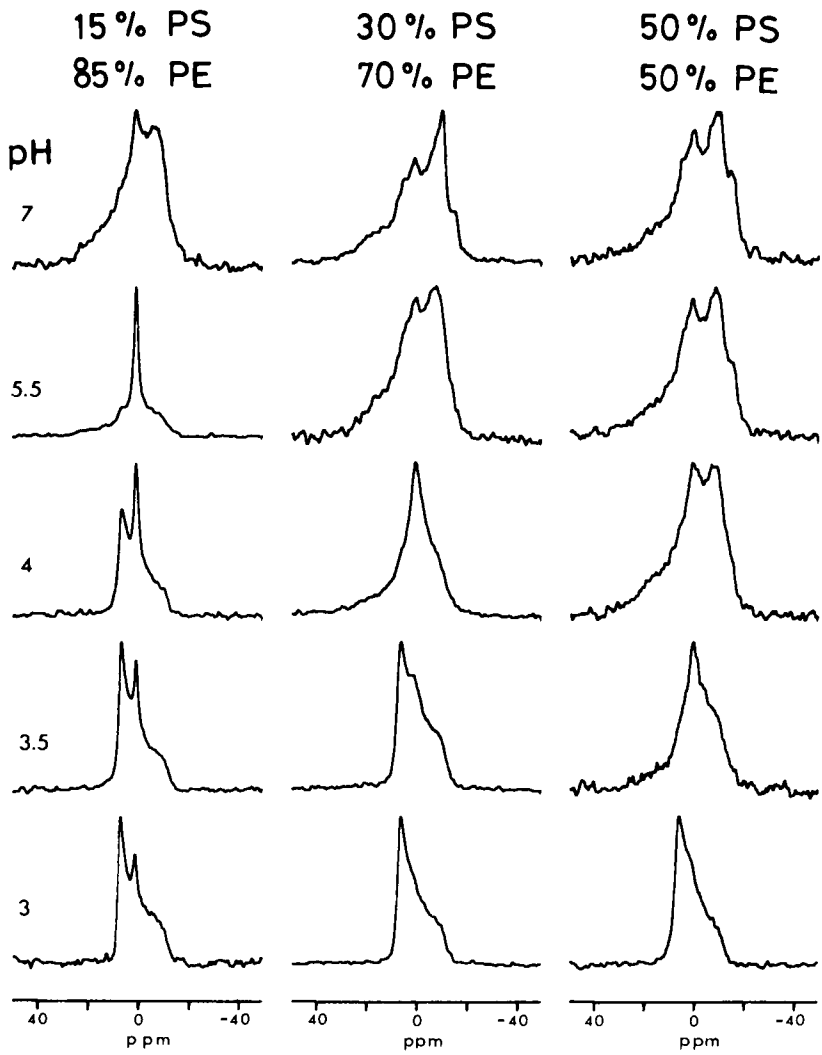

Fig. 12. 81.0 MHz ^{31}P-NMR spectra at 30°C obtained from aqueous dispersions of soya PE containing (*a*) 15% soya PS (*b*) 30% soya PS and (*c*) 50% soya PS at pH values 7.0, 5.5, 4, 3.5 and 3. The pH was adjusted as indicated in the legend of Fig. 11. Reproduced with permission from Tilcock and Cullis [32].

appreciable H_{II} phase component (see Hope and Cullis [37] for further details). These results suggest that when erythrocyte cytosol Ca^{2+} concentrations increase the inner monolayer will be unstable in the sense that a sizeable portion of the phospholipid would prefer nonbilayer structure. Such instability may be related to the fusion events involved in the "blebbing off"

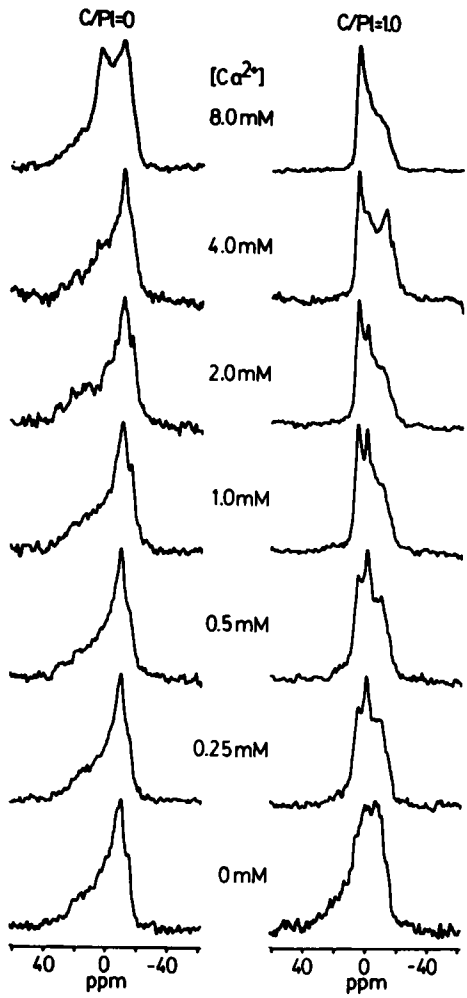

Fig. 13. 81.0 MHz ^{31}P-NMR spectra at 30°C obtained from aqueous dispersions of soya PE and soya PS (1:1) in the presence of 2 m*M* mg^{2+}, and dialyzed against various concentrations of $Ca_2{}^+$. The ratio C:PL refers to the molar ratio of cholesterol to phospholipid present. For full details of the dialysis procedure and other protocols, see Bally and Cullis [35].

processes [38] noted for erythrocytes upon ATP depletion, as discussed elsewhere [37]. These results also suggest a possible reason why the erythrocyte plasma membrane (and possibly other plasma membranes) exhibits lipid asymmetry. An outer monolayer of PC and SPM presents a rather inert permeability barrier to the extracellular environment, whereas an interior monolayer composed primarily of PE and PS will have structural

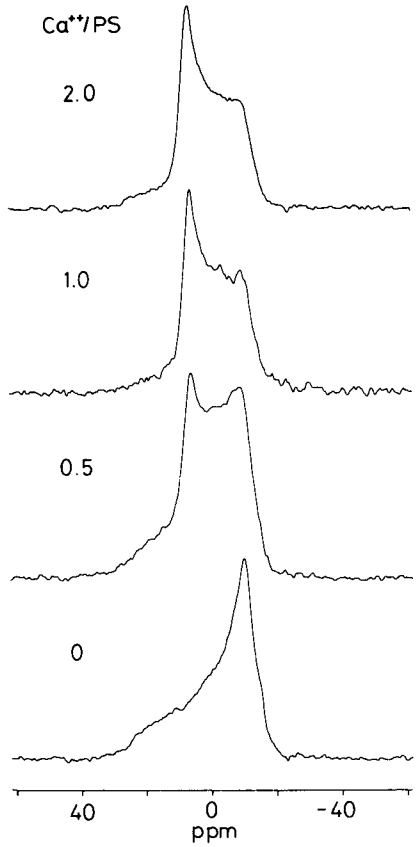

Fig. 14. 81.0 MHz ^{31}P-NMR spectra at 37°C arising from an aqueous dispersion of reconstituted "inner monolayer" lipid isolated from the human erythrocyte membrane. The lipid composition is PE:PS:PC:SPM (in the ratios 0.5:0.25:0.13:0.12) and containing equimolar cholesterol with respect to total phospholipid. The ratio Ca^{2+} = PS refers to the molar ratio Ca^{2+} to PS. Ca^{2+} was added as indicated in the legend to Fig. 10. Reproduced with permission from Hope and Cullis [37].

preferences which can be modulated locally. As we indicate in Section IVB such properties could play important roles in exocytotic events.

Influence of Protein on Membrane Lipid Structure

Most of the biochemical and physiological processes occurring in membranes are catalyzed or mediated by proteins which are often membrane bound. Given the fact that the majority of these proteins interact with lipids,

this interaction is potentially of great functional importance. In particular, as indicated in Section IV, some of these membrane mediated processes may involve the participation of nonbilayer lipids and associated structures and it is therefore important to establish the effects of proteins on membrane lipid structure. That proteins can have a large effect on the structural preferences of lipids can be inferred from a comparison of the phase behavior of the lipids in a biomembrane and in model systems composed of the extracted lipids. For example, whereas the lipids in the *E. coli* inner membrane [39] and the rod outer segment membrane are (predominantly) organized in a bilayer, the hydrated total lipids prefer hexagonal and inverted micellar structures [40]. This suggests that membrane proteins such as rhodopsin stabilize the bilayer. Alternatively the total lipids of the liver microsomal membrane adopt a bilayer configuration upon hydration, whereas ^{31}P-NMR studies of the intact membrane at 37°C reveal a fraction of the phospholipid experiencing isotropic motional averaging, possibly indicating the transient occurrence of nonbilayer lipid structures [41, 42]. This has led to the suggestion that proteins in this membrane, particularly cytochrome *P*-450 [42] induce nonlamellar structure for the endogenous lipids.

More precise information on the influence of proteins on lipid polymorphism can be obtained in reconstituted model systems. Reconstitution of glycophorin, the major asialoglycoprotein from the human erythrocyte membrane, with unsaturated PE has a profound bilayer stabilization effect [43]. Bilayer structure is maintained 50°C above the bilayer–hexagonal H_{II} transition temperature of the PE. Removal of the large sugar- and sialic acid-containing section of the protein (by trypsin treatment) does not affect this bilayer-stabilizing ability, demonstrating that the hydrophobic membrane-spanning portion of the protein is responsible for this effect. However not all intrinsic polypeptides have the same effect, as shown by experiments with gramicidin. This peptide is commonly used as a model for an intrinsic membrane protein and can form (by dimerization) a helical membrane-spanning channel. Incorporation of gramicidin in PE-containing model membranes leads to a strong bilayer destabilization [44]. The hexagonal H_{II} phase-promoting ability of this peptide is so strong that H_{II} phase structure can be induced even in saturated phosphatidylcholine model systems [44]. These bilayer destabilization effects are strongly dependent on the length and nature of the acyl chains suggesting that in order to accommodate the peptide in a bilayer the thickness of the hydrophobic part of the molecule has to match the length of the gramicidin dimer [44].

Cytochrome *c* is another example of a protein that can induce nonbilayer structures in model membrane systems. This highly basic "extrinsic" protein from the inner mitochondrial membrane experiences strong interactions with many negatively charged phospholipids [31] where lamellar structure is

maintained. Only in the case of cardiolipin (the major negatively charged inner mitochondrial membrane lipid) does this interaction result in the formation of the hexagonal H_{II} phase and structures which may be inverted micelles [31]. This observation is of interest for two reasons. First, it demonstrates specificity in lipid–protein interactions, and second, given the absolute requirement of cytochrome *c* oxidase for cardiolipin for activity [45] it can be suggested that nonbilayer structures formed by the cytochrome *c*–cardiolipin interactions may play a role in the functioning of the terminal part of the respiratory chain. This hypothesis is supported by the finding that the strongly cardiotoxic anticancer drug adriamycin specifically interacts with cardiolipin in model systems [46], blocks the formation of nonbilayer structures (47) and inhibits mitochondrial regulation [48].

Poly (L-lysine) is another highly basic polypeptide that has a pronounced effect on the structure of a cardiolipin dispersion. The strong electrostatic interaction of this polypeptide with cardiolipin leads to bilayer stabilization such that even in the presence of excess Ca^{2+}, cardiolipin remains organized in the lamellar phase [49]. Further, this polypeptide can induce phase separations in PE–CL mixed systems thereby triggering a bilayer– hexagonal H_{II} phase transition [49].

These preliminary studies demonstrate that lipid–protein interactions can isothermally modulate the phase preferences of lipids in a variety of ways consistent with the notion that nonbilayer structures may be of importance in biomembrane functioning. Other aspects of lipid–protein interactions in relation to lipid diversity will be discussed in section V.

Potential Roles of Nonbilayer Lipid Structures in Membranes

Membrane Fusion

Membrane fusion is an important and ubiquitous event in cell biology, occuring in processes such as fertilization, formation of polykaryocytes, exo- and endocytosis and the intracellular turnover and delivery of membrane components to name but a few. The detailed mechanism involved is not well understood. As indicated in Fig. 15, two events are vital for fusion to proceed—close apposition, which is likely to be a protein-mediated event, and the fusion event itself. Our initial interest in a potential role of nonbilayer lipid structures as intermediates in the fusion event was stimulated by the

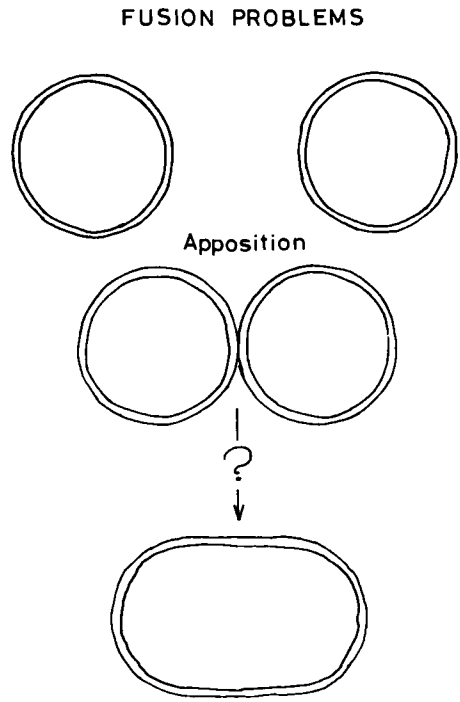

Fig. 15. Processes in membrane fusion phenomena.

observation that it is difficult (if not impossible) to imagine such an event occurring while bilayer lipid structure is maintained at the fusion interface. As a first approach to the problem, the properties of lipid-soluble fusogens (which enhance [50, 51] fusion between erythrocytes and other cells) were examined, with the precept in mind that these agents may enhance fusion by enabling endogenous lipids to adopt putative nonbilayer intermediates more easily.

The results obtained for the commonly employed fusogen glycerol monooleate (GMO) and the erythrocyte ghost membrane and model systems composed of the extracted lipids of the erythrocyte are illustrated in Figs. 16 and 17. Fig. 16 shows that the presence of equimolar (with respect to membrane phospholipid) or higher amounts of GMO associated with the intact erythrocyte (ghost) membrane results in appreciable formation of nonbilayer H_{II} phase phospholipid. Similar behavior is observed for reconstituted model systems of the erythrocyte phospholipids. In order for fusion to proceed between intact erythrocytes, equimolar or higher concentrations of membrane-associated GMO are required (see Fig. 17), and thus there is a

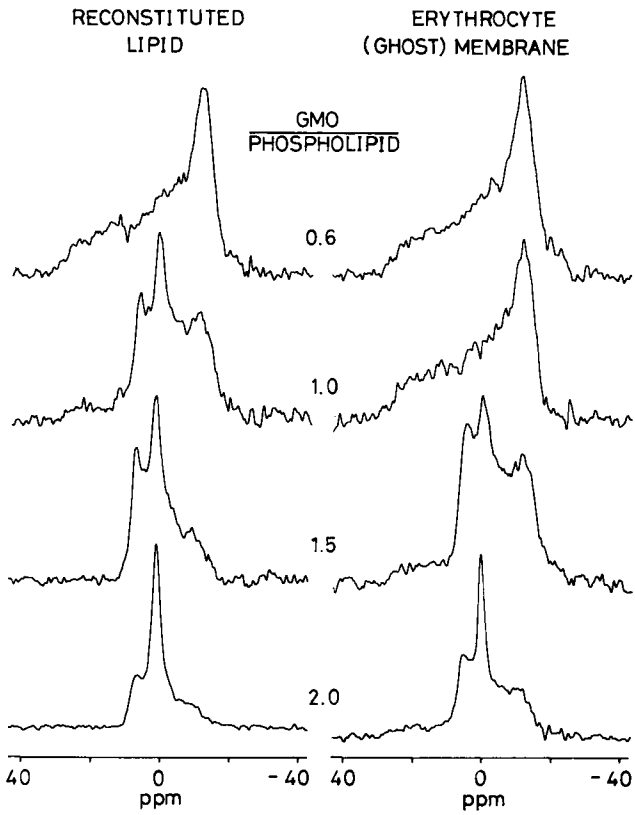

Fig. 16. 81.0 MHz ^{31}P-NMR spectra at 37°C obtained from (reconstituted) total erythrocyte lipid and erythrocyte (ghost) membrane in the presence of various concentrations of glycerol monoleate (GMO). For further details, see Hope and Cullis [51]. Reproduced with permission from Hope and Cullis [51].

strong correlation between the fusion event and the ability of endogeneous lipids to assume the nonbilayer (inverted cylinder) structure.

Although membrane-bound systems which undergo fusion *in vivo* do not contain equimolar (with respect to phospholipid) concentrations of fusogen, the fusogen results do suggest possible mechanisms of naturally occurring fusion. In particular they suggest that factors enhancing the ability of lipids to adopt inverted nonbilayer structures will also promote the fusion event. Given that Ca^{2+} is usually required for natural fusion [52], and that Ca^{2+} can trigger bilayer–H_{II} transitions in mixtures of acidic phospholipids with PE (Fig. 10), it is logical to suppose that the presence of Ca^{2+} allows the

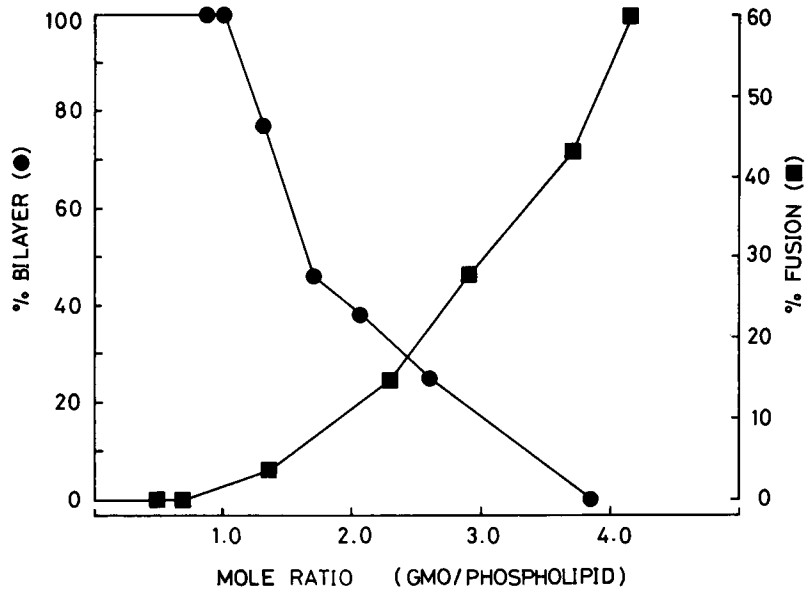

Fig. 17. A comparison of the extent of fusion between erythrocytes and the amount of phospholipid remaining in the bilayer phase in erythrocyte (ghost) membranes at various membrane concentrations of glycerol monoleate (GMO): ●—the percentage of membrane phospholipids in extended bilayers as indicated by ^{31}P-NMR; ■—the percentage fusion between erythrocytes following incubation for 2 hr with various concentrations of GMO which resulted in the indicated membrane-associated amounts of GMO. For further details, see Hope and Cullis [51]. Reproduced with permission from Hope and Cullis [51].

nonbilayer tendencies of endogenous lipid to be expressed, thus promoting the fusion event. Alternatively any other stimulus resulting in the same effect, such as lowering the pH in the case of systems containing PS and PA, should also promote fusion. These predictions appear to be justified, at least for model systems incubated in the presence of Ca^{2+}, as shown in the freeze-fracture micrographs of Fig. 18 (see also Verkleij *et al.* [25, 53]). These results, obtained for sonicated vesicle systems, composed of PE–PS [54], PE–PG [55], PE–PI [55], PE–CL [55], and PE–PA [55] illustrate two important points. First, in all cases the incubations result in the formation of larger structures, demonstrating that net fusion has occurred. Second, this fusion is accompanied by the observation of lipidic particles, and it is most interesting to note that these lipidic particles are often localized to regions corresponding to the fusion interface. This leads to the possibility that fusion proceeds via formation of intermediary inverted micellar structures; a possible model of this process is presented in Fig. 19.

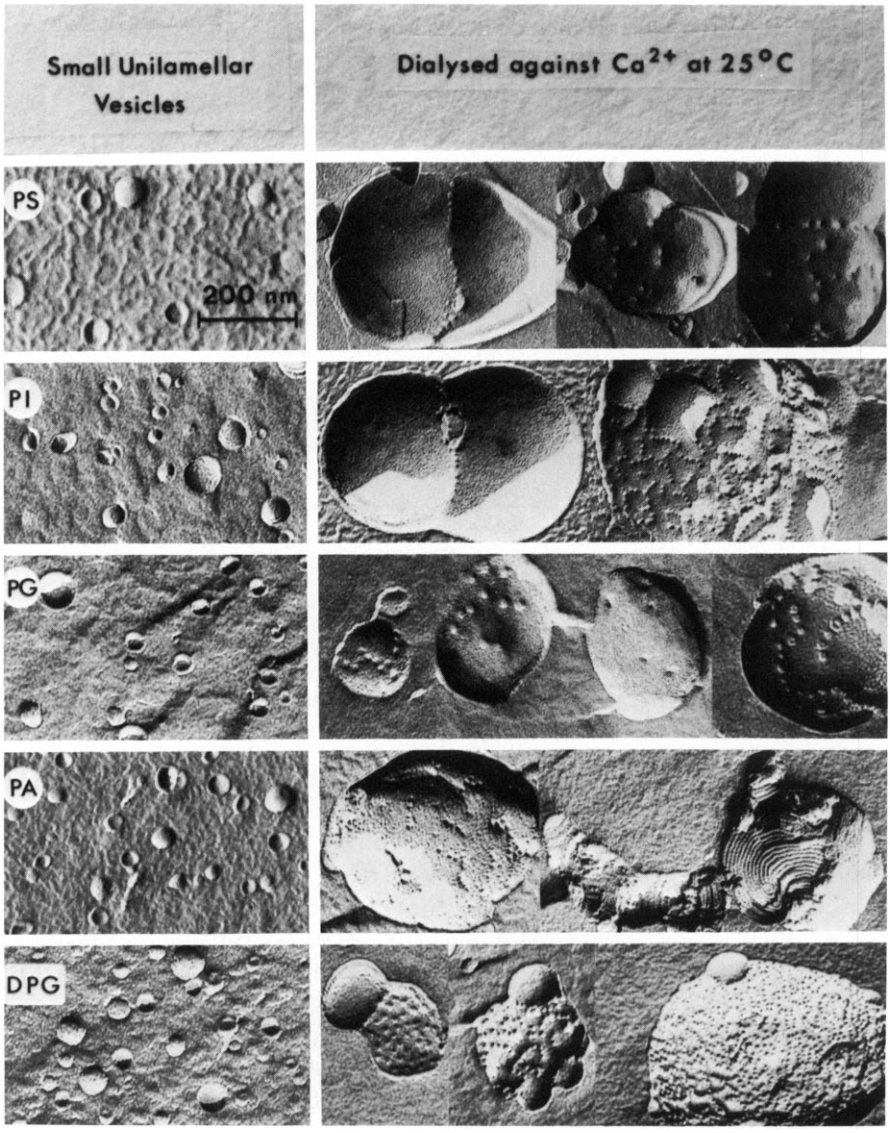

Fig. 18. Freeze-fracture micrographs showing Ca^{2+}-induced fusion of unilamellar vesicles consisting of soya phosphatidylethanolamine and a negatively charged phospholipid in the molar ratio of 4:1. Vesicles were prepared by sonication and subsequently dialysed at 25°C against buffer: 100 m*M* NaCl, 10 m*M* Hepes, pH 7.0 containing 10 m*M* Ca^{2+}. Samples were removed at various time intervals and frozen (in the presence of glycerol (25% v/v)) by plunging into a liquid–solid freon slush. Replicas were prepared employing standard procedures. The acidic phospholipids are: **PS** (egg phosphatidylserine), **PI** (soya phosphatidylinositol), **PG** (egg phosphatidylglycerol), **PA** (dioleoylphosphatidic acid) and **DPG** (beef heart cardiolipin).

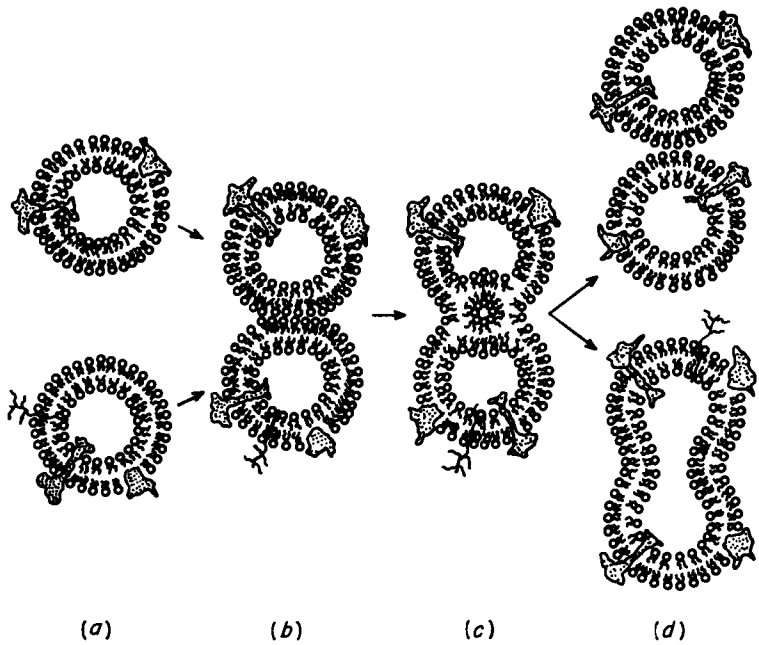

Fig. 19. Proposed mechanism of membrane fusion proceeding via an inverted cylinder or inverted micellar intermediate. The process whereby the membranes come into close apposition (*a*)–(*b*) is possibly protein mediated whereas the fusion event itself (*b*)–(*c*) is proposed to involve formation of an "inverted" lipid intermediate.

Exocytosis

An interesting expression of directed fusion *in vivo* is provided by the extracellular release of the contents of secretory granules, of which the Ca^{2+}-stimulated release of catecholamines from the chromaffin granules of adrenal medulla cells is particularly well characterized [56]. Our approach to studying the actual mechanism of exocytosis was based on the observation that release would be more efficient and controlled if Ca^{2+} stimulated granule–plasma membrane fusion rather than granule–granule fusion. Given our prejudice that nonbilayer lipid intermediates are required for fusion to proceed, it was reasoned that the inner monolayer lipids of the plasma membrane of the adrenal cells may play an active role. In particular, if this inner monolayer has a lipid composition approximately the same as that of the erythrocyte membrane, the influx of Ca^{2+} will destabilize this monolayer, enhancing the preference of the lipids for a nonlamellar organization. Such a reorganization appears to proceed as an *inter*bilayer event [57], hence the

instability of the inner monolayer lipids could be relieved by formation of inverted micellar (lipidic particle) structures with the outer monolayer of closely opposed granules, thus initiating the fusion process.

The model system employed to test such speculation is shown in Fig. 20, and consists of chromaffin granules incubated with sonicated vesicles composed of lipids which undergo structural reorganization in the presence of Ca^{2+}. Ca^{2+} is added to this mixture subsequently, and the release of chromaffin granule contents assayed. As shown in Fig. 21, these protocols could result in immediate and total release of granule contents for Ca^{2+} concentrations well below those which produced no release by themselves. Thus lipid mixtures such as PE–PS (as well as mixtures approximating the erythrocyte inner monolayer [57, 58]) can act as adjuncts for the Ca^{2+}-stimulated release of granule contents. These and other considerations (see Nayar *et al.*, [58] for full detail) lead us to propose a model for the exocytotic event as shown in Fig. 22.

Transbilayer Transport

The most important function of a membrane is to act as a selective permeability barrier between two aqueous compartments. It is generally as-

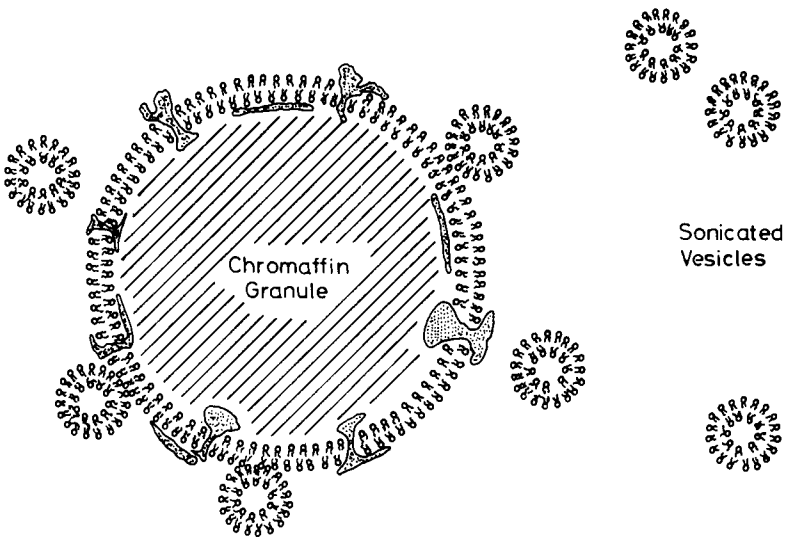

Fig. 20. Incubation procedure followed for incubation of chromaffin granules (large granule fraction) with sonicated vesicles. For further details, see Nayar *et al.* [58].

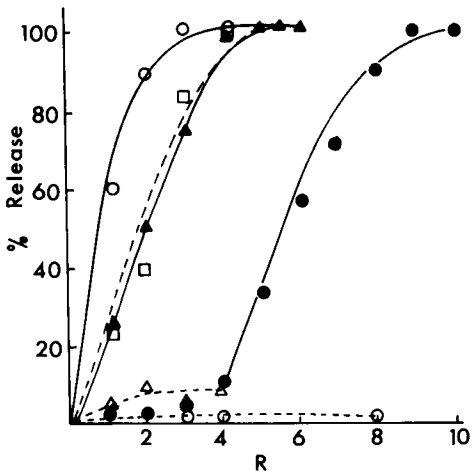

Fig. 21. Release of chromaffin granule contents after incubation (15 min) in the presence of increasing amounts of sonicated vesicles of varying composition, followed by the introduction of 5 m*M* Ca^{2+} after 10 min: ▲—soya PE:soya PS (3:1) vesicles; △—soya PC:soya PS (3:1) vesicles; □—egg PS vesicles; ○—beef heart cardiolipin vesicles; ●—vesicles with the "inner monolayer" lipid composition (PE:PS:PC:SPM in the ratios 0.5:0.25:0.13:0.12; equimolar cholesterol with respect to phospholipid); ○---○—vesicles with the "outer monolayer" composition. For details of experimental protocol, see Nayar *et al.* [58].

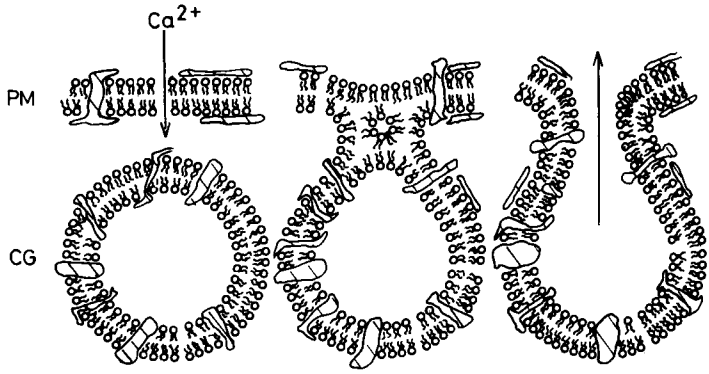

Fig. 22. Proposed mechanism of exocytotic release of chromaffin granule contents *in vivo*. PM refers to the chromaffin cell plasma membrane whereas CG denotes the chromaffin granule.

sumed that the lipid bilayer has an isolating function and only permits the free diffusion of small molecules and water. Specific permeability or translocation processes are thought to be mediated by proteins or polypeptides which may provide a pore or mobile carrier for particular substances. Although there is substantial experimental support for these hypotheses, the possibility that the lipids themselves may participate in translocation processes has not been seriously considered. This is because it is difficult to imagine how large polar molecules can move across a continuous lipid bilayer.

Nonbilayer lipids and the structures they can form give new possibilities however [57]. In particular lipids present in "inverted" structures such as the hexagonal H_{II} phase or inverted micellar structures can reside in a low energy configuration *within* a hydrophobic domain such as the interior of a lipid bilayer. Thus these structures with a hydrophilic interior and a hydrophobic exterior have some characteristics normally ascribed to a carrier molecule such as valinomycin. In Fig. 23 we depict a possible mechanism whereby the dynamic formation of inverted structures (inverted micelles) in a bilayer can act as a permeability pathway for both lipids and polar molecules within the aqueous compartment. Such transient events could be triggered by changes in the local concentrations of hexagonal H_{II} lipid species or by molecules which upon interaction with a lipid increase the tendency of that lipid to adopt an inverted structure.

An example of this latter process is the Ca^{2+} or cytochrome *c* interaction with cardiolipin which results in a transition from a lamellar to an inverted structure for a portion of the lipid. Two predictions can be made for systems displaying such behavior. First, transbilayer transport of lipids (flip–flop) which is usually a very slow process [59] should be greatly increased. Second, if the bilayer–nonbilayer transition is triggered by a modulator molecule, this molecule together with the lipid should be moved across the membrane via a carrier type of transport process. The first prediction has been tested in model systems containing unsaturated PE [60] and CL [61] where nonbilayer lipid structures have been induced by variations in temperature [60] and divalent cation concentrations [61]. Under both conditions phospholipid flip-flop was found to be greatly increased over that of control systems which did not contain "nonbilayer" lipid. Additional support comes from the observation that biological membranes in which phospholipid flip-flop is rapid (e.g., bacterial [59] and microsomal [62, 63] membranes) have a lipid composition and ^{31}P-NMR characteristics that are consistent with the presence of some nonbilayer lipid structures. Enhanced phospholipid flip-flop resulting from discontinuities in lipid packing around integral membrane proteins as well as at phase boundaries in mixed lipid systems is discussed elsewhere [2].

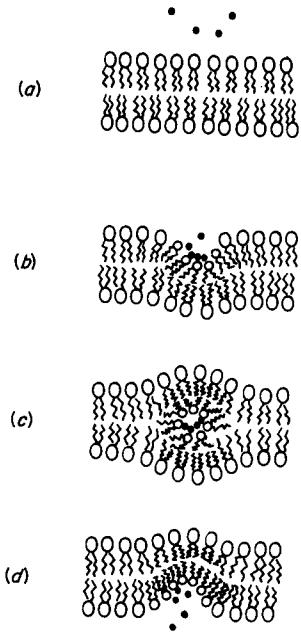

Fig. 23. A model of facilitated transport of Ca^{2+} (or other divalent cations) via formation of an intermediate intrabilayer inverted micellar cation—phospholipid complex—see part (*c*). The head-group of the charged phospholipid (e.g. CL or PA) interacting with the cation is depicted as being smaller in order to indicate a reduction in the area per phospholipid molecule in the headgroup region arising from reduced interheadgroup electrostatic repulsion in the presence of the cation. Reproduced with permission from Cullis *et al.* [57].

There is also evidence to support the prediction that nonbilayer lipid structures can act as carriers for molecules that trigger bilayer–nonbilayer transitions. For example, Mn^{2+} can induce the hexagonal H_{II} phase in pure cardiolipin dispersions [8] and rapidly permeates into mixed PC–CL liposomes, which also exhibit lipidic particle structure [61]. Furthermore, PA (which can undergo a bilayer to hexagonal transition upon interaction with divalent cations) can act as a carrier of cations across liposomal bilayers [64]. A further example concerns the addition of cytochrome *c* to multilayered cardiolipin liposomes resulting in the appearance of cytochrome *c* in the inner shells of the structure [31]. It has also been reported that phospholipids can form inverted micellar complexes which are soluble in organic solvents with a variety of polar compounds [65]. This is also consistent with carrier potential. The ability of a lipid to form such complexes with divalent cations has been related to the ability of the cation to induce the hexagonal H_{II} phase in an aqueous dispersion of that lipid [57]. In the case of biological

membranes the possible involvement of nonbilayer lipids in divalent cation transport has been proposed for two systems. These include Ca^{2+} influx following hormone–receptor interactions (this has been suggested to be mediated by PA [66, 67]) and the possibility that cardiolipin might participate in the Ca^{2+} transport system in mitochondria. This latter proposal is supported by the observation that ruthenium red, the classical inhibitor of this transport system, effectively blocks the formation of inverted structures by Ca^{2+} in cardiolipin-containing model membranes and blocks Ca^{2+} uptake into organic solvents by cardiolipin [57].

Intermembrane Communication

There are increasingly convincing indications that many different types of cell and organelle membranes are intimately connected with other membrane systems. Examples include the inner and outer membranes of *E. coli*, the inner and outer membranes of mitochondria, as well as continuities between the endoplasmic reticulum and the outer mictochondrial membrane, the nuclear membrane, and the Golgi [68, 69]. Such continuities could correspond to situations of arrested fusion (see Fig. 19(c)). Although

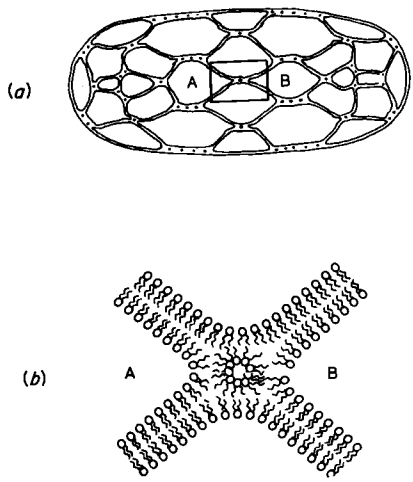

Fig. 24. A "honeycomb" structure compatible with ^{31}P-NMR, freeze-fracture and permeability results derived from systems containing mixtures of lipids which assume bilayer and hexagonal (H_{II}) phase structure in isolation. Compartmentalization within a continuous membrane structure is emphasized for compartments A and B in the expanded diagram of part (*b*).

direct evidence supporting such a conjecture is not available, freeze-fracture and ^{31}P NMR does suggest that lipidic particles (inverted micelles), formed between two closely opposed bilayers, can be long lived structures. In particular we have summarized evidence [57] indicating that multilamellar mixtures of bilayer and H_{II}-phase lipid can assume structures similar to that depicted in fig. 24. It is clear that such structures offer unique possibilities for maintaining compartmentalization within a continuous membrane structure.

A Rationale for Lipid Diversity—The Shape Concept

The ability of lipids to assume nonlamellar organizations offers new interpretations of the functional roles of lipids in biomembranes. In particular, lipids such as PC and SPM may be assigned functional roles related to maintenance of lamellar structure and an intact permeability barrier, whereas nonlamellar lipids such as PE allow formation of inverted micelles or cylinders which may play functional roles in fusion and related phenomena. Conversely, acidic phospholipids provide the possibility of isothermal transitions between lipid structures in appropriate lipid mixtures (as triggered by divalent cations or pH), thus providing mechanisms for isothermal regulation and control of membrane-mediated processes relying on nonbilayer intermediates. However there are a variety of factors that would suggest that this is not a complete story. First, the number of membrane functions potentially relying on nonlamellar lipid organization is not large (fusion, some transbilayer transport including flip-flop, and compartementalization within a continuous membrane structure) with membrane fusion being the only one for which relatively strong circumstantial evidence is available. Thus one is still left with the unsatisfactory situation indicated in the Introduction; the number of lipid species appears to vastly exceed the number of functional roles potentially involving expressions of nonlamellar lipid structure. Remaining questions that are not considered include the reasons for acyl chain diversity within a single phospholipid class, reasons why two or more lipid species preferring a bilayer organization are present in a single membrane (e.g. PC and SPM), questions concerning the role of cholesterol (which could play an adjunct role in fusion, but is not present in many membranes that undergo fusion), reasons why different acidic phospholipids are present in different membranes and so on. Although some of these differences could

be caused by differences in metabolic pathways, it is difficult to imagine that the enormous lipid diversity would not serve other roles. In order to answer these questions one must examine basic molecular factors that lead to the phase structure preferred by individual lipid species.

Initially, it seems that the macromolecular structure assumed by lipids in hydration is sensitive to a balance between the cross-sectional areas subtended by the polar and apolar regions respectively [70]. In the case of PE and PC, for example, the smaller headgroup of PE and relatively limited hydration of this headgroup as compared to PC may be expected to lead to a more "cone" shaped molecule compatible with H_{II} phase organization (see Fig. 25). The effective cross-sectional area of the head-group would be expected to be sensitive to electrostatic repulsion, which is consistent with the protonation of PS inducing H_{II} phase structure and with Ca^{2+} triggering bilayer to H_{II} transitions in CL. Perhaps the clearest demonstration of the

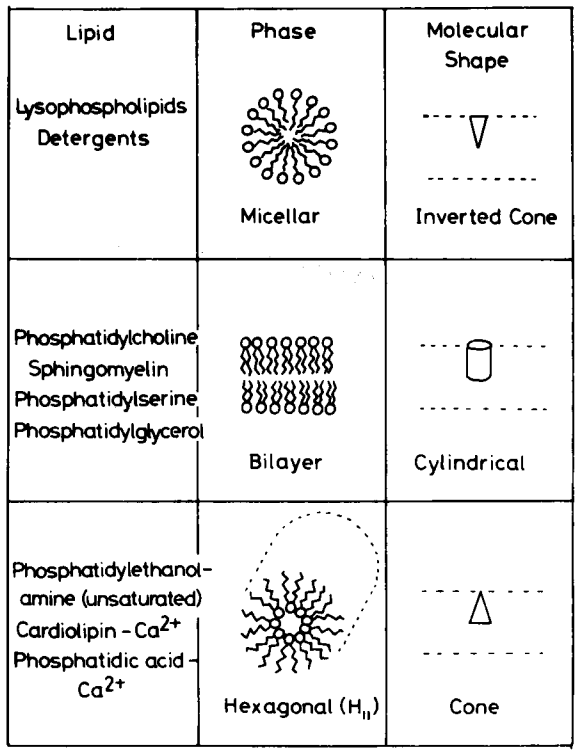

Fig. 25. Polymorphic phases and corresponding dynamic molecular shapes of component lipids. Reproduced with permission from Cullis and deKruijff [2].

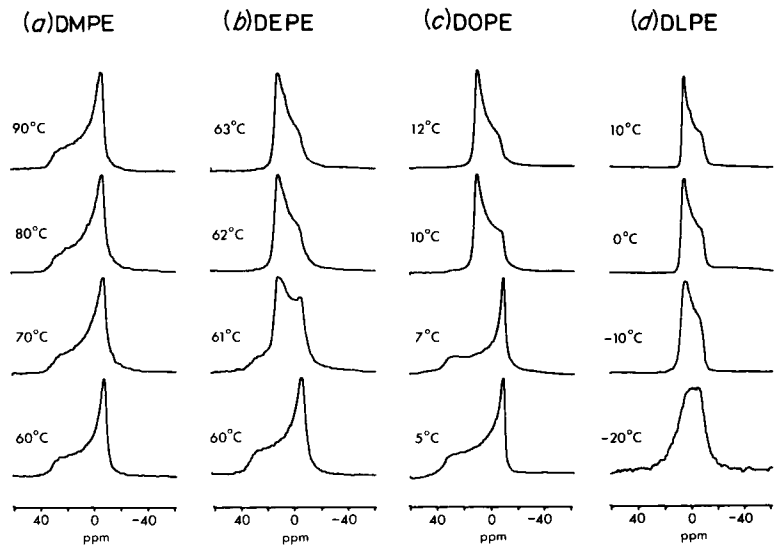

Fig. 26. 81.0 MHz ^{31}P-NMR spectra obtained from aqueous dispersions of different species of synthetic PE at various temperatures: (*a*) 14:0/14:0 PE (DMPE); (*b*) 18:1_t/18:1_t PE (DEPE); (*c*) 18:1_c/18:1_c PE (DOPE); (*d*) 18:2_c/18:2_c PE (DLPE). For further details of phospholipid synthesis and other protocols, see Tilcock and Cullis [90].

shape of the lipid molecule dictating the phase assumed is given by the effects of increasing the acyl chain unsaturation for PE. As shown in Fig. 26, a saturated PE remains in a bilayer organization at temperatures up to 100°C, whereas 18:1_t/18:1_t PE undergoes a bilayer to H_{II} transition at T_{BH} = 60°C. This T_{BH} is lowered for 18:1_c/18:1_c PE to 15°C and to ~ -15°C for 18:2_c/18:2_c PE. This increased affinity for the H_{II} organization as the acyl chain components assume a progressively larger cross-sectional area is most consistent with the shape hypothesis.

The proposal that the molecular shapes of lipids directly influence the structure formed is also supported by results obtained from mixed lipid systems. For example, from the simplistic divisions of Fig. 25 one may suggest that mixtures of cone (H_{II} phase) lipid with inverted cone (micellar) lipid should exhibit bilayer structures at appropriate ratios. This is indeed the case [71], as illustrated in Fig. 27 for mixtures of egg PE with several detergents commonly employed for membrane solubilization. Such behavior may be rationalized according to the shape hypothesis as shown in Fig. 28.

There are other examples of mixed systems composed of nonbilayer components which can assume a net bilayer structure in a manner consistent with the shape concept. These include mixtures of lyso-PC (LPC) with cho-

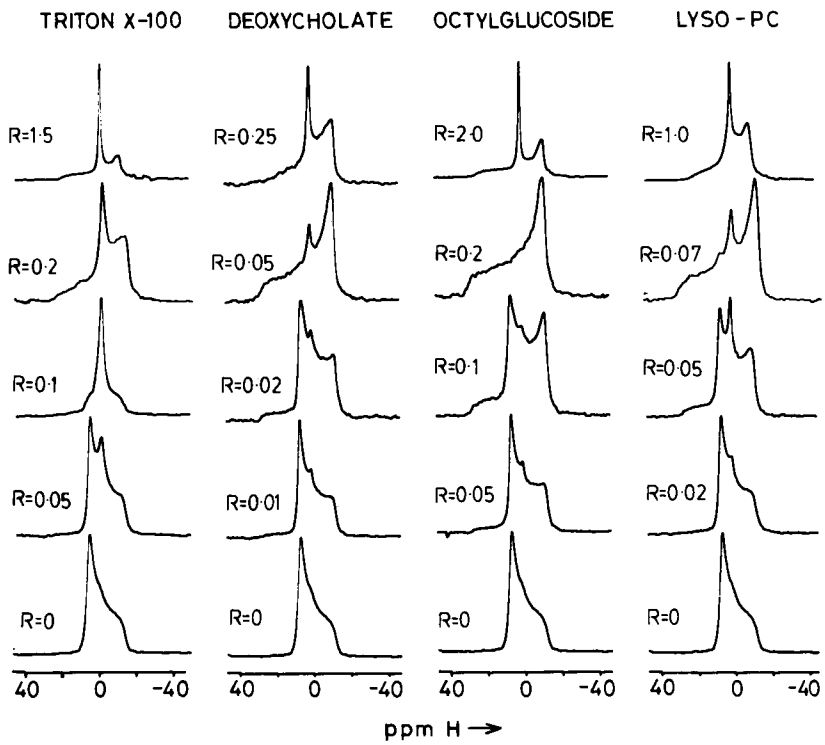

Fig. 27. 81.0 MHz ^{31}P-NMR spectra of egg yolk PE at 37°C in the presence of increasing amounts of various detergents. The detergent was added in appropriate amounts to the phospholipid in chloroform. Subsequently, the chloroform was evaporated and the lipid hydrated in 1 ml of standard buffer (see legend to Fig. 2). For details of protocol, see Madden and Cullis [71].

lesterol [72, 73] and fatty acids [74]. Again this behavior may be rationalized as resulting from complementarity between the inverted cone shape of LPC and cone-shaped cholesterol and fatty acids. That both cholesterol and fatty acids exhibit a net cone shape is suggested by the observation that both these agents can induce H_{II} phase structure in previously bilayer systems [18, 19, 50]. A further example is given by representative anaesthetics which can also stabilize the bilayer for unsaturated (egg) PE [75].

From the point of view of rationalizing lipid diversity in membranes, it is apparent that the shape concept offers some new possibilities for the functional roles of lipids. In particular, as originally proposed by Israelachvili and co-workers [39], it is likely that integral membrane proteins have somewhat irregular shapes within the bilayer. As indicated in Fig. 29, the availability of lipids with diverse geometry could provide possibilities for optimal packing

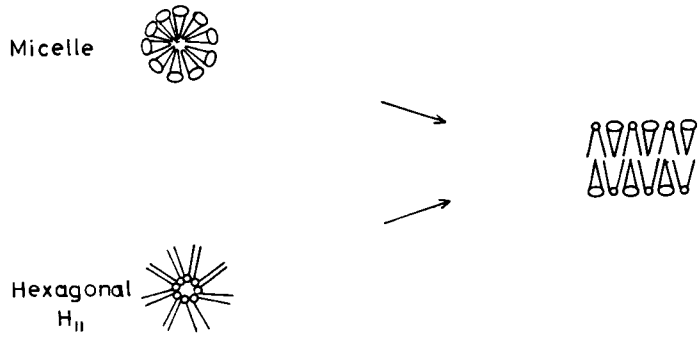

Fig. 28. Combination of "cone" shaped (H_{II} phase) lipids with "inverted cone" (micellar) lipids to produce a net bilayer structure.

and sealing at the protein interface. Such conjectures are consistent with investigations of transbilayer transport in reconstituted glycophorin–lipid model systems. Incorporation of glycophorin in DOPC bilayers results in increased phospholipid flip-flop [76, 77] and permeability to shift reagents [78]. However incorporation of small amounts of cone or inverted cone shaped lipid (e.g., PE or LPC) [78] or reconstitution of the protein with total

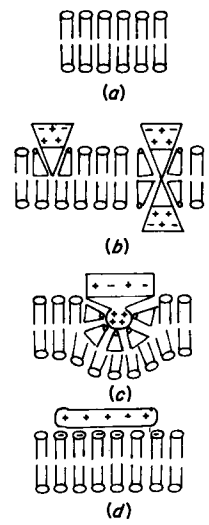

Fig. 29. Potential roles of lipid as a result of shape and/or charge in maintaining the architecture of (bilayer) lipid–protein membrane systems: (*a*) maintenance of bilayer structure, PC, SPM; (*b*) sealing at lipid–protein interface, PE, MGDG; (*c*) penetration (anchoring) of polar protein, CL, PA; (*d*) association of basic protein, PS, PG, PI.

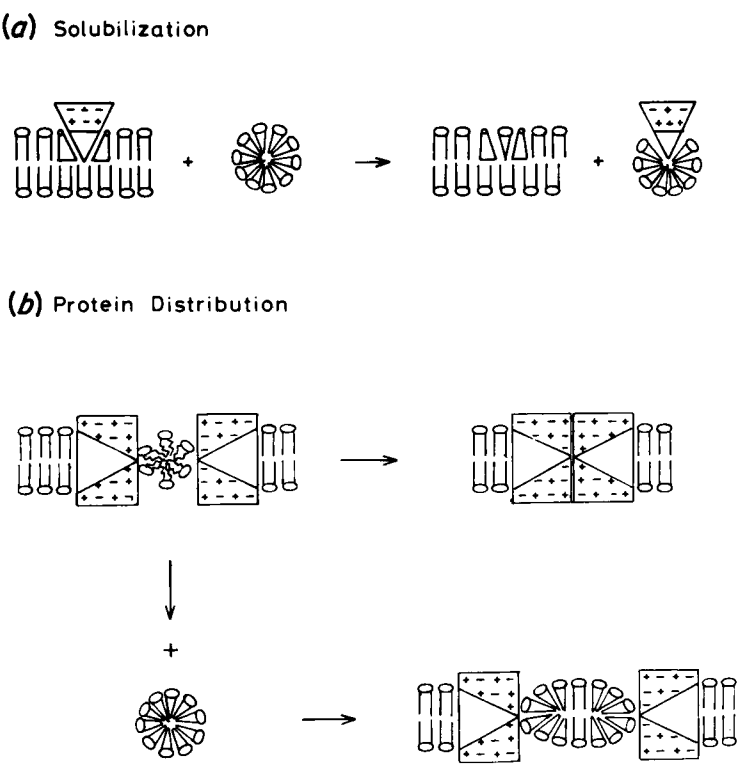

Fig. 30. Modulation of protein–protein interactions by the availability of lipids of various shapes: (*a*) dimer formation as a result of distribution of polar and apolar residues (see Israelachvili [84]) (*b*) production of stable monomers due to the availability of "inverted cone" (micellar) lipids.

erythrocyte lipids [79] results in sealed systems. Such observations give a rather different perspective on boundary or annular lipids than is currently in vogue. Alternative roles of lipids within the context of maintaining a stable semipermeable bilayer protein–lipid membrane are indicated in Fig. 29.

These and previous observations suggest that biological membranes regulate their lipid composition so as to obtain a variety of lipids with an appropriate distribution of various shapes. This proposal is consistent with the elegant experiments of Wieslander *et al.* [80] and Silvius *et al.* [81] on the *Acholeplasma laidlawii* membrane. Further, it may be noted that alterations in the lipid composition of *E. coli* [82] and other systems according to growth temperature, which have previously been interpreted as reflecting a need to

conserve a certain membrane "fluidity", can equally well be taken to support the notion that lipid shape is the conserved quantity.

Before closing this section on the potential roles of lipids with varying molecular shapes, it is interesting to note that biomembranes usually contain only two major varieties—namely, those with cylindrical shapes (bilayer) and those with cone shapes (H_{II} phase). In this context, detergent lipids are members of a general class of amphiphilic molecules commonly designated as anesthetics [83]. Low membrane levels of such agents have many remarkable, and as yet inexplicable, effects on membranes and their mechanism of action poses a major problem in membrane biology. This is because a large body of evidence suggests that the anesthetic potency of a given compound is directly related to its solubility (partition coefficient) in a hydrocarbon environment [83], whereas the result of its presence clearly is to affect the ability of some membrane proteins (e.g., the Na^+ channel or other facilitated transport proteins) to function. Thus one is led to believe that the Na^+ channel, for example, exists as a lipid–protein complex, and anesthetics somehow disrupt or replace the lipid participating in that complex. The observation that anesthetics have, as a general property, shapes different than other membrane components leads to the possibility that they may affect membrane function by virtue of that shape. An example among many possibilities is given in Fig. 30, which is an extension of a model due to Israelachvilli [84] who suggested that membrane protein aggregation may result from an appropriate distribution of polar and apolar residues. In Fig. 30 we suggest that anesthetic type lipids could affect such oligomer formation by virtue of these shapes, thus modulating protein function.

Closing Remarks

In closing, the spirit in which this chapter is written should perhaps be emphasized. The fundamental observation on which the many conjectures made here are based is that lipids in membranes do not, as a general rule, assume a bilayer organization upon hydration. In our view this leads to the strong possibility that those lipids preferring nonlamellar organization play functional roles which are not necessarily related to maintenance of bilayer structure. We have pointed out that many factors known to regulate membrane-mediated phenomena (divalent cations, pH, membrane protein, anesthetics, etc.) can strongly affect the macromolecular structures assumed under conditions which are not unduly removed from the physiological

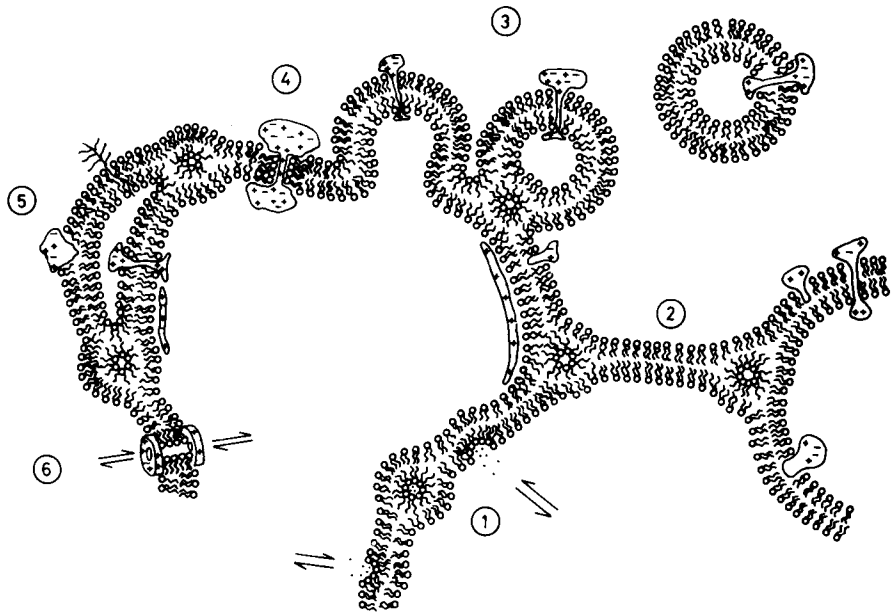

Fig. 31. A metamorphic mosaic model of biological membranes illustrating various structures and processes suggested by the ability of lipids to assume nonbilayer configurations. In part 1 transbilayer transport of polar molecules (e.g., divalent cations) is facilitated by intermediary formation of inverted micelles, whereas part 2 indicates membrane continuity between membrane bound compartments. In part 3 a process of budding off of a membrane bound vesicle is illustrated, as discussed elsewhere. The protein in part 4 is shown to assume a transmembrane configuration without the requirement for an apolar sequence of amino acids. The protein penetrates the membrane through a (short) cylinder of phospholipid. In part 5 compartmentalization is depicted within a continuous membrane system, whereas part 6 indicates possibilities of transmembrane transport where hexagonal (H_{II}) phase lipids form an aqueous pore through the membrane. This lipid configuration is stabilized in an orientation perpendicular to the plane of the surrounding bilayer by doughnut-shaped proteins with hydrophilic and hydrophobic sides, which could also serve as selectivity filters. Reproduced with permission from Cullis *et al.* [57].

situation. In addition we show that for a function such as fusion, which logically requires some nonlamellar intermediate, there are strong correlations between the availability of nonlamellar organizations and the expedition of fusion. Although the picture that emerges is by no means complete, the lamellar–nonlamellar characteristics of lipids lead to recognition of a new basic shape property of lipids, leading in turn to a different understanding of reasons for lipid diversity. Although none of the functional roles of lipids have been unambiguously identified in terms of their molecular shape, it

clearly provides a rich hypothesis on which future experimentation may be based. A synopsis of these possibilities is given in Fig. 31.

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