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Lipid Polymorphism and Membrane Function

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I. INTRODUCTION

One of the fundamental problems in membrane biology is that of lipid diversity. The number of chemically different membrane lipids is much larger than other key biological building blocks such as nucleotides, amino acids, and carbohydrates. For instance, a relatively simple biomembrane such as that of the red blood cell contains well over a hundred different lipid species.

Except for some metabolic and receptor-type functions, current models of biological membranes do not take into account this lipid diversity. In the fluid mosaic model (Singer and Nicolson, 1972), the lipids are thought to form a fluid semipermeable bilayer which acts as a matrix for the functional membrane proteins. On the basis of our present understanding, a single unsaturated phosphatidylcholine (PC) species could easily satisfy such demands.

The incomplete nature of such membrane models is reinforced by two basic membrane phenomena. First, biological membranes are highly dynamic structures which are continuously involved in a variety of biochemical processes during which transient departures from bilayer structure must occur. Examples include the transbilayer transport of lipids and proteins as well as membrane fusion, among others. Second, although the ability of lipids to adopt a variety of phases has been recognized

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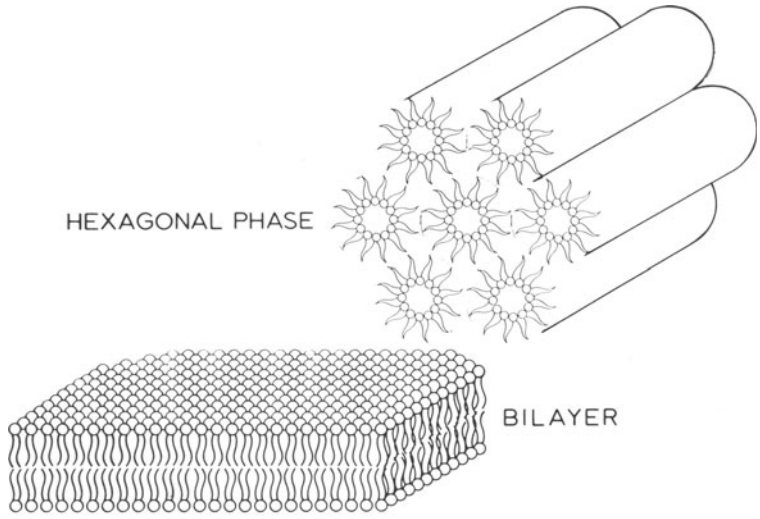

Figure 1. Schematic representation of the bilayer and hexagonal H_{II} phase.

for quite some time (Lucy, 1964; Luzzati *et al.*, 1968; Reiss-Husson, 1967; Shipley, 1973), only in recent years has it become apparent that several major membrane lipids assume the nonlamellar hexagonal, type II (H_{II}) phase (see Figure 1) when dispersed in isolated form in excess buffer under physiological conditions (pH, ionic strength, and temperature). This phase consists of hexagonally organized lipid cylinders in which the polar head groups of the lipid molecules surround a narrow aqueous channel. It is obvious that the presence of such lipids in membranes poses important questions concerning their structural and functional roles.

In this chapter, we present an inclusive review of the various aspects of lipid polymorphism. It will be shown that the ability of lipids to adopt different structures and the rationalization of this behavior via the "molecular shape" concept offers attractive possibilities for understanding the reasons for lipid diversity and provides new perspectives on structure–function relationships in biomembranes.

II. MEMBRANE LIPID POLYMORPHISM: TECHNICAL ASPECTS

Small angle X-ray diffraction is the classical technique to elucidate the macroscopic organization of (phospho) lipid aggregates (for reviews see Luzzati, 1968; Shipley, 1973). In lipid systems that possess some form of long-range order, analysis of the relationship between first and higher order reflections can provide an unambiguous means for lipid phase determination.

A number of authors (Luzzati *et al.*, 1968; Shipley, 1973; Reiss-Husson, 1967; Luzzati, 1968; Rand *et al.*, 1971; Janiak *et al.*, 1976; Harlos and Eibl, 1980) have shown that, in agreement with theory, in multibilayer systems, the spacings of the first and higher order reflections relate as 1 : 1/2 : 1/3. Depending on the sample

particulars, sometimes fourth, fifth, or even higher order reflections can be seen. The intensity of the various reflections is highly variable and depends also on the nature of the sample. In fact, in some multilamellar systems, only the first order reflection is observed (Harlos and Eibl, 1980; Marsh and Seddon, 1982), which makes a conclusive assignment of the phase rather difficult. The largest first-order spacing corresponds with the lamellar repeat distance, which is comprised of the bilayer thickness and the thickness of the water layer in between the bilayers.

Alternatively, sharp reflections in the ratio $1 : 1/\sqrt{3} : 1/2 : 1\sqrt{7}$ are also commonly encountered in lipid preparations (Luzzati *et al.*, 1968; Shipley, 1973; Reiss-Husson, 1967; Luzzati, 1968; Rand *et al.*, 1971; Janiak *et al.*, 1976; Harlos and Eibl, 1980; Marsh and Seddon, 1982) and found to be typical for the two-dimensional periodicity of hexagonally packed cylinders. Experimentally, the $1/\sqrt{7}$ and higher order reflections are seldom found. For naturally occurring lipids, the hexagonal H_{II} phase is found most often, rather than the hexagonal, type I (H_I) phase, where the polar head groups are at the periphery of the cylinders. The largest spacing of the hexagonal H_{II} phase (d) relates to the diameter of the lipidic cylinders (a) as $d = (\sqrt{3}/2).a$.

When a three-dimensional periodicity is present in a lipid preparation, often many reflections can be found (Luzzati *et al.*, 1968; Shipley, 1973; Rivas and Luzzati, 1969; Larsson *et al.*, 1980; Lindblom *et al.*, 1979). Provided that enough reflections are observed, an attempt can be made to characterize the lattice type and space group to which the structure belongs. Frequently, a cubic symmetry is found for lipid structures. As a possible model for some cubic lipid phases, an interesting structure based upon deformed bilayer units that are attached to create two different continuous aqueous networks has been proposed (Larsson *et al.*, 1980; Lindblom *et al.*, 1979).

When the long-range order is distorted or not present at all, the sharp Bragg reflections are replaced by continuous scattering profiles that show far less detail and are much more difficult to interpret in an unambiguous way. This particularly complicates the analysis of the phase state in samples that contain different phases in which only one of the phases is ordered. In this case, X-ray diffraction can totally overlook the presence of the disordered phase.

The application of X-ray techniques to biological membranes is hampered by the fact that the exposure times are often long compared to the biological stability of the preparations. Furthermore, only some specialized membrane systems such as nerve myelin, retinal rod outer segments, and chloroplasts are composed of ordered arrays of membranes, facilitating interpretation of the data. In general, biological membranes lack this order and only the scattering profile can be used to evaluate the properties of the membrane (for review see Blaurock, 1982).

Among the electron microscopic techniques to study lipid polymorphism, freeze-fracturing has been shown to be the most valuable method. This can be attributed to (1) the reliable fixation procedure by fast freezing, (2) the fact that beam damage in the microscope is excluded by the replication method, (3) the understanding of the fracture mechanism, (4) the resolution power of about 30 Å (Zingsheim, 1972; Verkleij and Ververgaert, 1978), and (5) like any electron microscopic method, it provides direct visualization of the macromolecular structure in contrast to the spectroscopic

and diffraction methods, which give averaged information originating from many sites simultaneously. This feature is also evident in the study of lipid polymorphism in that it has revealed unique structural details such as lipidic particles, transitional intermediates, and overall morphology and heterogeneity of the various systems.

Pure lipid phases characterized by X-ray diffraction have been visualized by freeze-fracturing (Deamer *et al.*, 1970). Liposomes consisting of concentric bilayers show alternating smooth fracture faces (Figure 2). The hexagonal H_{II} phases show distinct fracture faces composed of long parallel lines (giving a ribbed appearance) which occur along at least two fracture planes at angles of approximately 120° to each other (see Figure 2). The repeat distances of the bilayer and the different diameters of hexagonal H_{II} phase agreed well with X-ray repeat distances, which clearly demonstrates that the structure is well preserved during the freezing method. However, it is clear from other studies that because of temperature-induced disorder–order or hexagonal H_{II}-lamellar phase transitions, the high-temperature phase may not be preserved with the current quenching procedures. However, with the development of ultrarapid freezing devices, one is able to prevent alterations in the lipid organization although phase transitions occurring at temperatures higher than 20–30°C may not be prevented. A second argument to use fast-freezing devices is that one does not need to use cryoprotectants which could affect lipid structures (Boni *et al.*, 1981). Therefore, it is highly recommended to use ultrarapid quenching methods, e.g., spray-freezing

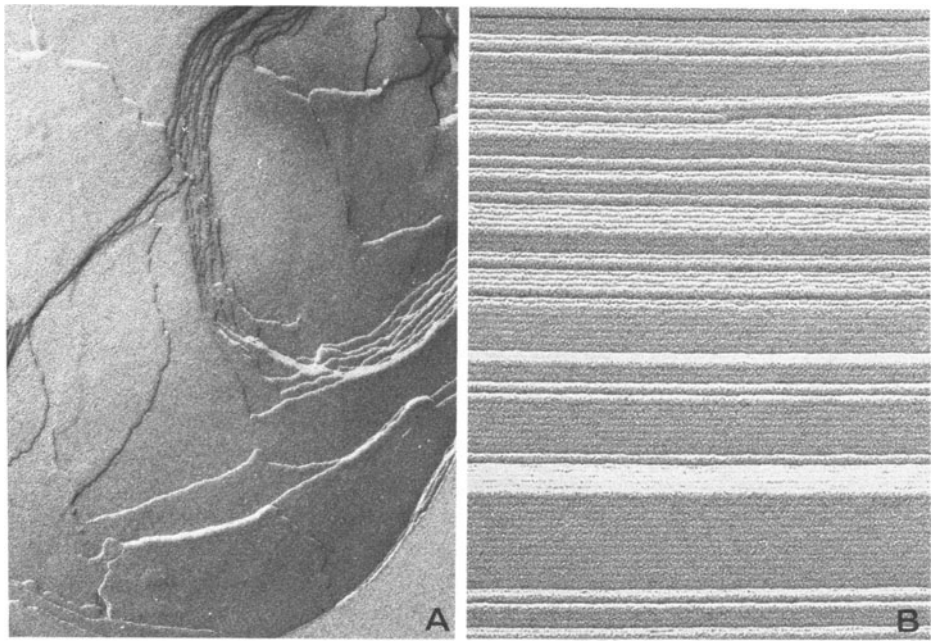

Figure 2. Freeze-fracture electron microscopy of (A) a multilamellar liposome (egg-PC) and (B) a hexagonal H_{II} phase (18 : 1_c/18 : 1_c-PE). Final magnification ×100,000.

(Bachmann and Schmitt, 1971), the copper black method (Heuser *et al.*, 1979), or the jet-freezing method (Moor *et al.*, 1976) to study the various lipid phases.

The introduction of nuclear magnetic resonance (NMR) techniques, especially ^{31}P NMR, has been greatly advantageous in studying lipid polymorphism. They allow for a convenient and quantitative discrimination between the most important phases found for hydrated membrane lipids. Samples can be heterogeneous, long-range order is not required, and these techniques can be equally well applied to model and biological membranes. Since NMR signals contain contributions both from structural and motional origin, the use of NMR to obtain structural information is extrapolative. Therefore, we will first relate the different types of ^{31}P NMR lineshapes to their corresponding phospholipid structures and give some insight in the theories describing the various lineshapes (for a detailed review see Seelig, 1978).

The lineshape of the ^{31}P NMR signal of phospholipids is determined both by the chemical shielding (or shift) anisotropy of the lipid phosphate moiety and ^{1}H–^{31}P dipolar interactions. The dipolar interactions complicate interpretation of the various lineshapes and therefore are most often reduced by applying strong proton-decoupling irradiation.

Dry lipid powders represent a completely immobilized system and are used as a reference state. Figure 3 shows a dry powder proton-decoupled ^{31}P NMR spectrum of 16 : 0/16 : 0-PC · H_2O. Since the electron density around the phosphorus nucleus is not isotropic but depends on the bonding pattern, different orientations of the phosphate segment in a magnetic field will result in different shielding and hence give rise to resonances at different frequencies. This phenomenon is known as chemical shift anisotropy (CSA). In a dry lipid powder, all possible orientations of the phosphate segment occur. Therefore, the spectrum in Figure 3 is a superposition of resonance frequencies corresponding with all those orientations and weighed by the distribution function of all microcrystalline regions. The resonance frequencies of the edges and the peak of the dry powder spectrum correspond with the principal elements of a static axially asymmetric tensor, which describes the CSA. Inspection of these principal tensor elements of various phospholipids from natural and synthetic origin, including PCs (Griffin, 1976; Kohler and Klein, 1977; Herzfeld *et al.*, 1978; Van Echteld *et al.*, 1981b), phosphatidylethanolamines (PEs) (Kohler and Klein, 1977; Herzfeld *et al.*, 1978), phosphatidylserines (PSs) (Kohler and Klein, 1977; Hope and Cullis, 1980), phosphatidylglycerols (PGs) (Farren and Cullis, 1980), phosphatidylinositol (PI) (Nayar *et al.*, 1982), sphingomyelin (Cullis and Hope, 1980), and lyso-PC (Van Echteld *et*

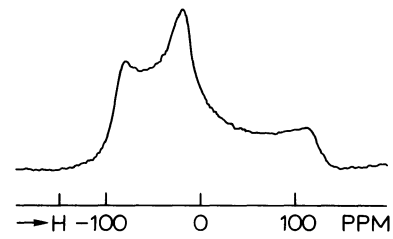

Figure 3. Dry powder proton-decoupled 81.0 MHz ^{31}P NMR spectrum of 16 : 0/16 : 0-PC · H_2O at 25°C. For experimental details see Van Echteld *et al.* (1981a,b). In this and other spectra of 0 ppm position corresponds to the chemical shift of the ^{31}P NMR resonance of sonicated egg-PC vesicles.

al., 1981b), reveals a great similarity with the exception of the phosphomonoester phosphatidic acid (PA) (Kohler and Klein, 1977). This similarity indicates very similar local conformation in the phosphate region of all these lipids. Also, other immobilized systems such as the calcium salts of certain PGs (Farren and Cullis, 1980) and PSs (Hope and Cullis, 1980) next to hydrated PCs and PEs at very low temperatures (Herzfeld *et al.*, 1978) give rise to powder-type ^{31}P NMR spectra with principal values of the CSA tensor similar to those observed for the dry phospholipid powders.

The hydration of phospholipids leads to the onset of motion, which partially averages the CSA. Hydrated phospholipids organized in extended bilayers give rise to a ^{31}P NMR spectrum which is characteristic for a shielding tensor that is axially symmetric around a director axis. Figure 4 shows an example of such a spectrum with a high field peak and a low field shoulder. An insight in the nature of this lineshape can be obtained by orienting the phospholipid bilayers between glass plates such that the long axes of the molecules are perpendicular to the glass plates. Stepwise rotation of these oriented bilayers in the magnetic field gives rise to individual narrow resonances with an angular-dependent chemical shift (McLaughlin *et al.*, 1975, 1981; Seelig and Gally, 1976; Hemminga and Cullis, 1982). The high field peak of the unoriented sample has been found to correspond with phospholipid molecules, which have their long axes perpendicular to the magnetic field ($\sigma_\perp$), whereas the shoulder resonance position corresponds with a parallel orientation ($\sigma_\parallel$). In general, the resonance frequency $\Delta\nu$ of phospholipid molecules having director axes at an angle α with the magnetic field is given by (Seelig, 1978; Cullis and De Kruijff, 1978a)

$$\Delta\upsilon(\alpha) = -2/3\,\Delta\sigma\left(\frac{3\cos^2\alpha - 1}{2}\right)\text{ ppm} \tag{1}$$

where the residual chemical shift anisotropy is:

$$-\Delta\sigma = \Delta\upsilon(0°) - \Delta\upsilon(90°) = \sigma_\parallel - \sigma_\perp$$

When these resonance frequencies $\Delta\upsilon(\alpha)$ are plotted vs. $(3\cos^2\alpha - 1)/2$ and taking α, the angle between the bilayer normal and the magnetic field, a straight line results for several phospholipids studied (McLaughlin *et al.*, 1981; Hemminga and Cullis, 1982; Cullis and De Kruijff, 1978a). This proves that the director axis coincides with

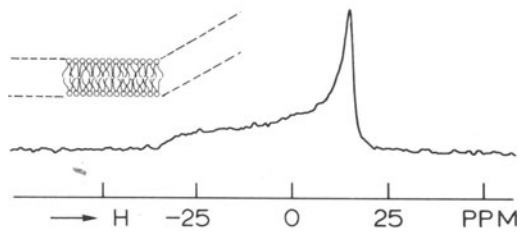

Figure 4. Proton-decoupled 81.0 MHz ^{31}P NMR spectrum of an aqueous dispersion of dierucoylphosphatidylcholine at 25°C. For experimental details see Van Echteld *et al.* (1982).

the bilayer normal and that the phosphate segment (and likely the whole molecule) rapidly rotates ($\tau_c \leq 10^{-6}$ sec) around the bilayer normal.

The lineshape of the spectrum in Figure 4 can be understood when realizing that, in liposomal lipid dispersions, molecules are found in every direction and therefore the spectrum will be a superposition of individual resonance lines from Eq. (1), weighed by the probability of finding lipid molecules with their long axes at angles α. This can be visualized by regarding the low number of lipid molecules in a large spherical bilayer with their long axes parallel to a given magnetic field as opposed to the high amount of lipid molecules with their long axes perpendicular to the field direction.

Up until now, for all naturally occurring membrane phospholipids organized in liquid-crystalline lamellar phases, ^{31}P NMR lineshapes have been observed such as shown in Figure 4. The $\Delta\sigma$-values of the ^{31}P NMR spectra of various phospholipids are all of the same magnitude and range from approximately 55 to 30 ppm (Seelig, 1978; Cullis and De Kruijff, 1978a). Comparable values of $\Delta\sigma$ are found for the ^{31}P NMR spectra of some biological membranes (McLaughlin *et al.*, 1981; Cullis, 1976a; De Kruijff *et al.*, 1976a; Seelig *et al.*, 1981). These data together with the observations that the ^{31}P NMR lineshape and $\Delta\sigma$ of bilayer organized phospholipids are hardly affected by the presence of membrane proteins, cations, cholesterol, fatty acid composition, and temperature demonstrate that the structure of the phosphate region is highly conserved (Seelig and Seelig, 1980). The structural stability of that part of the lipid molecule is also born out by ^{2}H NMR (Seelig and Seelig, 1980), neutron (Büldt *et al.*, 1978), and X-ray (Hauser *et al.*, 1981) diffraction studies. Suggestions (Thayer and Kohler, 1981) that changes in local structure can give rise to spectral changes resembling those noticed during polymorphic phase transitions (which will be discussed later) are theoretically valid, but in the absence of any evidence for the occurrence of such changes, rather speculative.

However, factors that do influence the ^{31}P NMR lineshape include the inter- and intramolecular ^{1}H–^{31}P dipolar interactions. Experimentally, these dipolar contributions are suppressed by applying proton decoupling. Unfortunately, this decoupling is imperfect in situations where dipolar couplings are relatively strong as for gel-state lipids. This results in substantial signal intensity at the edges of the spectrum beyond the resonance positions $\sigma_{\parallel}$ and $\sigma_{\perp}$ and a less well-defined lineshape.

Another important parameter which may determine the ^{31}P NMR lineshape of lamellar organized phospholipids is the reorientation rate of the molecules. For isotropic rotational motion of a spherical vesicle, this reorientation rate can be described by a correlation time τ_c that is related to the vesicle size by (Cullis, 1976b; Burnell *et al.*, 1980a)

$$\frac{1}{\tau_c} = \frac{6}{r^2}(D_t + D_{\text{diff}}) \tag{2}$$

where $D_t = kT/8\pi r\eta$ (η = medium viscosity) is the vesicle-tumbling-dependent part describing Brownian rotational diffusion and D_{diff} is the rate of lateral diffusion of the lipid molecules in the bilayer. When the reorientation rate of the phospholipid molecules is sufficiently fast, i.e., $\tau_c < 1/\Delta\sigma$, motional narrowing of the ^{31}P NMR spectrum will occur, until a final situation is reached where isotropic motional averaging will

lead to a narrow symmetrical "high resolution" resonance line. From Eq. (2) it can be seen that τ_c is dependent on the vesicle radius. To gain insight in the influence of the vesicle size on the ^{31}P NMR lineshape, spectra were simulated for different radii, which are shown in Figure 5. It can be seen that the asymmetrical lineshape with a high field peak and a low field shoulder only appears for vesicles with radii greater

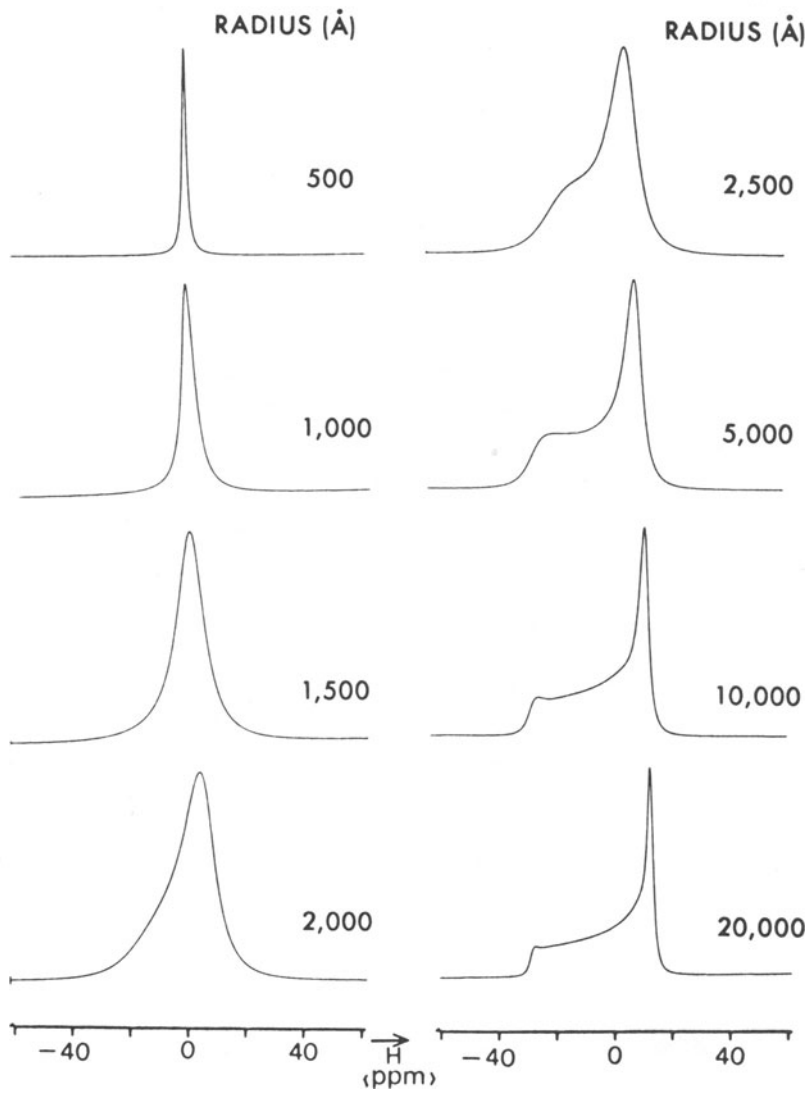

Figure 5. Simulated ^{31}P NMR spectra of 18 : 1_c/18 : 1_c-PC vesicles of different sizes at 30°C. The spectra were simulated using $\Delta\sigma$ = 3550 Hz, η = 0.008 P, orientationally independent Lorentzian full line width at half height = 60 Hz, $D_{diff} = 6.2 \cdot 10^{-7}$ cm^2/sec and r as indicated. Reproduced with permission from Burnell *et al.* (1982a).

than 2000 Å (assuming lateral diffusion rates in the order of 5×10^{-7} cm^2/sec) (Burnell *et al.*, 1980a).

The narrow symmetrical lineshape seen in Figure 6, resulting from isotropic motional narrowing, is not restricted to lipid bilayer vesicles with small radii, but such ^{31}P NMR spectra will result in all those situations where the phospholipid molecules can reorient themselves fast ($\tau_c < 10^{-5}$ sec) such as in micelles, inverted micelles, and cubic phases. Examples of these "isotropic" structures are presented in Figure 6.

For phospholipids organized in the hexagonal H_{II} phase, lateral diffusion of the lipid molecules around the cylinders which are the constituents of this phase will result in an additional averaging of the CSA. This can be understood in the following way: when a hexagonal phase cylinder is oriented with the cylinder axis parallel to the magnetic field, all the long axes of the phospholipid molecules are oriented perpendicular to the magnetic field and hence a resonance will be observed at $\sigma_\perp$ ppm. However, when the cylinder axis is perpendicular to the magnetic field, a completely different situation arises, since now a cylindrical distribution of phospholipid molecules is seen by the spectrometer. In general, it can be shown (Seelig, 1978; Cullis and De Kruijff, 1978a) that the resonance frequency $\Delta\nu(\beta)$ of phospholipid molecules in a particular cylinder having its axis at an angle β with the magnetic field direction is given by

$$\Delta\nu(\beta) = 1/3\ \Delta\sigma \left(3\frac{\cos^2\beta - 1}{2}\right) \text{ ppm}$$

The difference between the two extreme orientations is then

$$\Delta\nu(\beta = 0°) - \Delta\nu(\beta = 90°) = 1/2\ \Delta\sigma \tag{3}$$

For a random distribution of hexagonal cylinders, the probability of finding cylinder axes perpendicular to the magnetic field is higher than the probability of finding cylinder

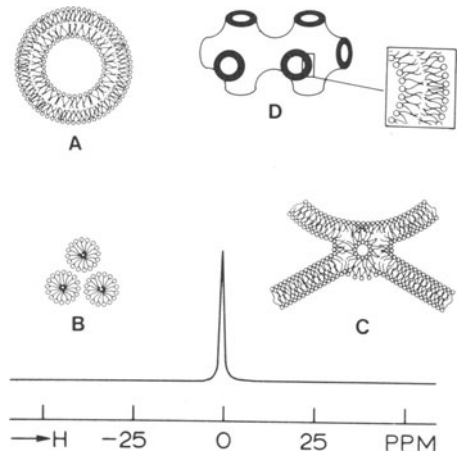

Figure 6. Various phospholipid structures that can give rise to a narrow symmetrical ^{31}P NMR lineshape at 0 ppm. (A) Sonicated vesicle, (B) Micelles, (C) inverted micelle, and (D) example of a cubic phase (Larsson *et al.*, 1980).

axes parallel to the magnetic field. Therefore, it can be shown in an analogous way as for the bilayer situation that for the hexagonal phase in the superposition of the individual resonance lines at all angles β a peak will arise at $\Delta\nu(= 90°)$ and a shoulder at $\Delta\nu(\beta = 0°)$, resulting in a reversed asymmetry, compared to the bilayer spectra, and a reduction in the separation of the spectral edges by a factor of two as shown in Figure 7. Experimentally, this separation between peak and shoulder has indeed been found to be approximately equal to 1/2 $\Delta\sigma$ (Cullis and De Kruijff, 1978a; Cullis *et al.*, 1980a). These observations indicate that there are few (if any) conformational and motional differences between polar head groups in bilayer and hexagonal H_{II} phases at least as far as the phosphate segment is concerned.

^{2}H NMR has been widely used in membrane research (see for reviews Seelig and Seelig, 1980; Seelig, 1977). Chemical or biochemical techniques allow for a selective replacement of ^{1}H by ^{2}H in lipid molecules, which is not expected to affect the molecular organization in the membrane. Since virtually all parts of a lipid molecule can be labeled, ^{2}H NMR has provided detailed insight on the conformational and motional properties of polar region, glycerol backbone, and acyl chains of membrane lipid molecules.

Briefly, the deuterium quadrupole interactions in a sample of hydrated specifically ^{2}H-labeled lipids, organized in large bilayer membranes, give rise to a characteristic ^{2}H NMR spectrum, which is dominated by two distinct peaks as shown in Figure 8. The separation of the two peaks is the ^{2}H quadrupole splitting $\Delta\nu_Q$ which can be used to calculate the ^{2}H order parameter S_{CD} according to

$$\Delta\nu_Q = (3/4)\left(\frac{e^2qQ}{h}\right)S_{CD} \tag{4}$$

where e_q^2Q/h is the static quadrupole coupling constant which has been found to be 170 kHz for aliphatic C-D bonds (Burnett and Muller, 1971) and 175 kHz for olefinic C-D bonds (Achlama and Zur, 1979). S_{CD} is defined as $(3\cos^2\theta - 1/2)$, where θ is the instantaneous angle between the C-D bond and the bilayer normal and the bar denotes the time average.

Analogous to what is found with ^{31}P NMR, the reorientation rate of the lipid molecules influences the ^{2}H NMR spectrum and $\Delta\nu_Q$ is dependent on the size of the lipid structures. A final situation is reached when isotropic motional averaging results in a collapse of the quadrupole splitting and the spectrum only shows a single resonance.

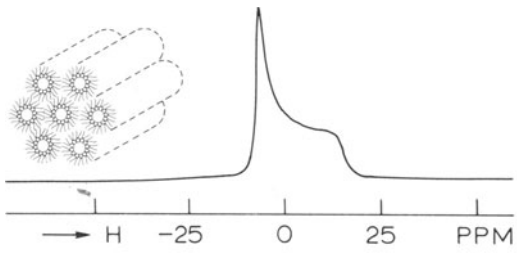

Figure 7. Proton decoupled 81.0 MHz ^{31}P NMR spectrum of an aqueous dispersion of 18 : 1_c/18 : 1_c-PE at 25°C. For experimental details see Van Echteld *et al.* (1981a,b).

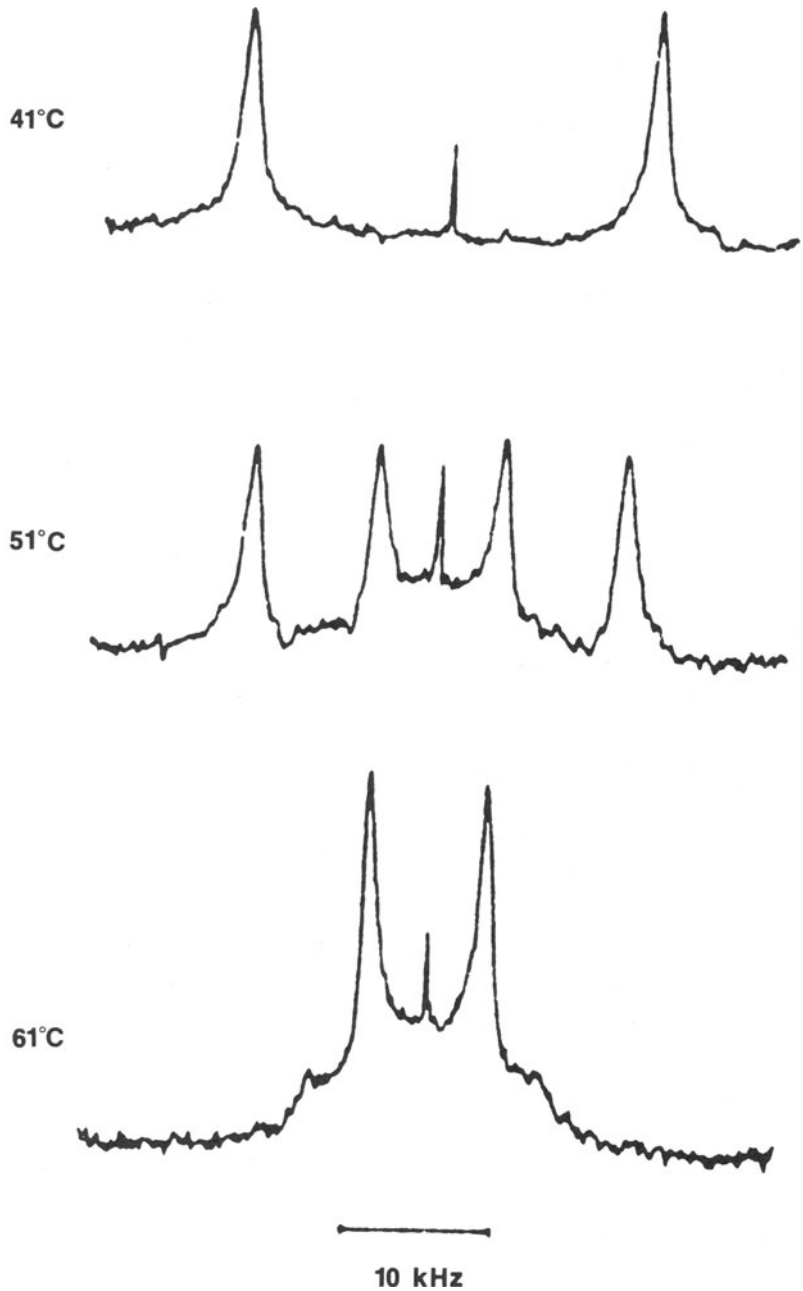

Figure 8. ^2H NMR of [9,10-^2H]-18 : 1_f/18 : 1_r-PE in 0.01 M Na_3PO_4, pH 7.0, 0.1 M NaCl, 0.001 M EDTA, 41°C bilayer, 51°C coexistence of bilayer and H_{II} phase, and 61°C H_{II} phase. Reproduced with permission from Gally *et al.* (1980).

Such isotropic motion for part of the lipid molecules has been observed for mixtures of acyl chain labeled 18 : 1_c/18 : 1_c-PE and 18 : 1_c/18 : 1_c-PC and has been found to closely correlate with results obtained with ^{31}P NMR (Akutsu and Seelig, 1981). However, since minor conformational changes may have large influences on ^{2}H NMR spectra of lipids, the observations of a singlet without the concomitant observation of an "isotropic" peak in the ^{31}P NMR spectrum (Akutsu and Seelig, 1981) indicates a local conformational change, rather than isotropic motion of the whole molecule.

Lipids in a hexagonal H_{II} phase can also be readily detected by ^{2}H NMR, since it can be shown that in such a situation the quadrupolar splitting will be described by

$$\Delta\nu_Q = (3/8)(e^2qQ/h) \cdot S_{CD} \tag{5}$$

so that in a transition from a lamellar to a hexagonal phase the splitting will be reduced by a factor of two, provided that there is no change in S_{CD}. This is illustrated in Figure 8 for ^{2}H labeled 18 : 1_t/18 : 1_t-PE.

The use of ^{2}H NMR in studying lipid polymorphism has been relatively limited. However, it may be very useful to surmount some of the shortcomings of ^{31}P NMR in this respect. For instance, not all membrane lipids are phospholipids and thus are not detected by ^{31}P NMR, whereas those lipids are in principle accessible by ^{2}H NMR. Also, specific labeling allows for the determination of individual phase behavior in (phospho)lipid mixtures (Tilcock *et al.*, 1982). Finally, in those situations where ^{31}P NMR data are not straightforward to interpret, such as for PA (Cullis and De Kruijff, 1976), ^{2}H NMR may give more unambiguous results.

III. PHASE PREFERENCES OF MEMBRANE LIPIDS

In this section, we will classify the major membrane lipids according to their structural preferences. Before doing so, it is important to realize two aspects of lipid polymorphism. First, early studies indicated that virtually every lipid can adopt a large variety of phases dependent upon water content and temperature (Luzzati, 1968). As nonlamellar structures were often observed at low (nonphysiological) water contents and high temperatures, the physiological relevance of lipid polymorphism was not obvious. Here, we only consider those structures adopted by isolated membrane lipids in excess (>50% by weight) water, at "physiological" (0–40°C) temperatures. Second, as will be discussed in detail in the following sections, the phase preferences of membrane lipids depend on a variety of factors such as fatty acid composition, pH, and the presence of divalent cations. Therefore, any given classification is somewhat arbitrary as a particular lipid can adopt different structures under different conditions.

It is remarkable that the large majority of the membrane lipids will adopt only two types of structure in isolation, the lamellar or the hexagonal H_{II} phase. Micellar phospholipid organizations have only been observed for minority membrane lipids such as gangliosides and lysophospholipids.

Table 1 summarizes the phase preferences of various membrane lipid classes. It

Table 1. Phase Preferences of Membrane Lipid Classes

	Lamellar	H_{II}	Micellar
Zwitterionic phospholipids			
Phosphatidylcholine	+	−	−
Sphingomyelin	+	−	−
Phosphatidylethanolamine	+	+	−
Negatively charged phospholipids			
Phosphatidylserine	+	+	−
Phosphatidylglycerol	+	+	−
Phosphatidylinositol	+	−	−
Phosphatidic acid	+	+	−
Cardiolipin	+	+	−
Glycolipids			
Monoglucosyldiglyceride	−	+	−
Monogalactosyldiglyceride	−	+	−
Diglucosyldiglyceride	+	−	−
Digalactosyldiglyceride	+	−	−
Cerebroside	+	−	−
Cerebroside sulfate	+	−	−
Gangliosides	−	−	+
Lysophospholipids	−	−	+

should be noted that this table is incomplete in that information is given only for commonly occurring major membrane lipid species. When a lipid can adopt more than one structure depending on the experimental conditions, it is classified as such.

The quantitatively most important type of H_{II} lipids are PE (Cullis and De Kruijff, 1979) and monoglucosyl- (Wieslander *et al.*, 1981b) and monogalactosyldiglycerides (Shipley, 1973; Sen *et al.*, 1982). It is intriguing that in bacterial membranes there appears to be an interrelationship between the amount of PE and the proportions of these glycolipids (Minnikin *et al.*, 1971), despite large differences in their chemical structures, suggesting a regulated requirement for H_{II}-favoring lipids in these membranes.

As will be shown in subsequent sections, the observation that the bilayer-preferring diglucosyl- and digalactosyldiglycerides (Shipley, 1973; Sen *et al.*, 1982; Wieslander *et al.*, 1981a) are metabolically directly coupled to the monoglucosyl and galactosyl derivatives offers fascinating regulatory possibilities for the net structure preferences of certain membrane systems.

Both lamellar and hexagonal H_{II} phases have been observed in the total lipid extracts of biological membranes. The total lipid extract of the human erythrocyte has a ^{31}P NMR spectrum which is typical of extended bilayers (Cullis and Grathwohl, 1977), despite the presence of PE (20% of phospholipid), which prefers the H_{II} phase in isolation (Cullis and De Kruijff, 1978b). In contrast, X-ray (Huynk, 1973), freeze-fracture, and ^{31}P NMR (De Grip *et al.*, 1979) studies demonstrate the presence of H_{II} phase structure for an aqueous dispersion of the total rod outer segment lipids at 37°C.

In addition, ^{31}P NMR studies often reveal the presence of an "isotropic" component such as observed for total *E. coli* lipids (Burnell *et al.*, 1980b). As will be shown in later sections, in these cases other inverted lipid structures are also present.

IV. THE HEXAGONAL H_{II} PHASE

Of the two major structures adopted by hydrated membrane lipids, the lamellar phase is the most familiar and its properties have been reviewed in great detail and will not be explicitly dealt with here. However, since many of the ideas developed over the last years concerning the structural and functional aspects of nonbilayer structures rely on the fact that many membrane lipids prefer the hexagonal H_{II} phase, a closer look at the properties of this phase is warranted.

A distinction can often be made visually between lipids in lamellar and hexagonal H_{II} organizations. Whereas bilayer-forming lipids in excess buffer usually form homogeneous milky dispersions readily, hexagonal H_{II} type lipids often do not disperse at all or form large aggregates. This phase separation is likely related to the low hydration capacity of these lipids (Hauser *et al.*, 1981). With regard to the nature of the interface between the bulk water and the H_{II} phase-two questions come into mind. First, are the aqueous channels present in the hexagonal H_{II} phase in open contact with the surrounding aqueous phase? Second, what is the lipid organization at the interface between the hydrophobic cylinder and the water?

For unsaturated PEs in the H_{II} organization, it has been found that the addition of Mn^{2+} broadened the entire ^{31}P NMR signal of the phospholipids beyond detection (C. J. A. Van Echteld, unpublished observations) demonstrating that Mn^{2+} can interact with all lipid head groups. This suggests that the aqueous tubes are open to the bulk aqueous phase. Freeze-fracture electron microscopy on similar systems indicates that the outermost tubes of the hexagonal H_{II} phase in excess water are covered with a monolayer of lipid (Van Venetië and Verkleij, 1981), thereby shielding the hydrophobic acyl chains from the aqueous phase. Another interesting feature revealed by these studies is that in some cases the tubes (often 10^4–10^5 Å long) are straight and in other cases highly curved (Van Venetië and Verkleij, 1981; Verkleij *et al.*, 1980). There is as yet no explanation for this phenomenon. The intertube distance (tube diameter) of hexagonal H_{II} phase lipids have been determined from both freeze-fracture electron microscopy and X-ray. Values obtained from some selected systems are presented in Table 2.

Typically, the tube diameters for PEs range from 70 to 74 Å and appear to be rather independent of the fatty acid composition. For complexes of cardiolipin (CL) and 18 : 1_c/18 : 1_c-PA with various cations, the tube diameter ranges from 52 to 75 Å, depending on the type of cation. A unique feature of the hexagonal H_{II} phase is that the phospholipid molecules reside in very curved monolayers. For instance, in the most highly curved lamellar system (sonicated vesicles) the inner diameter is still approximately 170 Å. This property, together with the notion that the molecules in the hexagonal H_{II} phase have an inverted orientation, forms the basis for the potential ionophoric properties of these types of phospholipids (see Section VIII).

Table 2. Repeat Distances of Selected Hexagonal H_{II} Phase Lipid Systems

	Repeat distance (nm)	
	Freeze-fracture	X-ray[h]
18 : 1_c/18 : 1_c-PE (20°C)	7.4[a]	
18 : 1_t/18 : 1_t-PE (60°C)		7.0[b]
18 : 3_c/18 : 3_c-PE (20°C)	7.7[c]	
20 : 4/20 : 4-PE (20°C)	4.4[c]	
22 : 6/22 : 6-PE (20°C)	4.3[c]	
Tetrahymena PE (10°C)		7.4[d]
Soya PE (5°C)		7.3[e]
CL, Ca^{2+}-salt (20°C)	5.2[a]	5.3[f]
CL, Mg^{2+}-salt (20°C)	6.5[a]	
CL, Mn^{2+}-salt (20°C)	7.5[a]	
18 : 1_c/18 : 1_c-PA, Ca^{2+}-salt (20°C)	5.2[g]	
18 : 1_c/18 : 1_c-PA, Mg^{2+}-salt (20°C)	5.7[g]	
18 : 1_c/18 : 1_c-PA, Mn^{2+}-salt (20°C)	7.4[g]	

[a] Van Venetië and Verkleij (1981).
[b] De Kruijff, (unpublished observations).
[c] Dekker *et al.* (1983).
[d] Ferguson *et al.* (1982).
[e] Hui *et al.* (1981).
[f] Rand and Sengupta (1972).
[g] Verkleij *et al.* (1982).
[h] To obtain the tube diameter multiply by $\sqrt{3}/2$.

The acyl chains in hexagonal H_{II} phases adopted by hydrated membrane lipids are in a liquid-crystalline state. From ^{31}P NMR (Seelig, 1978; Cullis and De Kruijff, 1979) and ^{2}H NMR (Seelig, 1977; Burnell *et al.*, 1980b) studies, it can be inferred that the lipid molecules undergo lateral diffusion around the aqueous cylinders at rates comparable to that in the liquid-crystalline lamellar phase. These studies further indicate that the molecular order in the polar head group region is very similar for lipids organized in the hexagonal H_{II} and the liquid-crystalline lamellar phase (Seelig, 1977; Gally *et al.*, 1980; Taylor and Smith, 1981). In contrast, ^{2}H NMR (Tilcock *et al.*, 1982; Gally *et al.*, 1980), electron spin resonance (ESR) (Hardman, 1982), and Fourier transform infrared spectroscopy (FT-IR) (Mantsch *et al.*, 1981) studies indicate that the acyl chains are progressively more disordered towards the terminal methyls, consistent with the strong curvature of the lipid monolayers surrounding the aqueous channels.

V. MODULATION OF MEMBRANE LIPID POLYMORPHISM

An important aspect of lipid polymorphism is that the macroscopic structure adopted by lipids depends very much on the experimental conditions. As regulation of lipid structure is of crucial importance for potential functional roles of nonlamellar

lipid structures in membranes, we will review in this section the various ways membrane lipid polymorphism can be modulated.

A. One-Lipid Systems

1. Temperature and Fatty Acid Composition

Temperature is an important experimental parameter which determines the macroscopic structure of hydrated membrane lipids. This is illustrated in Figure 9, which shows the ^{31}P NMR spectra of 18 : 1_t/18 : 1_t-PE dispersed in excess aqueous buffer at pH 7.0. At 30°C, this phospholipid is organized in a gel state lamellar phase as is shown by the characteristic lineshape and the large line width, which results from incomplete removal of the strong dipolar ^{1}H–^{31}P coupling (Seelig and Gally, 1976). At 40°C, the acyl chains melt and a ^{31}P NMR lineshape typical of liquid-crystalline lamellar phospholipids is observed. Above 50°C, a second spectral component appears which gradually increases in intensity with increasing temperature such that at 70°C, the entire spectrum consists of this spectral component. From the chemical shift position of the dominant high-temperature spectral feature, the reversal of the asymmetry in the spectrum, and the reduced width, it can be concluded that the phospholipid molecules are organized in cylindrical structures around which rapid diffusion of the molecules occurs. In conjunction with freeze-fracture and X-ray data, these results demonstrate that 18 : 1_t/18 : 1_t-PE adopts the hexagonal H_{II} phase above 50°C. When the temperature is increased through the 50–70°C temperature range, these transitions are also manifested in differential scanning calorimetry (DSC) scans of the same

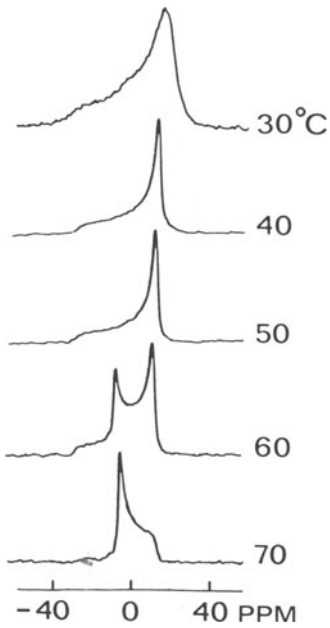

Figure 9. 81.0 MHz ^{31}P NMR spectra of 18 : 1_t/18 : 1_t-PE in 100 mM NaCl, 10 mM Tris/HCl, and pH 7.0. For experimental details see Van Echteld *et al.* (1981a,b).

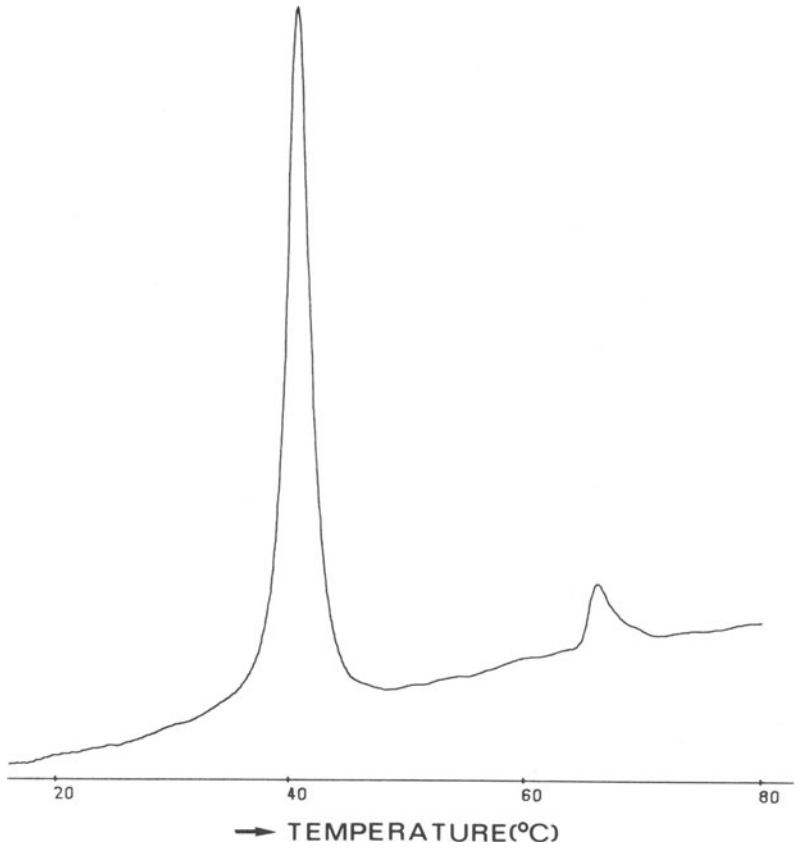

Figure 10. Heating scan (2°C/min) of 18 : 1_t/18 : 1_t-PE in 100 mM NaCl, 10 mM Tris/HCl, pH 7.0, recorded on a Setaram high-sensitivity calorimeter.

dispersions (Figure 10). The large endothermic transition at 40°C ($\Delta H = 6.9 \pm 0.1$ kcal/mole) originates from the melting of the acyl chains, whereas the small endotherm at 66°C ($\Delta H = 0.4 \pm 0.1$ kcal/mole) corresponds to the bilayer $\rightarrow H_{II}$ transition. The low heat content of the latter transition must be related to the fact that in both phases, the acyl chains are in the liquid-crystalline state. From the functional point of view, it is intriguing that there is such a low energy barrier between these macroscopically very different structures.

Temperature-dependent bilayer $\rightarrow H_{II}$ transitions have been observed in a large variety of both synthetic and natural PEs (Table 3). The bilayer $\rightarrow H_{II}$ transition depends strongly on the fatty acid composition of the lipid. Increasing unsaturation results in decreased bilayer $\rightarrow H_{II}$ transition temperatures. However, it should be realized that there is no direct correlation between membrane "fluidity" and H_{II} formation. For instance, whereas for 18 : 1_t/18 : 1_t- and 18 : 1_c/18 : 1_c-PE there is approximately a 15–20°C interval between the gel $\rightarrow$ liquid-crystalline and the bi-

Table 3. Bilayer–Hexagonal H_{II} Transition Temperatures of PEs

Product	Temperature (0°C)	Remarks
Synthetic		
Saturated (diester)		
18 : 0/18 : 0-PE	105[a]	1 M NaCl
Saturated (diether)		
1,2-Dihexadecyl-*sn*-glycero-3-phosphoethanolamine	87[a], 88[b]	1 M NaCl
1,2-Ditetradecyl-*rac*-glycero-3-phosphoethanolamine	93.5[a]	1 M NaCl
Saturated (ether-ester)		
1-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine	102[b]	
Unsaturated		
16 : 1_c/16 : 1_c-PE	~0[c]	
18 : 1_c/18 : 1_c-PE	10[d]	
18 : 1_t/18 : 1_t-PE	55[d], 60–63[e], 65[f]	
18 : 2_{cc}/18 : 2_{cc}-PE	0–25[g], < − 15[e]	
18 : 3_{ccc}/18 : 3_{ccc}-PE	0–30[g]	
20 : 4/20 : 4-PE	< − 30[g]	
22 : 6/22 : 6-PE	< − 30[g]	
Mixed species		
16 : 0/18 : 1_c-PE	75[g]	
Natural species		
Egg	25–30[d], 32–45[b], 28[h], 28[i]	
Egg (from egg-PC via phospholipase D)	50[i], 63[b]	
E. coli	55–60[d]	Wild-type
Endoplasmic reticulum	7_j	Rat liver
Sarcoplasmic reticulum	− 10[k]	Rabbit
Inner mitochondrial membrane	10[l]	Muscle
Erythrocyte membrane	8[d]	Rat liver
Soya	− 10[m]	Human
Soya (from soya-PC, via phospholipase D)	0 to − 20[n]	
Bovine white matter ethanol phospholipid	18[b]	Containing plasmalogens

[a] Harlos and Eibl (1981).
[b] Boggs *et al.* (1981).
[c] Van Dyck *et al.* (1976).
[d] Cullis and De Kruijff (1978b).
[e] Tilcock and Cullis (1982).
[f] Ghosh and Seelig (1982).
[g] Dekker *et al.* (1983).
[h] Hardman (1982).
[i] Mantsch *et al.* (1981).
[j] De Kruijff *et al.* (1980b).
[k] Cullis *et al.* (1982).
[l] Cullis *et al.* (1980b).
[m] Cullis and De Kruijff (1978a).
[n] Cullis and Hope (1980).

layer → H_{II} transitions, no H_{II} phase has been observed up to 70°C above the gel → liquid-crystalline transition of 14 : 0/14 : 0-PE. Furthermore, in a series of disaturated PEs, the gel → liquid-crystalline transition temperature increases with increasing chain length, whereas the bilayer → H_{II} phase transition decreases in temperature with increasing chain length (Boggs *et al.*, 1981). Hydrated PEs isolated from biological membranes are typically organized in the H_{II} phase at physiological temperatures. Very similar temperature- and fatty-acid-composition-dependent bilayer → H_{II} transitions have been observed for monoglucosyldiglyceride isolated from *A. laidlawii* (Wieslander *et al.*, 1981b). Although no information is available on the exact kinetics of the bilayer → H_{II} transition in PEs, freeze-fracture experiments have shown that it is very difficult to preserve the hexagonal H_{II} phase when the bilayer → H_{II} transition occurs above 30°C. In these cases, only lamellar phases are observed. From the freezing rates, it can be estimated that the hexagonal → lamellar transition is completed in the order of milliseconds (Van Venetië *et al.*, 1981). Studies employing ^{31}P NMR demonstrate that there is no detectable hysteresis in this transition, and also indicate (using oriented PE multilayers) that the tubes of the hexagonal H_{II} phase run parallel to the bilayers from which they are formed, suggesting that the transition is a highly cooperative interbilayer fusion process (Cullis *et al.*, 1980a). The bilayer → H_{II} transition of 18 : 1_c/18 : 1_t-PE is accompanied by an increase in repeat distance from 55 to 70 Å (De Kruijff, unpublished observations). Assuming that the length of a lipid molecule is 20 Å in each phase, then the interbilayer aqueous space is 15 Å thick, whereas the aqueous cylinder in the H_{II} phase has a diameter of 30 Å. This corresponds to very similar water contents of 27 and 25% (vol./vol.) for both these phases. Thus, no massive, long-range water movements have to occur during the transition. That the water structure at the lipid–water interface probably is important for the transition is indicated by the decrease in transition temperature induced by glycerol in 18 : 1_t/18 : 1_t-PE dispersions (Van Echteld, unpublished observations). Therefore, the use of this cryoprotectant in freeze-fracture studies on such systems is questionable.

2. *Electrostatic Interactions*

The molecular packing of negatively charged phospholipids in particular is extremely sensitive to the ionic composition of the aqueous phase. It is therefore not surprising that lipid polymorphism is dependent on the magnitude and nature of electrostatic lipid head group interactions.

At neutral pH, when PEs are uncharged, the unsaturated species prefer the H_{II} phase. Upon increasing the pH towards the pK of the amine, thereby increasing the net negative charge and corresponding interheadgroup repulsion, the bilayer phase is preferred (Cullis and De Kruijff, 1978b; Hardman, 1982). Similarly, lipids such as PA and PS are negatively charged at neutral pH and form bilayers. Lowering the pH below the respective pKs reduces the charge density resulting in H_{II} phase formation (Hope and Cullis, 1980; Farren *et al.*, 1983). Increasing NaCl concentrations will decrease the lamellar to hexagonal H_{II} phase transition temperature of PEs (Harlos and Eibl, 1981).

Divalent cation–lipid interactions are of special interest for a number of reasons.

In the case of negatively charged lipids, these interactions are very strong and can result in the formation of divalent cation–lipid salts with unique structural properties. Moreover, many membrane functions appear to be highly dependent on the presence of Ca^{2+} in particular suggesting that such interactions might play important structural and functional roles.

Aqueous dispersions of PS, PG or PI are organized in a lamellar phase at neutral pH. The addition of divalent cations such as Ca^{2+} will result in strong Ca^{2+} binding and, at high concentrations, in lipid precipitation. However, in these precipitates, the phospholipid-Ca^{2+} complexes remain organized in a lamellar arrangement (Hope and Cullis, 1980; Farren and Cullis, 1980; Papahadjopoulos *et al.*, 1975a; Verkleij *et al.*, 1974). In some cases, such as the more saturated species of PS and PG, the Ca^{2+} salt forms anhydrous cochleate or cylindrical structures in which the acyl chains are in the gel state (Hope and Cullis, 1980; Farren and Cullis, 1980; Papahadjopoulos *et al.*, 1975a; Verkleij *et al.*, 1974).

It is important to note that although bilayer structure is maintained in all these cases, such interactions can play important roles in lamellar → nonlamellar transitions in mixed lipid systems.

Divalent cations can also directly influence the macroscopic organization of two other negatively charged lipids, CL and PA, and we will discuss these effects in more detail. CL is in many respects a unique membrane lipid (for review see Ioannou and Golding, 1979). Chemically, it can be defined as a "double" phospholipid containing four fatty acids and at neutral pH, two charged phosphates. In eukaryotic cells, it is found exclusively in the inner mitochondrial membrane where it is the only major (20 mole% of the total lipids) negatively charged phospholipid. In addition, CL is found in several bacterial membranes. The mitochondrial species is highly unsaturated and specifically enriched in linoleic acid, whereas the bacterial CL is much more saturated. Beef heart CL, when dispersed in salt solutions at neutral pH, is organized in a lamellar configuration (Rand and Sengupta, 1972; Cullis *et al.*, 1978a; De Kruijff *et al.*, 1982b). Upon gradually increasing the Ca^{2+} concentration, via dialysis methods such that the liposomes are never exposed to high local Ca^{2+} concentrations, the following events occur. The lamellar phase is maintained up to a free Ca^{2+} concentration of 1 mM, whereas in the 1–3-mM free Ca^{2+} concentration range the lipids precipitate and a transition to the hexagonal H_{II} phase is observed (De Kruijff *et al.*, 1982b; see Figure 11). This structural transition is endothermic (ΔH = 1.8 kcal/mole) and is accompanied by a marked increase in Ca^{2+}-binding from a maximum of 0.35 Ca^{2+}/CL in the bilayer phase to the stoichiometric value of 1.0 Ca^{2+}/CL in the H_{II} phase (De Kruijff *et al.*, 1982b). Addition of Ca^{2+} from a concentrated stock solution, thereby transiently exposing the liposomes to local high Ca^{2+} concentrations, results in the formation of a so-called "isotropic" intermediate structure, which will be discussed in detail in Section VI.

Removal of Ca^{2+} from the hexagonally organized Ca^{2+}–CL (1 : 1) complex by addition of ethylenediaminetetra-acetic acid (EDTA) or by dialysis results in the formation of large unilamellar vesicles (De Kruijff *et al.*, 1982b) and an "isotropic" structure (De Kruijff *et al.*, 1982b), respectively. The Ca^{2+}-induced H_{II} phase formation in beef heart CL can be effectively blocked by the cardiotoxic anticancer drug

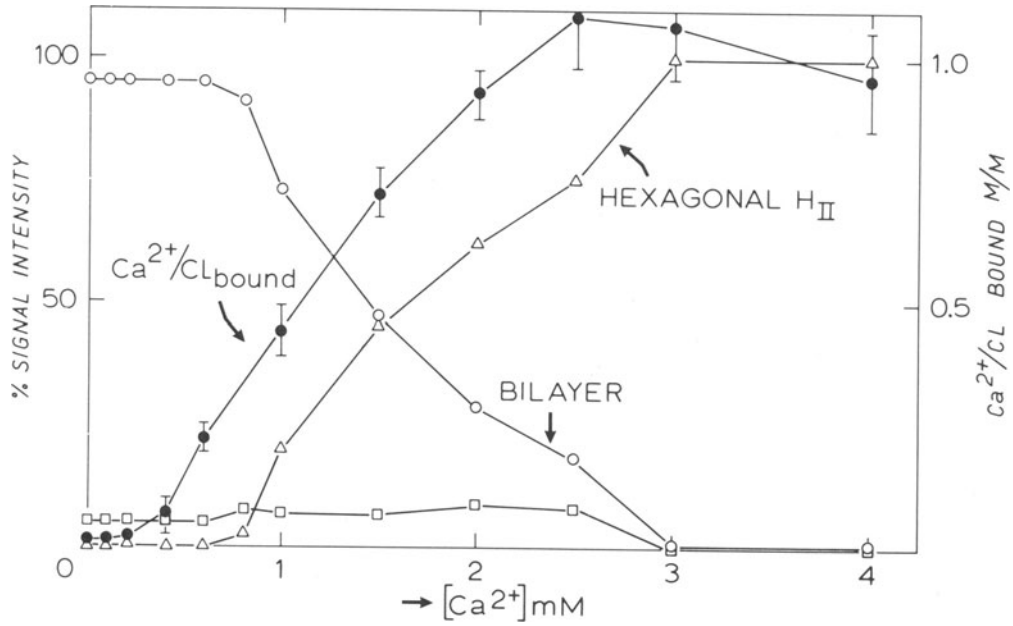

Figure 11. Ca^{2+} binding to and structure of CL liposomes. CL liposomes in 100 mM NaCl, 10 mM Tris/HCl, and pH 7.0 were dialyzed against excess Ca^{2+}-containing buffer, then the Ca^{2+} binding and the structure of the lipids were determined. For experimental details see De Kruijff *et al.* (1982b).

adriamycin (Goormaghtigh *et al.*, 1982) and the specific inner mitochondrial Ca^{2+} transport inhibitor ruthenium red (Cullis *et al.*, 1980a), suggesting that nonlamellar structures formed by CL might play functional roles in the inner mitochondrial membrane (see also Section IX-C).

Other divalent cations such as Mg^{2+}, Mn^{2+}, and Ba^{2+} also interact strongly with CL, leading to the formation of the respective salts (Van Venetië and Verkleij, 1981; Rand and Sengupta, 1972; Vasilenko *et al.*, 1982a) which show temperature-dependent lamellar → H_{II} phase transitions (Figure 12, see Table 2 for H_{II} tube dimensions). The transition temperatures increase in the order $Mg^{2+} < Ca^{2+} < Ba^{2+}$, which is also the order of the size of the dehydrated cations, suggesting, together with other data (Shaw and Schulman, 1965), that the H_{II}-promoting ability of divalent cations is related to the formation of an intramolecular phosphate–metal–phosphate complex in which the two phosphates are pulled together resulting in a decreased head group size (De Kruijff *et al.*, 1982b).

For the more saturated bacterial CL, the bilayer → H_{II} transition of the divalent cation–CL salts occurs at a much higher temperature (Vasilenko *et al.*, 1982a), a result which is consistent with the phase behavior of PEs (see Figure 12). In contrast to beef heart CL at physiological temperatures the lamellar phase is always preferred.

PA, a key intermediate in phospholipid metabolism, occurs in small amounts in many membranes and undergoes a rapid turnover which in some cases is related to

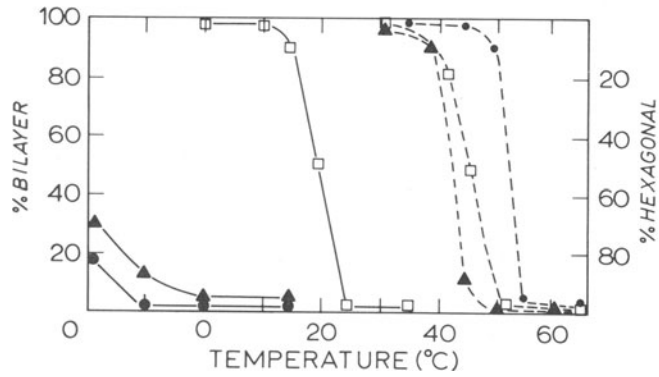

Figure 12. Temperature dependency of the bilayer → H_{II} transition of various CL salts. Beef heart CL: ▲———▲, Ca^{2+}-salt; ●———●, Mg^{2+}-salt; and □———□, Ba^{2+}-salt. *B. subtilis* CL: ▲- - -▲, Ca^{2+}-salt; ●- - -●, Mg^{2+}-salt; □- - -□, Ba^{2+}-salt. Reproduced with permission from Vasilenko *et al.* (1982a). For further details see this reference.

hormonal stimulation (Van den Bosch, 1974; Salmon and Honeyman, 1980; Putney *et al.*, 1980). As a phosphomonoester, it has two ionizable groups with pKs of 3.5 and 8.0, respectively (Koter *et al.*, 1978). 18 : 1_c/18 : 1_c-PA forms bilayers when the molecule is singly or doubly charged (Verkleij *et al.*, 1982; Farren *et al.*, 1983). A lamellar organization is also found for the Ca^{2+} and Mg^{2+} salts above pH 8.0 and below pH 4.0 (Verkleij *et al.*, 1982; Farren *et al.*, 1983). However, at neutral pH, when the molecule only bears one negative charge, Ca^{2+} addition results in H_{II} phase formation. Similar effects have been reported for egg-PA (Papahadjopoulos *et al.*, 1976). This finding offers intriguing regulatory possibilities as the stimulation of nonlamellar structures by divalent cations is dependent on the charge of the lipid molecules which in turn will be dependent on environmental conditions.

3. *Anaesthetics*

Anaesthetics are a broad class of molecules which greatly differ in chemical structure but which have as common properties their affinity for membranes and their inhibitory action on the Na^+ channel in excitable membranes (Seeman, 1972). The correlation between solubility in an apolar environment and anaesthetic potency (Seeman, 1972) suggests a nonaspecific type of membrane interaction. Several models have been put forward for their mode of action, most of which evoke some pertubation in fluidity at a protein–lipid interface (Lee, 1976).

The observation that lipid polymorphism is greatly affected by the presence of these molecules has led to the formulation of alternative molecular mechanisms (Cullis *et al.*, 1980c). Several effects can be observed dependent on the type of lipid or anaesthetic. In the case of beef heart CL (Verkleij *et al.*, 1982; Cullis *et al.*, 1978a) or 18 : 1_c/18 : 1_c-PA (Verkleij *et al.*, 1982a), under conditions where divalent cations

would promote H_{II} formation, the local anaesthetic dibucaine and chlorpromazine produce similar effects. This is shown in Figure 13 for 18 : 1_c/18 : 1_c-PA. At pH 6.0, in the absence of the drug, freeze-fracturing reveals bilayer structure. Addition of stoichiometric amounts of chlorpromazine results in lipid precipitation and the formation of the hexagonal H_{II} phase in conjunction with lipidic particles (the structural features of this latter component will be discussed in Section VI). It should be emphasized that these effects are specific for these types of lipids and are not observed for other negatively charged lipids. Furthermore, other positively charged amphipatic molecules such as adriamycin militate against hexagonal H_{II} phase formation (Goormaghtigh *et al.*, 1982).

In PE systems, the local anaesthetic chlorpromazine, dibucaine, tetracaine, and procaine stabilize bilayer structure (Hornby and Cullis, 1981), their relative anaesthetic potency being mirrored by their relative ability to stabilize bilayer structure. Of the neutral anaesthetics such as alkanes and alcohols, differential effects have been observed. Whereas ethanol and butanol stabilize bilayer structure for egg-PE, the longer chain ($n > 6$) normal alcohols and alkanes strongly promote hexagonal H_{II} phase formation (Hornby and Cullis, 1981). It is suggested that the ability of these molecules

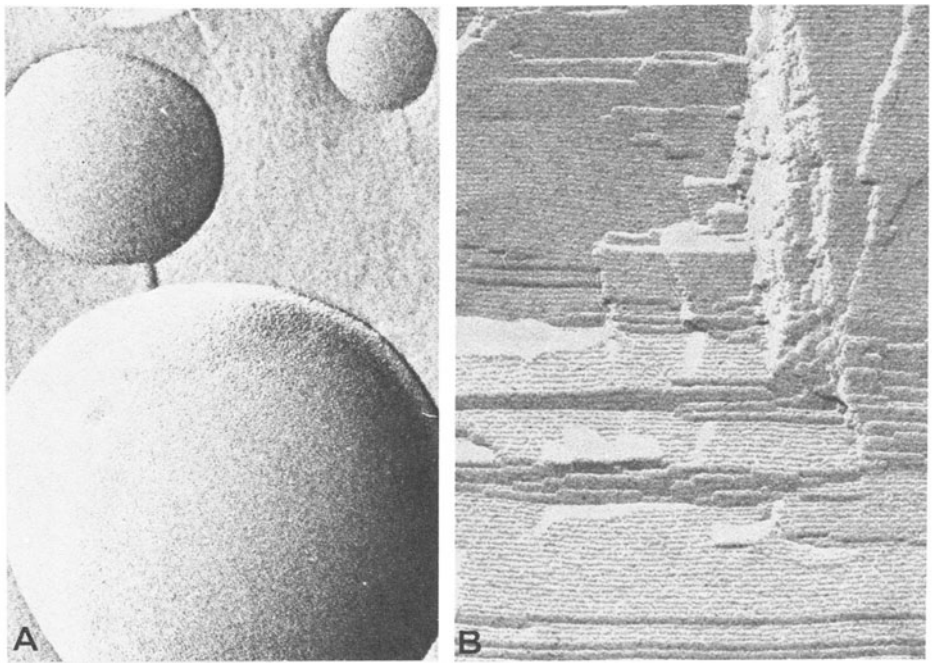

Figure 13. Freeze-fracture micrographs of 18 : 1_c/18 : 1_c-PA dispersed in 10 mM Mes, 100 mM NaCl at pH 6.0 before (A) and after (B) the addition of chlorpromazine at a ratio chlorpromazine/18 : 1_c/18 : 1_c-PA of 1 : 1. Final magnification ×100,000. Besides the H_{II} phase formation, addition of the anaesthetic also induces the formation of lipidic particles. For further details see Verkleij *et al.* (1982).

to affect the phase structure of PEs is related to their dynamic shape (Hornby and Cullis, 1981).

B. Mixed Lipid Systems

As biological membranes contain both bilayer-and H_{II}-forming lipids,it is essential to obtain insight into the phase properties of mixtures of these lipids under various physiologically relevant conditions. Most experimental work has been directed towards obtaining an understanding of the phase behavior of PE in mixtures with PCs, sterols, and negatively-charged lipids which we will review in that order.

1. PE–PC Mixtures

Incorporation of PC in unsaturated PE dispersions stabilizes the bilayer configuration of the PE. The degree of stabilization depends on the acyl chain composition, the phase state of the PC (gel vs. liquid-crystalline), and the temperature (Cullis and De Kruijff, 1979). In general, decreasing unsaturation or temperature results in a stronger preference for bilayer organization. Figure 14 illustrates this for soya egg-PE–PC mixtures at 30°C. In the absence of PC, the PE is hexagonally organized, whereas in the equimolar mixture, all lipids adopt the bilayer organization (Cullis and De Kruijff, 1978a). At intermediate concentrations, an isotropic ^{31}P NMR signal is observed. Such a signal is commonly observed in lipid systems intermediate between lamellar and H_{II} configurations and indicates the presence of structures in which isotropic motional averaging occurs (see Section VI). In the related 18 : 1_c/18 : 1_c-PE–18 : 1_c/18 : 1_c-PC system, as little as 25 mole% PC is sufficient to convert the PE into the bilayer phase (Cullis *et al.*, 1978b). In equimolar 18 : 1_c/18 : 1_c-PE–16 : 0/16 : 0-PC mixtures, the magnitude of the stabilization is strongly temperature-dependent (Cullis *et al.*, 1978b; Vasilenko *et al.*, 1982b). For example, the use of the thion analogue of PC (P═S instead of P═O), for which the ^{31}P NMR signal is completely separated from that of the normal species, allows the observation of the phase state of each lipid in these mixed systems (Vasilenko *et al.*, 1982b). It was observed that at low temperatures, where the PC is organized in the gel state, phase separation occurs between gel state 16 : 0/16 : 0-PC bilayers and a liquid-crystalline 18 : 1_c/18 : 1_c-PE H_{II} phase. Melting of the palmitic acid chains results in mixing of the two lipids and the formation of a lamellar phase. At higher temperatures, both molecules are incorporated into an "isotropic" structure. In mixtures of two PE species, the gel → liquid-crystalline phase transition can also induce similar structural transitions (Dekker *et al.*, 1983; Tilcock and Cullis, 1982). This is shown in Figure 15 for 16 : 0/18 : 1_c-PE–22 : 6/22 : 6-PE mixtures for which a gel state lamellar and a hexagonal H_{II} phase is observed below the gel → liquid-crystalline transitions of 16 : 0/18 : 1_c-PE. Upon melting of the 16 : 0/18 : 1_c-PE, mixing occurs in the liquid-crystalline bilayer producing a net bilayer structure whereas at higher temperatures, the lipids convert to the H_{II} phase. It may be noted that the other major zwitterionic membrane lipid with a choline head group, sphingomyelin, exerts similar strong bilayer stabilization in model membranes containing unsaturated PE (Cullis and Hope, 1980).

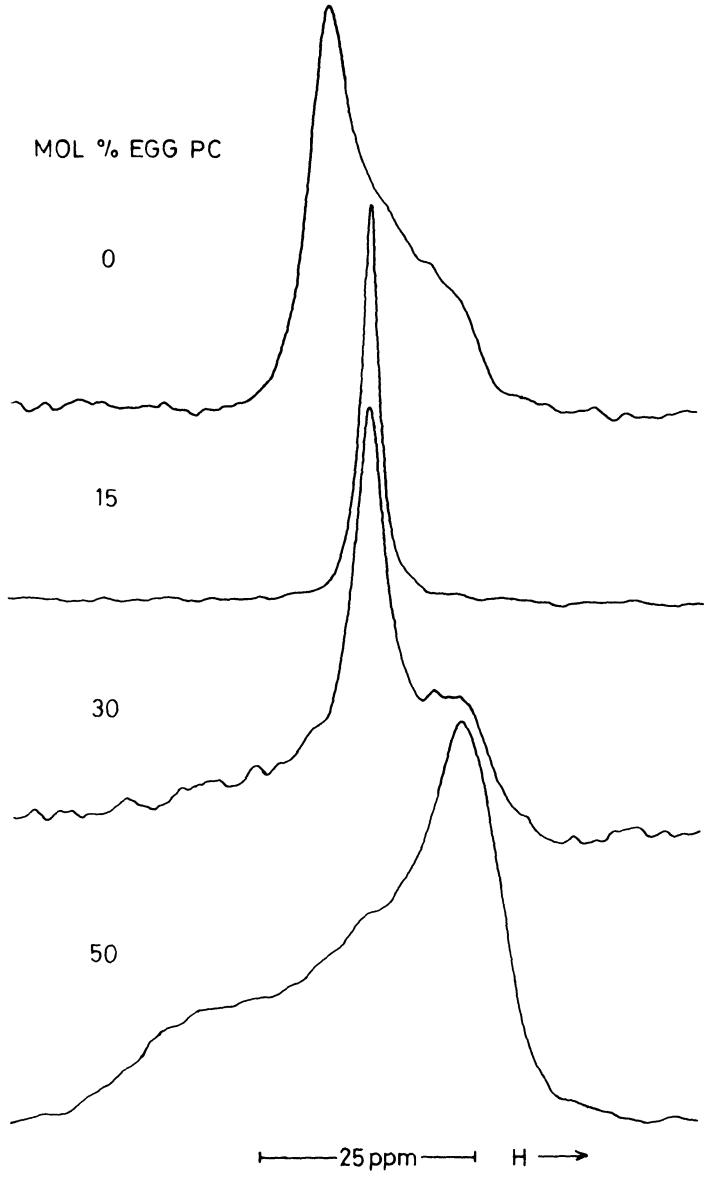

Figure 14. 36.4 MHz ^{31}P NMR spectra at 30°C of aqueous dispersions of soya-PE containing increasing amounts of egg-PC. Reproduced with permission from Cullis and De Kruijff (1976).

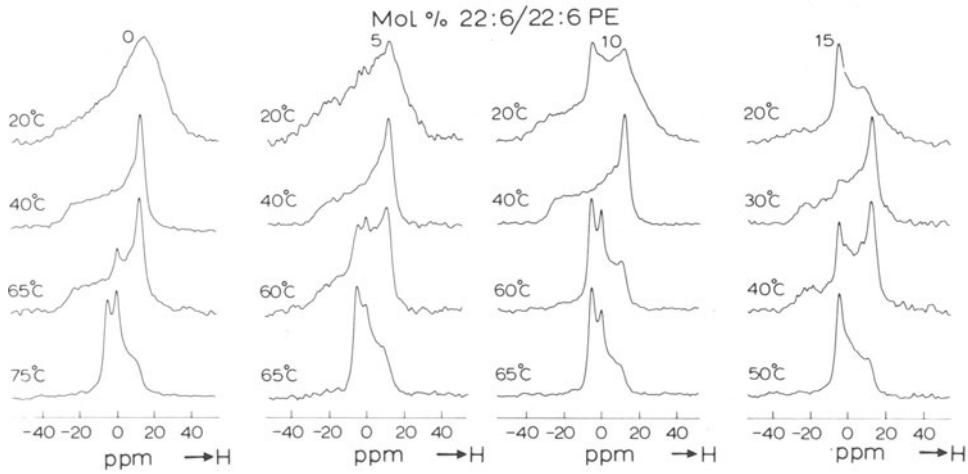

Figure 15. 36.4 MHz ^{31}P NMR spectra of aqueous dispersions of various mixtures of 16 : 0/18 : 1_c-PE and 22 : 6/22 : 6-PE. For further details see Dekker *et al.* (1983).

2. *Mixtures with Sterols*

Sterols are main constitutents of many biological membranes. Despite extensive effort, no clear picture has emerged of their functional role(s). Cholesterol, the most abundant mammalian sterol, can solidify or "condense" liquid-crystalline lipid systems and liquidify or expand gel state phospholipids leading to an intermediate state of "fluidity" (Demel and De Kruijff, 1976). For both effects, a 3β-OH group, a planar ring system, and an aliphatic side chain at C_{17} are required. *A priori,* there are several reasons to suppose that cholesterol–PE interactions might be special and could affect membrane structure. In the first place, there appears to be an inverse relationship between the occurrence of PE and sterols in membranes (Demel and De Kruijff, 1976). PE-rich membranes, such as occur in bacteria, have a very low sterol content whereas in mammalian plasma membranes, such as that of the human erythrocyte, the opposite is true. Second, in PE–PC mixtures under conditions of lateral phase separation, cholesterol preferentially interacts with PC (Van Dyck *et al.*, 1976). Third, from the preferential localization of cholesterol in the inner monolayer of sonicated vesicles (De Kruijff *et al.*, 1976b), it can be inferred that cholesterol will have a cone shape with the hydroxyl group at the smaller end of the cone. As cone-shaped lipids favor H_{II} phase formation (see Section VII), it can be expected that the incorporation of sterols in membranes will destabilize bilayer structure. Indeed, in mixed PE–PC systems, strong bilayer destabilization effects have been observed on incorporation of cholesterol. For instance, whereas 25 mole% 18 : 1_c/18 : 1_c-PC stabilizes bilayer structure at 40°C for 18 : 1_c/18 : 1_c-PE, the additional incorporation of cholesterol (equimolar with respect to PC) induces the H_{II} phase for all the phospholipids (Cullis *et al.*, 1978b). Using lower cholesterol concentrations and selective ^2H labeling (Tilcock *et al.*, 1982), it could be demonstrated (employing ^{31}P and ^2H NMR) that when both

lamellar and H_{II} phases were present, the phospholipid composition of both phases was very similar (Figure 16). This is a rather remarkable result, as it could have been expected that the H_{II} phase would be enriched in PE and the bilayer phase in PC. It has also been shown for other lipid mixtures that substantial amounts of the bilayer-preferring lipid can be incorporated into nonlamellar structures (Vasilenko *et al.*, 1982b; De Kruijff *et al.*, 1979).

The magnitude of the bilayer destabilization by sterols depends very much on their chemical structure (Gallay and De Kruijff, 1982). Figure 17 summarizes the effect of the incorporation of different sterols on the bilayer → H_{II} transition temperature of 18 : 1_t/18 : 1_t-PE. The bilayer destabilization potential is found to be directly related to the mean molecular area occupied by these sterols in pure and mixed monolayers at the air–water interface (Figure 17). Interestingly, those sterols which do not show condensing or liquifying effects in PC bilayers cause the strongest bilayer destabilization. Thus, observations such as the increased fragility of erythrocytes caused by replacing cholesterol with 3-ketosterols (Bruckdorfer *et al.*, 1969) could well result from the strong bilayer-destabilizing action of the latter molecule.

The effects observed upon incorporation of cholesterol in PE-containing lipid mixtures are paralleled by similar effects observed upon incorporation of cholesterol in monoglucosyl- and diglucosyl-containing lipid systems (Khan *et al.*, 1981). The strong bilayer-destabilization ability of cholesterol is illustrated by the observation that

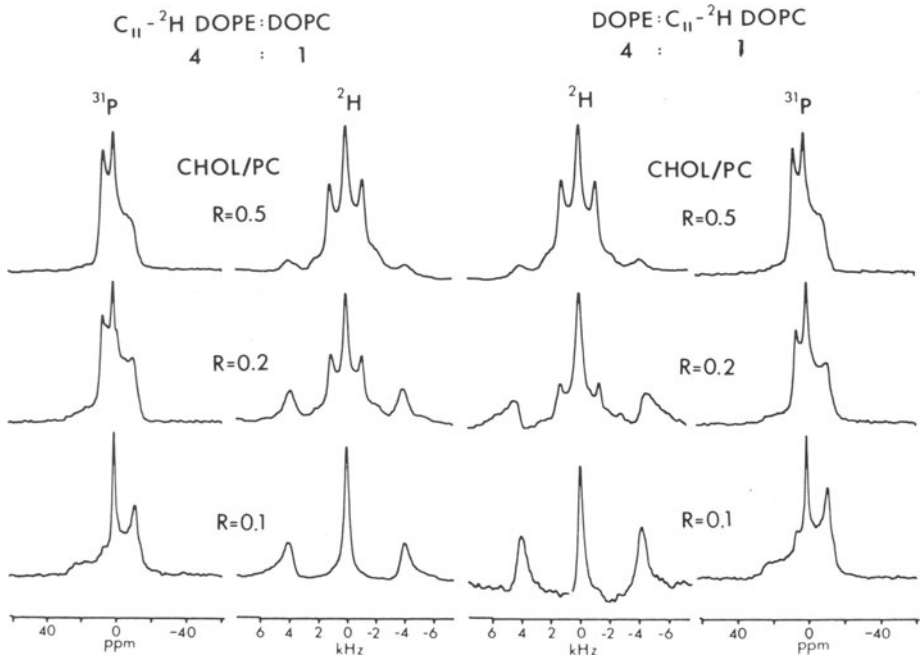

Figure 16. 81.0 MHz ^{31}P NMR and 30.7 MHz ^{2}H NMR spectra at 40°C arising from aqueous dispersions of mixtures of 18 : 1_c/18 : 1_c-PE, 18 : 1_c/18 : 1_c-PC and cholesterol. Reproduced with permission from Tilcock *et al.* (1982).

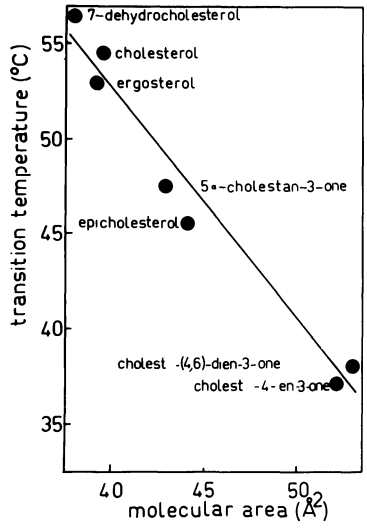

Figure 17. Relationship between the effect of equimolar sterols on the bilayer → H_{II} transition of 18 : 1_t/18 : 1_t-PE and the molecular area of the pure sterols in monolayers at the air–water interface. Reproduced with permission from Gallay and De Kruijff (1982).

incorporation of cholesterol in polyunsaturated PC species such as 18 : 3/18 : 3-PC, 20 : 4/20 : 4-PC, and 22 : 6/22 : 6-PC will induce H_{II} phase formation (Dekker *et al.*, 1983).

3. *Negatively Charged Lipid-Containing Mixtures*

The ability to isothermally modulate lipid structure is a prerequisite for possible functional roles of nonlamellar lipid structures in biomembranes. The results described in Section V-A.2, indicating that divalent cations can influence the molecular packing of negatively charged lipids, together with the observation that bilayer-forming lipids stabilize bilayer structure in PE-containing model membranes, suggest that in mixed PE negatively charged phospholipid systems the lipid structure will be very sensitive to the presence of divalent cations and the nature of the charged lipid.

This is certainly the case as illustrated in Figure 18, which shows the ^{31}P NMR characteristics of mixtures of unsaturated PE with unsaturated PS (Tilcock and Cullis, 1980), PG (Farren and Cullis, 1980), PI (Nayar *et al.*, 1982), and CL (De Kruijff and Cullis, 1980b) as a function of the molar ratio of Ca^{2+} to negatively charged lipid. The presence of 30 mole% or more of the charged lipid stabilizes bilayer structure of the PE. In the case of PS-containing systems, Ca^{2+} addition results in the formation of the gel state (lamellar) Ca^{2+}-PS salt. Phase separation occurs and the PE reverts to the H_{II} phase. In contrast, in the case of PG both lipids are incorporated into the H_{II} phase upon the Ca^{2+}–PG interaction despite the fact that the Ca^{2+}-PG salt favors a lamellar organization. On the other hand, Ca^{2+}–PI interactions appear to result in lateral phase segregation of PI into liquid-crystalline lamellar domains leaving the PE to adopt the H_{II} phase. Since CL itself favors the H_{II} phase in the presence of Ca^{2+} in mixtures with PE, the addition of Ca^{2+} results, as may be expected, in the formation of the H_{II} phase for all the lipids. Correspondingly, when the PE in the mixture with

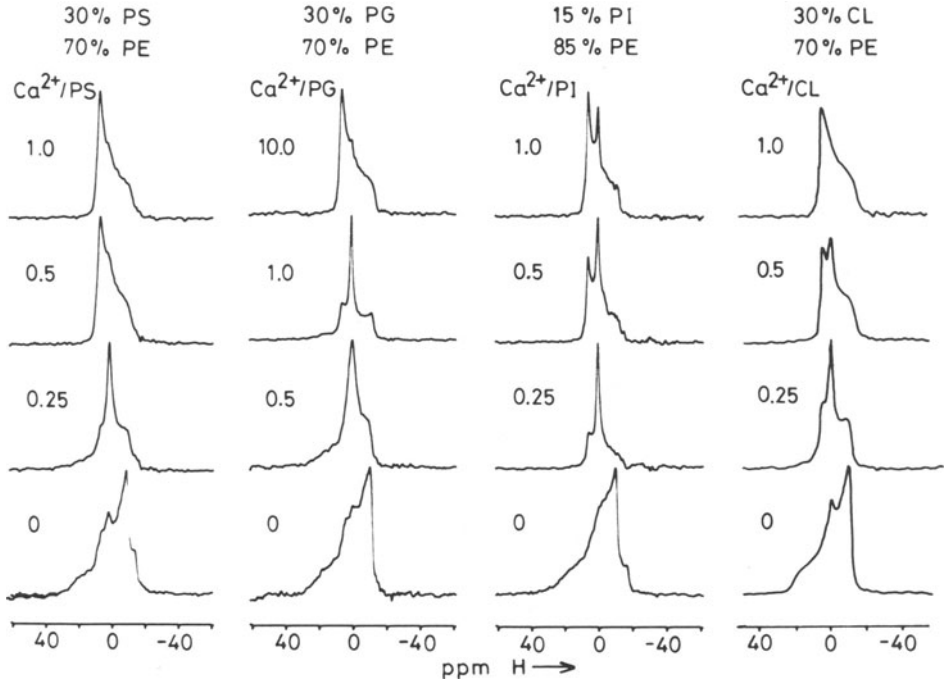

Figure 18. 81.0 MHz ^{31}P NMR spectra arising from mixtures of acidic phospholipids with soya-PE in the presence of various molar ratios of Ca^{2+}. For experimental details for PS-PE see Tilcock and Cullis (1980), for PG-PE see Farren and Cullis (1980), for PI-PE see Nayar *et al.* (1982), and for CL-PE see De Kruijff and Cullis (1980b). All previously published spectra reproduced with permission.

CL is replaced by PC, the H_{II} phase-stimulating capacity of Ca^{2+} is much reduced. In this latter case, next to the H_{II} phase in the PE-containing systems, a large isotropic ^{31}P NMR signal is observed with associated lipidic particles as detected by freeze-fracture (see Section VI). The detailed morphology of the hexagonal H_{II} phase in the divalent cation-PC/CL (1 : 1) system depends on the nature of the cation. Whereas in the presence of Ca^{2+}, one type of H_{II} phase cylinder is observed by freeze-fracturing (with an increased diameter as compared to the pure CL-Ca^{2+} H_{II} phase), addition of Mg^{2+} results in the occurrence of two types of H_{II} cylinders. One of these has the size observed for the H_{II} phase of the Mg^{2+}-CL salt, whereas the other exhibits an increased diameter, suggesting phase separation (Van Venetië and Verkleij, 1981).

It should be noted that not only the absolute amounts of Ca^{2+} (Figure 18), but also the Ca^{2+} concentrations needed to induce these structural changes, are different for the various negatively charged phospholipids. In several biological membrane lipid extracts, Ca^{2+} addition causes similar structural changes. For instance, an appreciable fraction of the total rat liver inner mitochondrial membrane lipids (composition 40% PC, 40% PE, and 20% CL) converts to the H_{II} phase upon addition of Ca^{2+} (Cullis *et al.*, 1980b). Similar effects have been noted for an equimolar mixture of the phos-

pholipids derived from the inner leaflet of the human erythrocyte membrane and cholesterol (Hope and Cullis, 1979).

C. Lipid–Protein and Lipid–Peptide Interactions

1. Extrinsic Proteins and Peptides

Poly-L-lysines have been commonly used as model peptides to gain insight regarding electrostatic peptide–lipid interactions (Hartmann and Galla, 1978; Papahadjopoulos *et al.*, 1975b). The high affinity of the basic poly-L-lysine for negatively charged lipids is also apparent in studies on the effect of this compound on lipid polymorphism (De Kruijff and Cullis, 1980a).

Addition of poly-L-lysine to CL liposomes results in strong peptide–lipid binding and immediate precipitation of the lipids. In this precipitate, the lamellar phase is maintained (Figure 19). Further, whereas Ca^{2+} addition in the absence of poly-L-lysine triggers a bilayer $\rightarrow H_{II}$ phase transition, the presence of the peptide completely blocks this transition (Figure 19), revealing a strong bilayer-stabilization effect of the poly-L-lysine in this system. Alternatively, if poly-L-lysine is added to mixed PE-CL

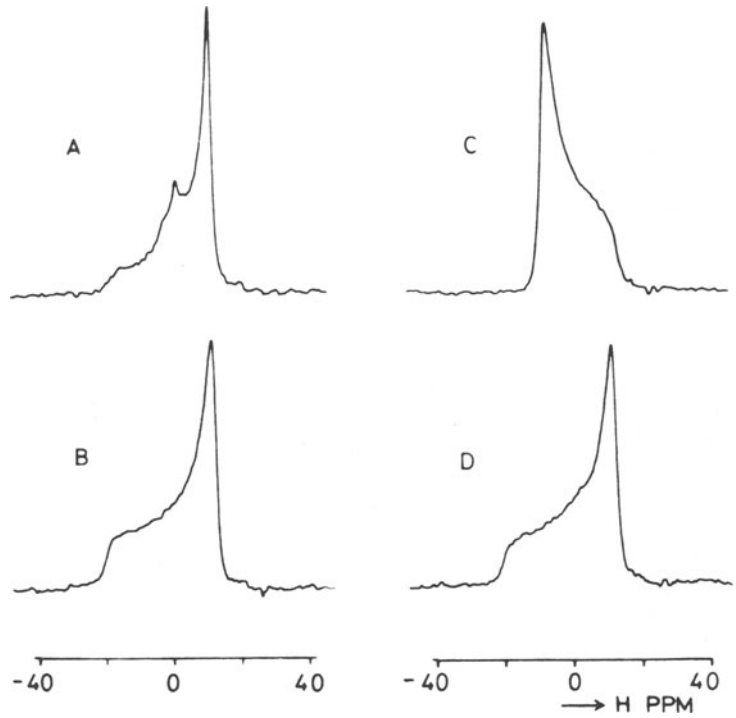

Figure 19. 81.0 MHz ^{31}P NMR spectra at 30°C of (A) 50 μmoles of CL in 1.0 ml buffer; (B) 50 μmoles of CL in 1.0 ml buffer, 5 min after the addition of 40 mg poly-L-lysine; and (C) as in (A) after addition of 100 μl 1 M $CaCl_2$. Reproduced with permission from De Kruijff and Cullis (1980a).

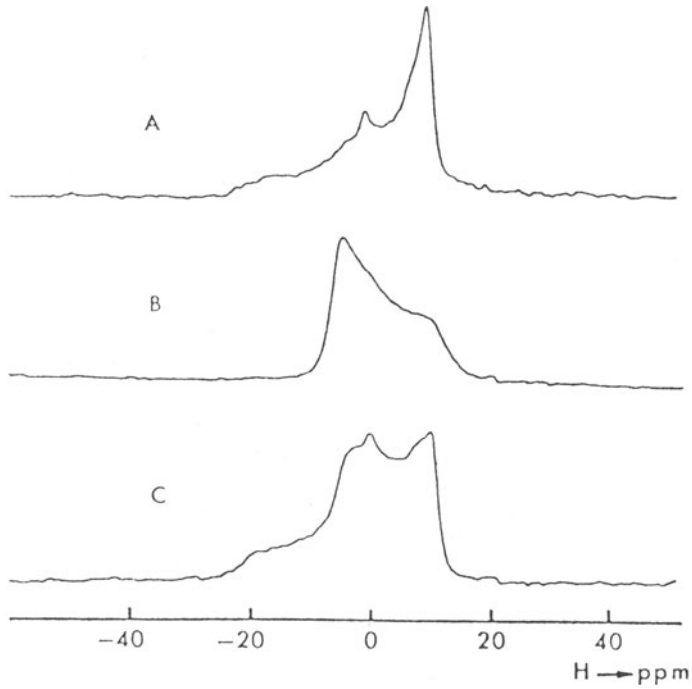

Figure 20. 81.0 MHz ^{31}P NMR spectra at 30°C of an aqueous dispersion of CL (A) in the presence of Ca^{2+} (B) and cytochrome *c* (C). Beef heart CL (50 μmoles) was dispersed in 1.0 ml 100 mM NaCl, 10 mM Tris/HCl, 0.2 mM EDTA, and pH 7.0. In (B) 0.1 ml 1 M $CaCl_2$ and in (C) 0.2 ml buffer containing 36 mg oxidized cytochrome *c* was added. For further details see De Kruijff and Cullis (1980b).

liposomes in which CL stabilizes bilayer structure of the PE component, a phase separation of the CL/poly-L-lysine complex occurs, leading to a bilayer → H_{II} transition of the PE (De Kruijff and Cullis, 1980a).

The inner mitochondrial membrane protein cytochrome *c* is another example of a highly basic protein which experiences strong interactions with a variety of negatively charged membrane phospholipids (Nicholls, 1974). It is interesting that only in the case of the inner mitochondrial CL does this interaction result in changes in macroscopic organization of the lipids (De Kruijff and Cullis, 1980b). ^{31}P NMR (De Kruijff and Cullis, 1980b), electron microscopy (De Kruijff and Cullis, 1980b; Borovjagin and Moshkov, 1973) and X-ray (Gulik-Krzywicky *et al.*, 1969) have shown that the protein can induce nonlamellar lipid structures in this case. This is shown in Figure 20 by ^{31}P NMR. The addition of cytochrome *c* to CL liposomes results in the formation of an isotropic component together with a spectral component indicative of the H_{II} phase.

2. *Gramicidin–Lipid Interactions*

The helical membrane spanning dimer of the hydrophobic pentadecapeptide gramicidin (Urry, 1971; Weinstein *et al.*, 1980) may serve as a model for the hydrophobic

segments of intrinsic membrane proteins (Chapman *et al.*, 1977). Due to its hydrophobicity, gramicidin can be easily incorporated in phospholipids. When gramicidin is incorporated in 18 : 1_t/18 : 1_t-PE it can be seen from Figure 21 that the onset of the formation of the hexagonal H_{II} phase is shifted to much lower temperatures (Van Echteld *et al.*, 1981a). At a gramicidin/18 : 1_t/18 : 1_t-PE ratio of 1 : 25 the transition is already completed at 50°C. This hexagonal H_{II} phase-promoting ability of gramicidin, which can be seen to be clearly concentration-dependent, has also been found for 18 : 1_c/18 : 1_c-PE (Van Echteld *et al.*, 1981a). A straightforward implication of these findings is the association of gramicidin with phospholipid molecules within the hexagonal H_{II} phase.

Most surprisingly, however, this strong influence of gramicidin on the structural organization of lipids is not restricted to H_{II} type of lipids that undergo a lamellar to hexagonal H_{II} phase transition by themselves, but has also been found with lipids that are known to pre-eminently organize themselves in bilayers such as 18 : 1_c/18 : 1_c-PC (Van Echteld *et al.*, 1981a). In Figure 22, it is shown that incorporation of a small amount of gramicidin in 18 : 1_c/18 : 1_c-PC gives rise to a small isotropic component in the ^{31}P NMR spectrum and a reduction of $\Delta\sigma$. These spectral changes are compatible with a reduction in size of the gramicidin-containing structures compared to the pure

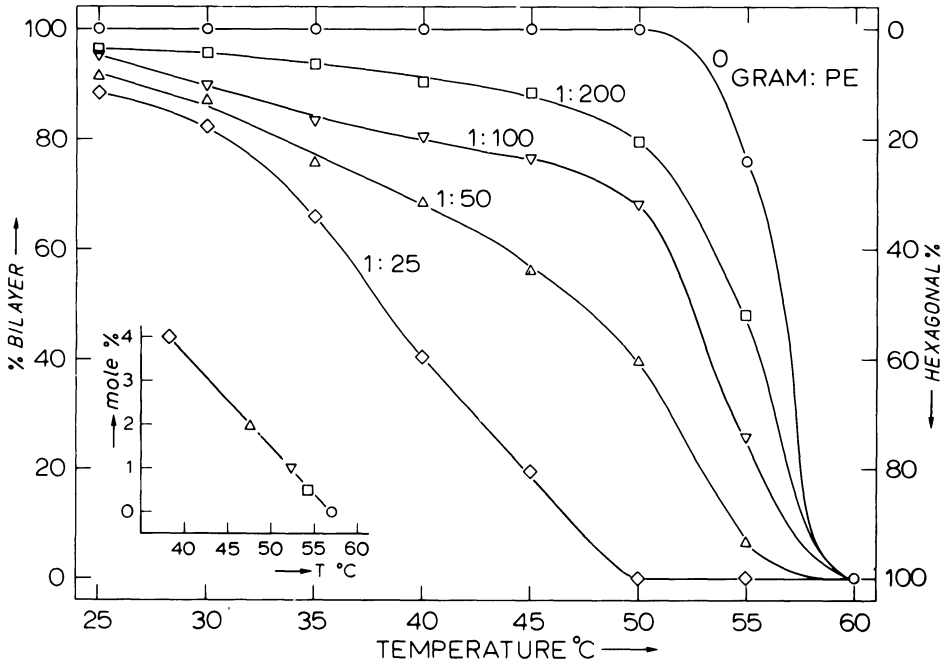

Figure 21. Effect of gramicidin on the bilayer → H_{II} transition of 18 : 1_t/18 : 1_t-PE. The gramicidin/18 : 1_t/18 : 1_t-PE molar ratios are indicated in the figure. The insert shows the relationship between gramicidin concentration (mole%) and the temperature at which 50% of the lipid is organized in the H_{II} phase. Reproduced with permission from Van Echteld *et al.* (1981a). See this reference for further details.

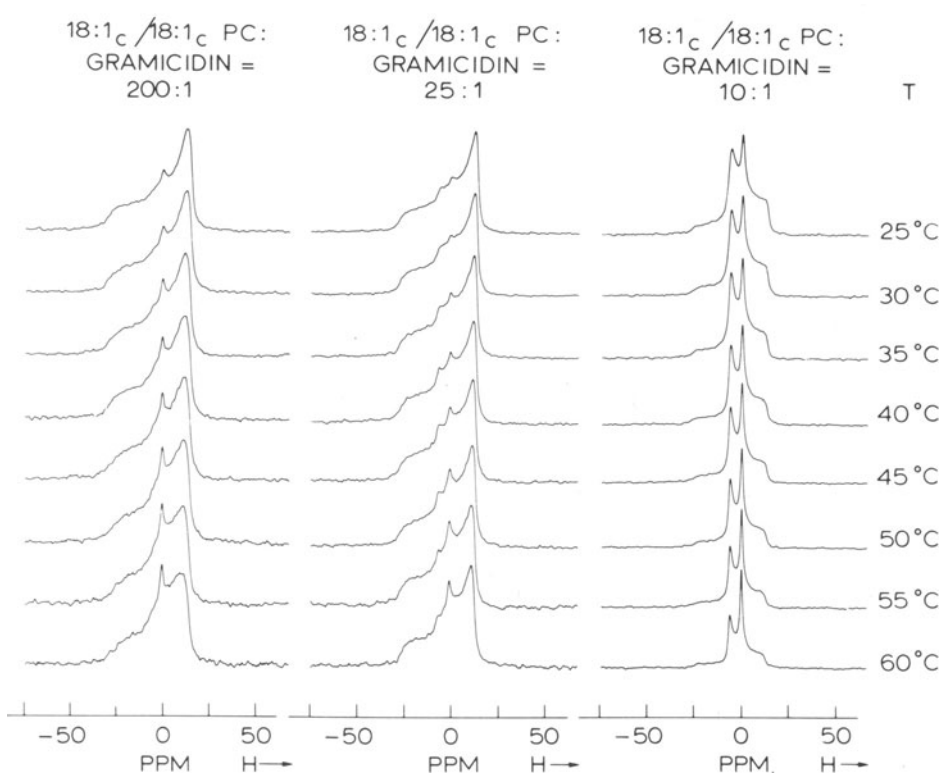

Figure 22. 81.0 MHz ^{31}P NMR spectra of 18 : 1_c/18 : 1_c-PC-gramicidin mixtures at various temperatures. Reproduced with permission from Van Echteld *et al.* (1981a). See this reference for further details.

lipid, which also was found with freeze-fracture electron microscopy. However, increasing the gramicidin content induces an additional spectral component indicating PC organized in a hexagonal H_{II} phase, which can be seen most clearly in Figure 22 as the resonance intensity at approximately -6 ppm. Freeze-fracturing of the same sample shows large areas with a typical striated fracture pattern (Van Echteld *et al.*, 1981a).

The hexagonal H_{II}-promoting ability of gramicidin could possibly be the result of a mismatch of the length of the gramicidin dimer with the thickness of the hydrophobic part of the bilayer. To test this latter hypothesis, the influence of gramicidin on PCs with varying fatty acid chain lengths has been investigated (Van Echteld *et al.*, 1982). As can be seen from Figure 23, the onset of the induction of the hexagonal H_{II} phase occurs when the fatty acid chain exceeds 16 carbon atoms. The isotropic peaks seen in this figure originate from lipid in smaller structures. The chain length-dependent formation of the hexagonal H_{II} phase by gramicidin is not restricted to unsaturated PCs. As shown in Figure 24, the onset of the induction of the hexagonal phase in liquid-crystalline-saturated PCs again coincides with a fatty acid chain exceeding 16 carbon atoms. Also, with PC from natural sources, e.g., egg-PC and soya-

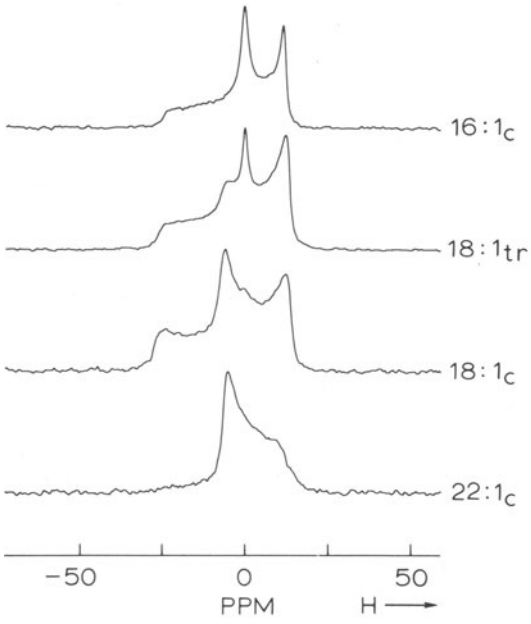

Figure 23. 81.0 MHz ^{31}P NMR spectra of aqueous dispersions of mixtures of gramicidin with various unsaturated PCs in a 1 : 10 molar ratio at 25°C. The fatty acids present in the various PC species are indicated in the figure. Reproduced with permission from Van Echteld *et al.* (1982). See this reference for further details.

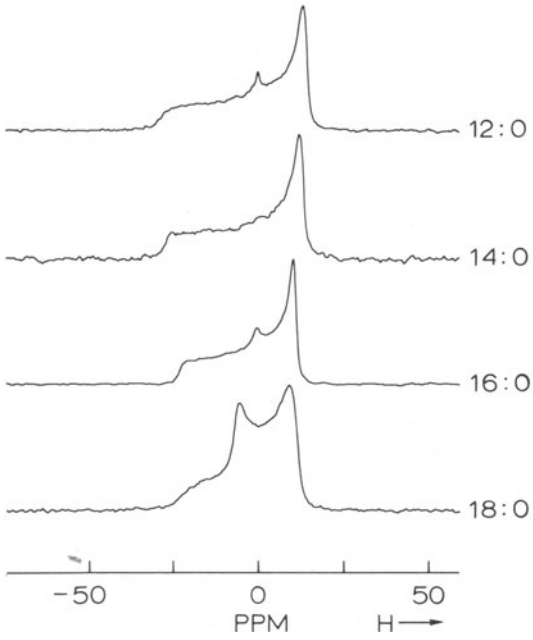

Figure 24. 81.0 MHz ^{31}P NMR spectra of aqueous dispersions of mixtures of gramicidin with various saturated liquid-crystalline PC species in a 1 : 10 molar ratio. Fatty acid composition as indicated in the figure. Recording temperatures: 12 : 0/12 : 0-PC at 30°C, 14 : 0/14 : 0-PC at 40°C, 16 : 0/16 : 0-PC at 50°C, and 18 : 0/18 : 0-PC at 60°C. Reproduced with permission from Van Echteld *et al.* (1982). See this reference for further details.

PC, induction of a hexagonal phase for part of the lipid molecules was found (Van Echteld *et al.*, 1982). From neutron diffraction studies (Büldt *et al.*, 1978), the thickness of the hydrophobic part of liquid-crystalline 16 : 0/16 : 0-PC may be estimated to be 30–31 Å, similar to the length of the gramicidin dimer of approximately 30 Å (Wallace *et al.*, 1981). When the length of the hydrophobic part of the phospholipid molecules exceeds this length, apparently an instable situation arises which results in the formation of a hexagonal H_{II} phase for part of the molecules. The longer the fatty acid chains, the more lipid molecules enter the hexagonal phase, probably as a result of a larger imbalance. To accommodate the gramicidin in the hexagonal H_{II} phase, a model has been proposed (Van Echteld *et al.*, 1982) in which the gramicidin dimer spans adjacent cylinders, thereby maintaining a similar orientation as in the bilayer situation.

Alternatively, the H_{II}-promoting ability could be related to the shape of gramicidin (or the dimer). The bulky tryptophan residues located at the C-terminal renders the molecule cone-shaped, thus favoring H_{II} phase formation (see Section VII). In agreement with this suggestion is the observation that in mixtures with lyso-PC gramicidin is organized in a lamellar phase (Killian *et al.*, 1983).

3. *Glycophorin–Phospholipid Interactions*

Glycophorin, the major integral sialoglycoprotein of the human erythrocyte membrane, is well characterized biochemically (Marchesi *et al.*, 1976), has been the subject of many physicochemical studies in reconstituted phospholipid systems, and has been found to cause extensive perturbation of membrane phospholipid hydrocarbon chain conformation and packing (Taraschi and Mendelsohn, 1980; Van Zoelen *et al.*, 1978; Rüppel *et al.*, 1982). Furthermore, it is known to carry receptors for several sugar-specific lectins such as wheat germ agglutinin (WGA) (Verpoorte, 1975), MN blood group substances (Marchesi *et al.*, 1972), influenza virus (Marchesi *et al.*, 1972), and malaria (Pasvol *et al.*, 1982) and therefore when incorporated into membranes offers systems amenable to effector–receptor studies.

The introduction of glycophorin into liposomes of 18 : 1_c/18 : 1_c-PC gives rise to unilamellar vesicles (1000–5000 Å diameter) with intramembranous protein particles as observed by freeze-fracture electron microscopy (Taraschi *et al.*, 1982a). When examined by ^{31}P NMR, these vesicles (300/1; moles lipid/mole protein) exhibit bilayer spectra over a wide range of temperatures (0–60°C) (Taraschi *et al.*, 1982a).

Reconstitution of glycophorin with 18 : 1_c/18 : 1_c-PE, which prefers the hexagonal H_{II} phase above 10°C, results in the formation of small, unilamellar bilayer vesicles (300–1500 Å diameter) with ill-defined intramembranous protein particles (Taraschi *et al.*, 1982a). In contrast to the 18 : 1_c/18 : 1_c-PC–glycophorin system where the lipid–protein ratio could be varied over a wide range, the 18 : 1_c/18 : 1_c-PE–glycophorin recombinants exclusively form a 25/1 (moles/mole PE : glycophorin) complex. The protein particles observed by freeze-fracture electron microscopy are much larger in the 18 : 1_c/18 : 1_c-PE–glycophorin vesicles than in the 18 : 1_c/18 : 1_c-PC–glycophorin system suggesting that the protein was more highly aggregated in this former system.

The temperature dependence of the ^{31}P NMR lineshapes arising from unilamellar vesicles of 18 : 1_c/18 : 1_c-PE and glycophorin is shown in Figure 25. At 0°C, a

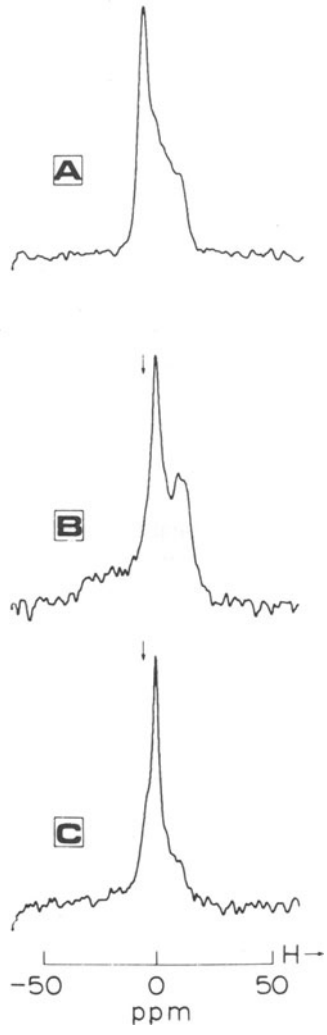

Figure 25. 36.4 MHz ^{31}P NMR spectra of 18 : 1_c/18 : 1_c-PE in the absence (A) 0°C and presence of glycerophorin (B) 0°C and (C) 25°C. Arrow indicates the position of the main spectral component of 18 : 1_c/18 : 1_c-PE in the H_{II} phase. Reproduced with permission from Taraschi *et al.* (1982a). See this reference for further details.

considerable amount of the phospholipid (50%) is in a structure allowing isotropic motion and the remaining lipid is arranged in a bilayer organization. The isotropic signal arises from the smaller glycophorin-containing vesicles present in the preparation. In comparison, 18 : 1_c/18 : 1_c-PE in isolation assumes a hexagonal H_{II} phase at this temperature. The spectra of the glycophorin–PE system become increasingly "isotropic" at higher temperatures (Taraschi *et al.*, 1982a); however, the signals remain devoid of hexagonal H_{II} phase characteristics. In summary, the combination of ^{31}P NMR and freeze-fracture electron microscopy demonstrates a strong bilayer-stabilizing capacity of glycophorin.

It may be noted that treatment of the vesicles with trypsin, which removes the large, bulky hydrophilic sugar residues [which are oriented (95%) towards the exterior

of the vesicles] but leaves the hydrophobic segment of the protein incorporated in the membrane (Tomita and Marchesi, 1975), does not cause any appreciable changes in vesicle structure. Thus, the intrinsic hydrophobic segment of glycophorin appears to be mainly responsible for the strong bilayer-stabilization effects (Taraschi *et al.*, 1982a).

Glycophorin has also been reported to stabilize bilayer organization in reconstituted vesicle systems containing CL, a lipid which undergoes an isothermal bilayer to hexagonal H_{II} phase transition in the presence of Ca^{2+} (Taraschi *et al.*, 1983). Trypsin treatment failed again to alter the bilayer-stabilizing capacity of this protein.

During the past 10 years, considerable attention has been focused on the complex contact events that occur at the eukaryotic cell surfaces. These phenomena are thought to be specifically mediated by complex carbohydrates which coat the surface of cells and are covalently linked to membrane lipids and proteins. Following the initial recognition of the effector, e.g., hormone, toxin, antibody, lectin, or virus, a chain of events transpire that eventually evoke a metabolic response by the cell (Nicolson, 1976). The mechanistic details of the events which occur following the recognition of the effector by the receptor and the resulting response remain largely a mystery. Although a number of theories have appeared (Nicolson, 1976; Edelman, 1976), none have directly addressed the possibility that the binding event and subsequent receptor rearrangement ("patching") may lead to an alteration in the molecular organization of the lipids in that region of the membrane in which the receptor glycolipids and glycoproteins are located. This hypothesis is appealing in that major membrane lipids can form nonbilayer structures of potential functional importance. In order to investigate these possibilities, glycophorin–WGA interactions have been characterized in model membrane systems (Taraschi *et al.*, 1982b).

The addition of WGA to cosonicated vesicles of 18 : 1_c/18 : 1_c-PC and glycophorin (200/1; moles lipid/mole protein), in which 100% of the receptor-bearing carbohydrates are externally oriented, induces aggregation but not fusion of the vesicles as indicated by both ^{31}P NMR and freeze-fracture electron microscopy. This aggregation can be completely reversed by *N*-acetyl-D-glucosamine, which is a (sugar hapten) inhibitor of WGA agglutination. It was therefore concluded that the binding and subsequent vesicle aggregation caused by WGA proceeds without a structural reorganization of the membrane phospholipid bilayer.

The effect of WGA on two 18 : 1_c/18 : 1_c-PE–glycophorin vesicle preparations (moles/mole PE : glycophorin; 200/1; 25/1) has been obtained from ^{31}P NMR and freeze-fracture electron microscopy (Figure 26). The 25 : 1 cosonicated recombinant gives rise to a narrow, symmetric ($\nu_{1/2}$ = 100 Hz), "high resolution" ^{31}P NMR spectrum which arises from phospholipids present in small bilayer vesicles as indicated by freeze-fracture electron microscopy (Taraschi *et al.*, 1982b). The addition of increasing amounts of WGA to the sample (up to WGA/glycophorin mole ratio = 1.8) induces a substantial broadening in the spectrum although the lipids maintain a bilayer organization (Figure 26). Some membrane reorganization does occur, most likely as a result of vesicle fusion; however, a gross morphological change from a bilayer to nonbilayer (hexagonal H_{II} phase) is not observed.

Cosonication of a glycophorin–18 : 1_c/18 : 1_c-PE (1 : 200 sample) results in a narrow, symmetric ($\nu_{1/2}$ = 130 Hz), ^{31}P NMR lineshape, typical of small bilayer vesicles

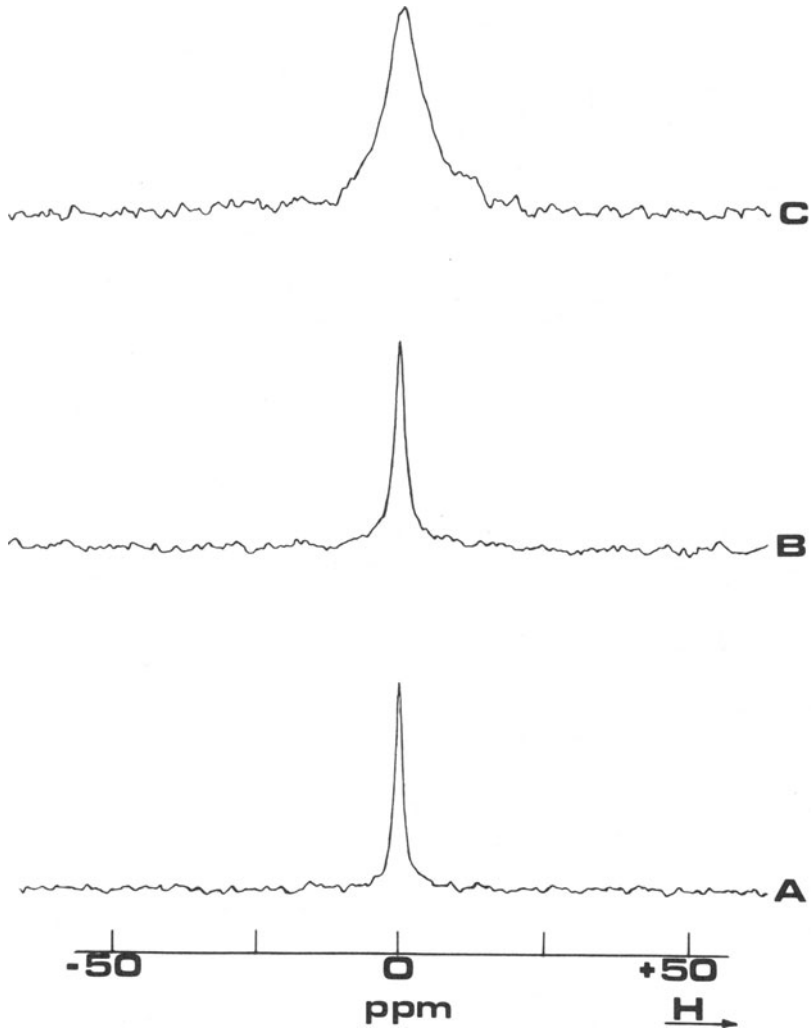

Figure 26. 81.0 MHz ^{31}P NMR spectra of sonicated 18 : 1_c/18 : 1_c-PE–glycophorin vesicles (25 : 1, moles lipid/mole protein; 15 μmoles 18 : 1_c/18 : 1_c-PE, 30.0 mg glycophorin) in the absence (A) and presence of 420 nmoles WGA (B) and 840 nmoles WGA (C) at 30°C. The molar ratios WGA/glycophorin were 0.7 (A) and 1.4 (B). For further details see Taraschi *et al.* (1982b).

(Figure 27). Titration with WGA causes drastic changes in the vesicles as indicated by both ^{31}P NMR and freeze-fracture electron microscopy, as higher levels of WGA (WGA/glycophorin, mole/mole = 1.0) induced a nearly complete (90%) bilayer to hexagonal H_{II} phase transition in the vesicle preparation as evidenced from ^{31}P NMR (Figure 27) and freeze-fracture experiments (Figure 28).

Treatment of the 25/1 and 200/1 18 : 1_c/18 : 1_c-PE–glycophorin samples with trypsin provides insight into the origin of the differing effects that WGA exerts on

these membrane recombinants. The ^{31}P NMR spectra obtained for the enzymatically treated vesicles are shown in Figure 29. In the case of the 200/1 sample, trypsin treatment triggers a nearly complete bilayer to hexagonal H_{II} phase transition (with 95% of the ^{31}P NMR signal arising from lipid in the hexagonal H_{II} phase), whereas the 25/1 sample showed only an increase in line width of the isotropic NMR signal. Thus, it was concluded that the large hydrophilic carbohydrate residues which present a steric barrier to fusion and consequently prevent hexagonal H_{II} phase formation were mainly responsible for the bilayer-stabilizing effect of glycophorin in the 200 : 1 vesicles (Taraschi *et al.*, 1982b). The absence of this transition in the 25/1 sample likely results from a direct bilayer stabilization by the intrinsic, hydrophobic portion of the protein.

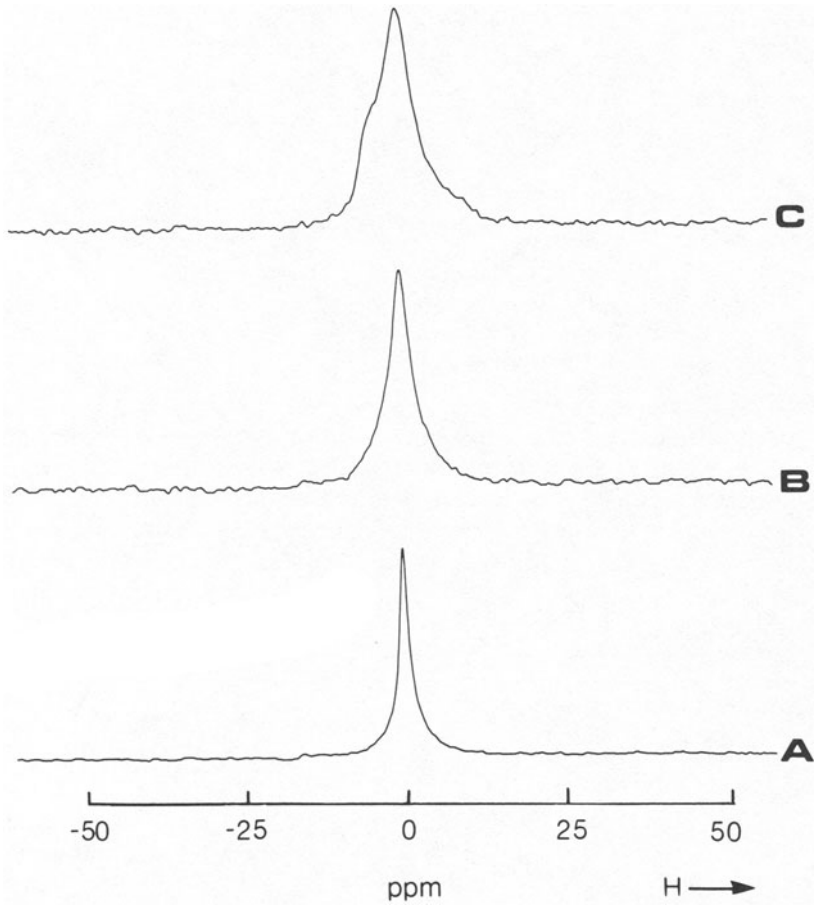

Figure 27. 81.0 MHz ^{31}P NMR spectra of cosonicated 18 : 1_c/18 : 1_c-PE–glycophorin vesicles (200 : 1, moles lipid / mole protein; 37.5 μmoles 18 : 1_c/18 : 1_c-PE, 7.5 mg glycophorin) in the absence (A) and presence of 100 nmoles WGA (B) and 200 nmoles WGA (C) at 30°C. The molar ratios WGA/glycophorin were 0.5 and 1.0 in (B) and (C), respectively. For further details see Taraschi *et al.* (1982b).

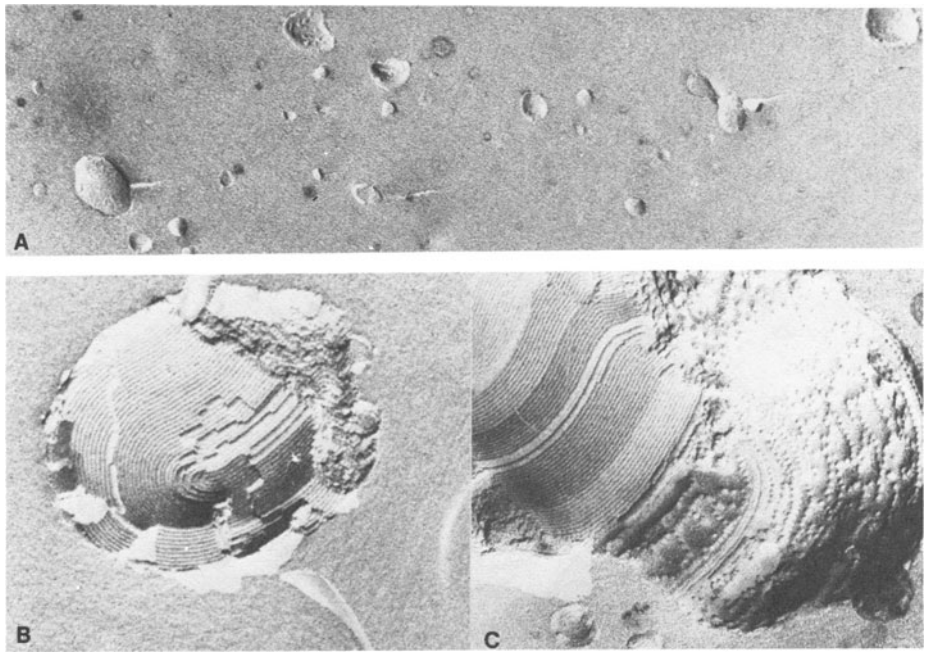

Figure 28. Freeze-fracture electron micrographs of cosonicated 18 : 1_c/18 : 1_c-PE–glycophorin vesicles (200 : 1, moles lipid/mole protein) in the absence (A) and presence (B,C) of 200 nmoles WGA (WGA/glycophorin 1.0). Final magnification × 100,000. Reproduced with permission from Taraschi *et al.* (1982b). See this reference for further details.

It is interesting to discuss these results further. Upon WGA binding to the 18 : 1_c/18 : 1_c-PE–glycophorin vesicles (200 : 1), crosslinking of the receptor head groups of glycophorin within the individual vesicles by the multivalent WGA results in aggregation of the protein into "patches," thus removing the distributed bilayer-stabilizing effect of glycophorin. As a result, large domains of lipid are freed from

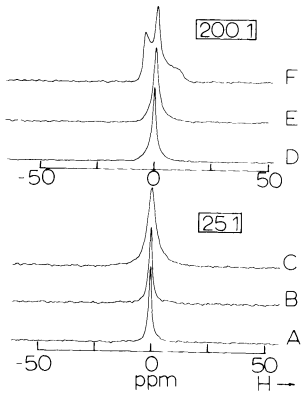

Figure 29. Effect of neuraminidase and trypsin treatment on the 81.0 MHz ^{31}P NMR spectra of cosonicated 18 : 1_c/18 : 1_c-PE–glycophorin vesicles. The molar ratios of 18 : 1_c/18 : 1_c-PE–glycophorin are indicated in the figure. Vesicles were treated with (B,E) neuraminidase (50 units/1 mg of protein, 2 hr, 37°C) followed by (C,F) trypsin treatment (5% wt./wt., 2 hr, 37°C). Reproduced with permission from Taraschi *et al.* (1982b).

the influence (both hydrophobic and steric) of the protein and may form intrabilayer hexagonal H_{II} phase cylinders in the patch or fuse with protein-free lipid areas present in adjacent vesicles to form the hexagonal H_{II} phase. It is also likely that intervesicle receptor crosslinking may act to aggregate the vesicles, allowing fusion (and consequent H_{II} phase formation) to proceed. It is interesting that lipidic particles (see Section VI) are observed by freeze-fracture electron microscopy in these preparations (see also Figure 28). In the light of the dramatic membrane destabilization resultant from WGA binding to membrane glycoprotein, it can be suggested that varying degrees of cellular-mediated protein aggregation in a membrane may trigger local structural reorganizations of the lipid, which may participate in and facilitate certain cellular functional processes.

VI. "ISOTROPIC" LIPID STRUCTURES AND LIPID PARTICLES

From the previous sections, it is clear that the hexagonal H_{II} phase is commonly observed for various hydrated membrane phospholipids. It is, however, hard to imagine that a membrane can contain extensive hexagonal H_{II} phase lipid and still maintain a permeability barrier. The possibility must be considered whether lipids can adopt alternative nonlamellar structures and if so, whether such structures are more compatible with membrane structure and function. As noted previously, many lipid systems at conditions intermediate between lamellar or H_{II} phase reveal a component experiencing "isotropic motion" as indicated by ^{31}P NMR. This is commonly observed in mixtures of bilayer and H_{II} phase-preferring lipids (see for instance Figure 14), including the total lipid extracts of various biological membranes. Although the isotropic signal can, in some cases, be attributed to the presence of small vesicles in the great majority of the systems studied, the phospholipids are organized in macroscopically large structures. From the NMR lineshape alone, it is impossible to derive information on the exact nature of these "isotropic" structures, however. Among various possibilities, lateral diffusion around highly curved bilayer surfaces or tumbling and diffusion in inverted micellar-like structures are the most attractive.

The X-ray data on these "isotropic" structures are limited, which is due to a large extent to the structural heterogeneity in such samples. Often, also, lamellar and/or hexagonal H_{II} structures are present as well as those structures allowing isotropic motion. In the case of an "isotropic" structure observed for glycolipids extracted from *A. laidlawii* cell membranes, evidence has been presented for a cubic type of phase (Wieslander *et al.*, 1981a,b) similar to an interwoven bilayer network system as shown in Figure 6.

A clearer picture on the nature of the "isotropic" structures has emerged from freeze-fracture studies (Sen *et al.*, 1982; De Kruijff *et al.*, 1979; Verkleij *et al.*, 1979a; Rand *et al.*, 1981) in such systems, which often reveal small spherical particles and pits (Figure 30). These "lipidic particles" are in some cases organized in a string or sometimes are randomly dispersed and have uniform sizes in a particular lipid system. The diameter can range from 80 to 130 Å, depending on the type of lipid (Van Venetië and Verkleij, 1981; De Kruijff *et al.*, 1979). When the H_{II} phase is also present, lipidic particles can often be seen in transitional regions where they seem to originate from

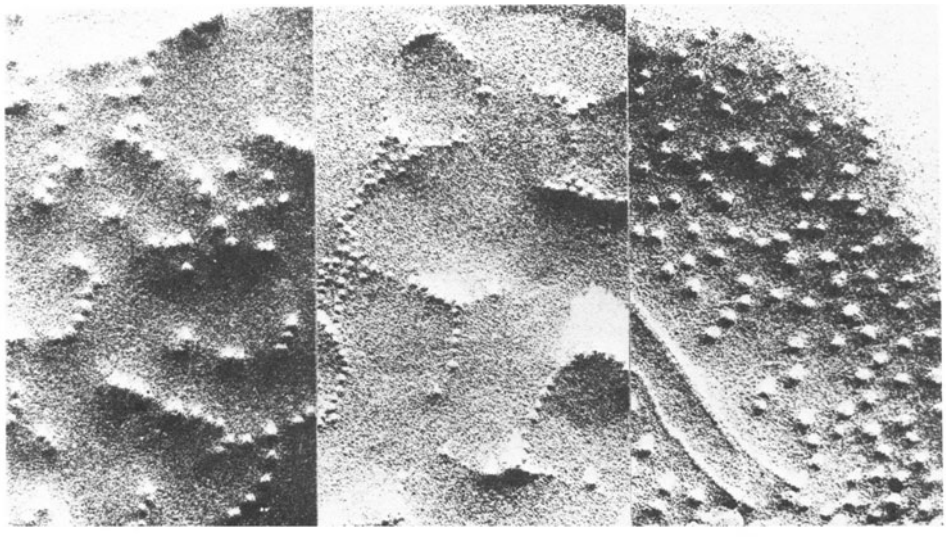

Figure 30. Freeze-fracture micrographs of lipidic particles in CL-(Ca^{2+})-PC (card/PC), monoglucosyldiglyceride-PC (MGDG/PC), and PE/PC/cholesterol (PE/PC/chol) mixtures. Reproduced with permission from De Kruijff *et al.* (1980a). See this reference for further details.

(or form) an H_{II} cylinder (Figure 31; Van Venetië and Verkleij, 1981; Verkleij *et al.*, 1980, 1982). In systems such as mixtures of 18 : 1_c/18 : 1_c-PC–18 : 1_c/18 : 1_c-PA in the presence of chlorpromazine, the particles are organized in a three-dimensional lattice (Verkleij *et al.*, 1982). Also, large irregular cusp-shaped particles have been noticed (Verkleij *et al.*, 1979a; Hui and Stewart, 1981; Verkleij and De Kruijff, 1981; Miller, 1980; De Kruijff *et al.*, 1980a; Borovjagin *et al.*, 1982; Rand *et al.*, 1981). Several models have been proposed for the molecular organization of the lipidic particles. Although conclusive evidence is lacking, the general consensus is that the homogeneously sized lipidic particles represent inverted micelles present in one bilayer (Figure 32b) or at the nexus of intersecting bilayers (Figure 32a). The larger cusp-shaped particles possibly represent intermembrane attachment sites without inverted micelles (Figure 32c). The inverted micelle, which can be considered to be a short H_{II} cylinder with closed-off ends, is a nonlamellar lipid structure which clearly is more compatible with membrane structure and function than the extended areas of H_{II} phase. It is not surprising that the observation of these particles has greatly contributed to our present ideas concerning lipid polymorphism in relation to membrane structure and to a renewed interest in the nature of intramembrane particles.

In model membrane systems containing lipidic particles, the bilayers often appear to form an interwoven network with particles present predominantly at the intersections of bilayers (Figure 33). Such honeycomb (Cullis *et al.*, 1980a) or sponge (Noordam *et al.*, 1980) structures in which membrane continuity exists with compartmentalization

of aqueous spaces are reminiscent of structures found in some biological membranes, which will be discussed in Section IX. Two other aspects of lipidic particles are of interest. First, those factors which can modulate bilayer $\rightarrow H_{II}$ transitions also can regulate the presence of these particles, which is a prerequisite for a possible functional role of these structures. Second, although the lipidic particles observed in model membranes are long-lived structures, it can be expected that similar structures might be (transiently) formed at discrete sites in a membrane dependent on local environmental conditions.

The amount of isotropic ^{31}P NMR signals arising from such systems is often considerably larger than the fraction of the lipids present in lipidic particles (De Kruijff *et al.*, 1979). This might indicate the presence of short-lived lipidic particles which might escape detection by freeze-fracture electron microscopy or might indicate that the isotropic signal originates from both lipids present in inverted micellar structures and from lipids present in strongly curved bilayers present in the honeycomb type of liposomal structure (Figure 33). It is intriguing to note that ^{31}P NMR has demonstrated that both bilayer and hexagonal H_{II} type lipids can be present in these isotropic structures in relative amounts identical to those found for the entire sample (De Grip *et al.*, 1979; Vasilenko *et al.*, 1982b; De Kruijff *et al.*, 1979).

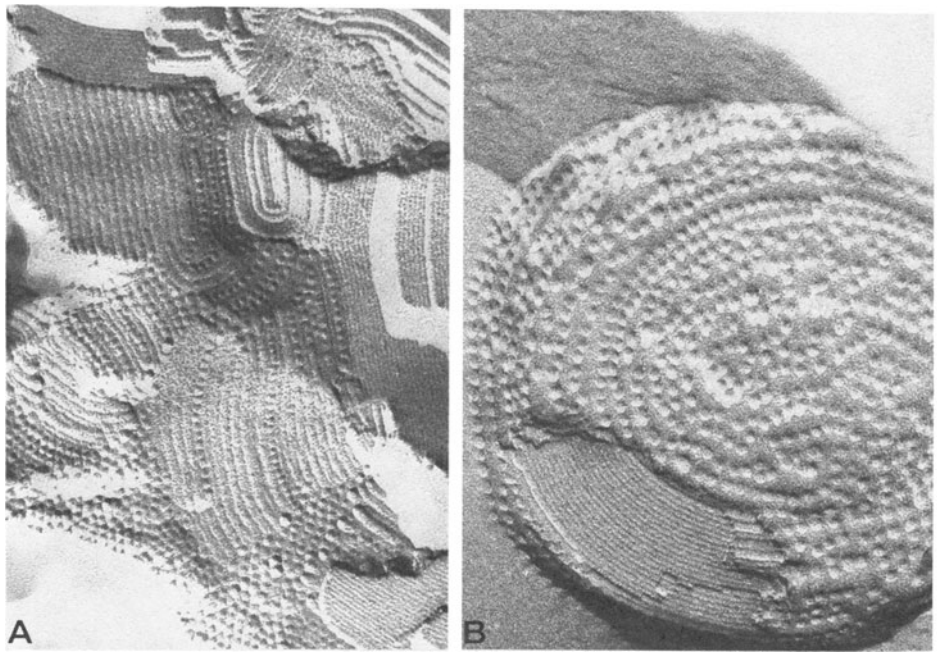

Figure 31. Freeze-fracture morphology of transitional regions between the H_{II} phase and lipidic particles in (A) chlorpromazine–18 : 1_c/18 : 1_c-PA mixtures (Verkleij *et al.*, 1982) and (B) 18 : 1_c/18 : 1_c-PC–CL (1 : 1) vesicles in the presence of 2 mM $MnCl_2$ (Verkleij *et al.*, 1980). Final magnification $\times$100,000. Reproduced with permission from Verkleij *et al.* (1980, 1982).

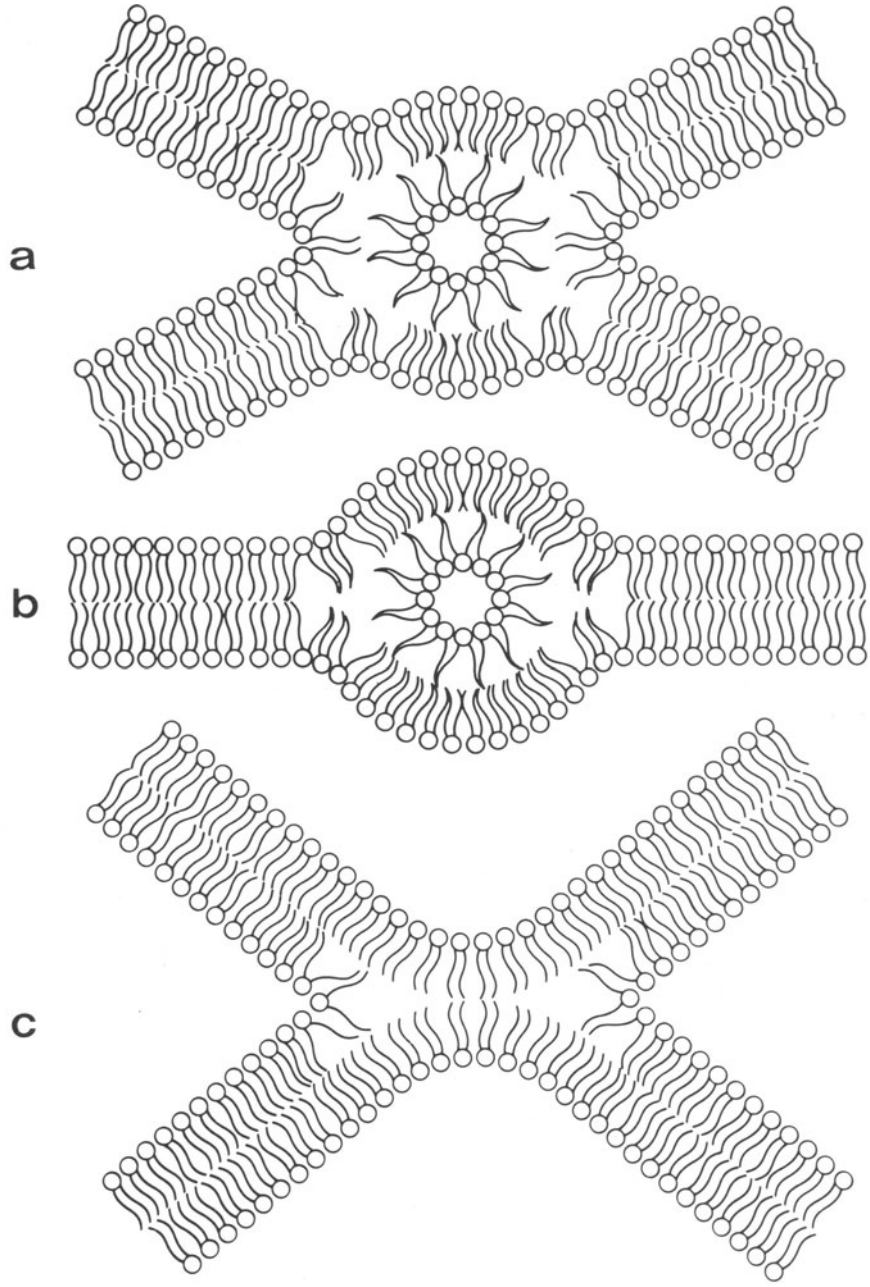

Figure 32. Inverted micelles at the nexus of intersecting bilayers (a) within one bilayer (b) and intermembrane attachment sites without inverted micelles (c).

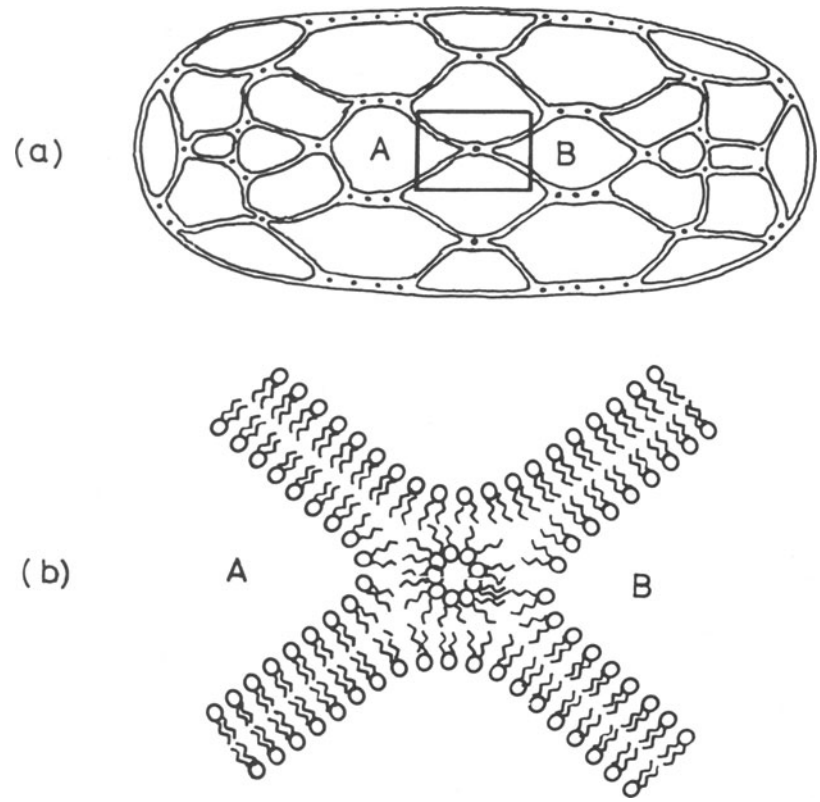

Figure 33. (a) A "honeycomb" structure compatible with ^{31}P NMR, freeze-fracture, and permeability results derived from systems containing mixtures of lipids which assume bilayer and H_{II} phase structure upon isolation. Compartmentalization within a continuous membrane structure is emphasized for compartments A and B in the expanded diagram (b).

VII. THE SHAPE CONCEPT: A RATIONALE FOR LIPID POLYMORPHISM

In order to explain the phase preferences of membrane lipids, a simple concept has been developed which relates the shape of the lipid molecule to the phase preferred by that lipid in aqueous dispersion (Cullis and De Kruijff, 1979). In this "molecular shape"-concept, lipids with a relatively large head group as compared to the cross-sectional area of the hydrocarbon chains are defined as "inverted cone"-shaped molecules. Such lipids include lysophospholipids and detergents in general, which pack optimally in micellar or H_I type configurations (Figure 34). The opposite case of "cone"-shaped lipids includes lipids where the head group is smaller and thus, inverted structures such as the H_{II} phase or inverted micelles are preferred. When the head

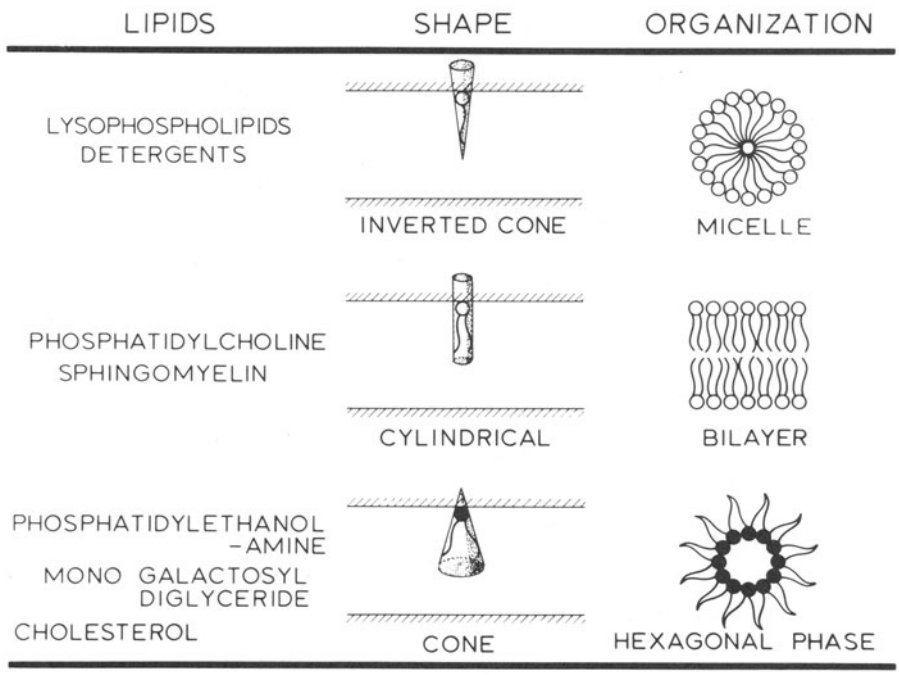

Figure 34. The molecular shape concept of lipid polymorphism.

groups and the acyl chains have comparable cross-sectional areas, the molecules have an overall cylindrical shape and organize themselves in bilayers. In some of the cubic phases which are intermediate between the lamellar and H_{II} phase, the shape of the molecules is also intermediate between that resulting in lamellar or H_{II} phase formation (Larsson *et al.*, 1980).

It should be noted that the shape concept is inclusive, and takes into account the following factors: (1) dynamic properties of the molecules (for instance fast rotation about the long axis), resulting in a time-averaged shape, (2) intra- and intermolecular interactions (in particular the strong intermolecular hydrogen bonding possible for PEs; Hauser *et al.*, 1981), and (3) the hydration properties of the lipid head groups.

Although the shape concept may initially appear naive, it describes surprisingly well many of the phase properties described in the previous sections. The temperature-dependent bilayer $\rightarrow H_{II}$ transitions can be understood in terms of an increase in hydrocarbon area with increasing temperature, resulting in an increased cone shape which eventually triggers the transition. Similarly, the more unsaturated PE species are more cone-shaped and thus have lower transition temperatures. Addition of Ca^{2+} to CL liposomes results in Ca^{2+}-binding which reduces the head group size because of dehydration, decreased electrostatic repulsion, as well as possibly decreasing the distance between the phosphates in one molecule because of intramolecular P-Ca-P salt formation (De Kruijff *et al.*, 1982b). Whereas monoglucosyldiglyceride prefers the H_{II} phase, the additional presence of another glucose in the head group to produce

diglucosyldiglyceride increases the head group size and results in a preference for bilayer structure.

The shape hypothesis also allows the structural preferences of certain mixed lipid systems to be understood. In an equimolar mixture of the inverted cone-shaped lyso-PC and the cone-shaped unsaturated PE, bilayer structure is observed (Figure 35; Madden and Cullis, 1982). Also, in mixtures with cholesterol (Van Echteld *et al.*, 1981b; Rand *et al.*, 1975) and fatty acids (Jain *et al.*, 1980), which both can induce H_{II} structures (Marsh and Seddon, 1982; Cullis and De Kruijff, 1979), the lyso-PC can form bilayers.

It is important to note that the shape–structure relationship is discontinuous. Although shapes appear to be continuously variable, structural transitions occur only at discrete steps. Nevertheless, within one type of structure, for instance the lamellar phase, the molecular shape of the components might still be important to particular properties of the system. For instance, the membrane spanning portion of integral proteins most likely will have an irregular contour. It can therefore be expected that in bilayers containing different lipid species, those lipids which partition into the protein–lipid interface are those which match the shape of that part of the protein (Israelachvili, 1977). In the case of a mismatch of shapes, packing defects could exist which might affect the barrier function of the bilayer. Alternatively, protein aggregation could possibly be modulated by matching protein and lipid shapes (Israelachvili, 1977). Some evidence for these hypotheses has accumulated in recent years in studies on reconstituted lipid protein systems. The general observation is that incorporation of integral membrane proteins into artificial bilayers made from one lipid species greatly increases the bilayer permeability towards small solutes. On the other hand, incorporation into mixed lipid systems results in "sealed" vesicles. This is shown in Figure 36 for glycophorin. Whereas the protein induces a large increase in glucose permeability through 18 : 1_c/18 : 1_c-PC bilayers, glycophorin incorporation in vesicles containing the total lipid extract of the erythrocyte (in which lipids with a large variety of dynamic

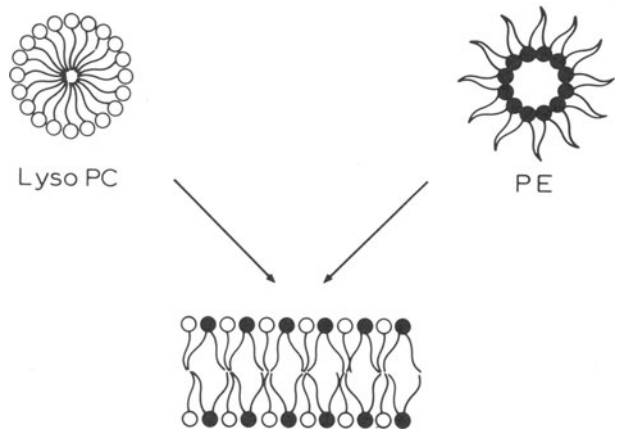

Figure 35. Molecular shape concept in mixed lipid systems.

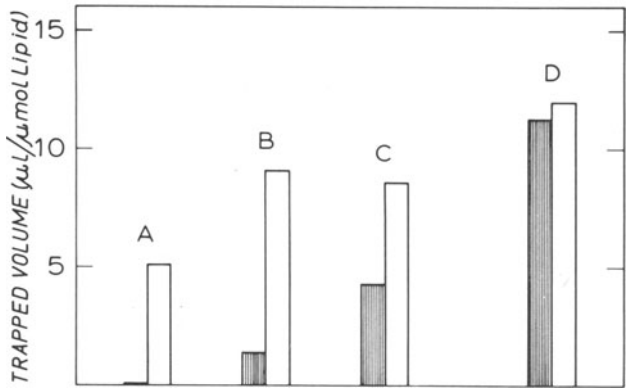

Figure 36. Glucose trap (shaded) and dextran trap (unshaded) in large unilamellar glycophorin-containing vesicles and protein-free vesicles made of various lipid mixtures. (A) Glycophorin-containing vesicles made of 18 : 1_c/18 : 1_c-PC (PC/glycophorin, 500 : 1, moles/mole). (B) Glycophorin-containing vesicles made of cholesterol-free erythrocyte lipids (lipid/glycophorin, 310 : 1, moles/mole). (C) Glycophorin-containing vesicles made of erythrocyte lipids in the presence of 45 mole% cholesterol (lipid/glycophorin, 330 : 1, moles/mole), and (D) protein-free 18 : 1_c/18 : 1_c-PC vesicles. Reproduced with permission from Van der Steen *et al.* (1981). See this reference for further details.

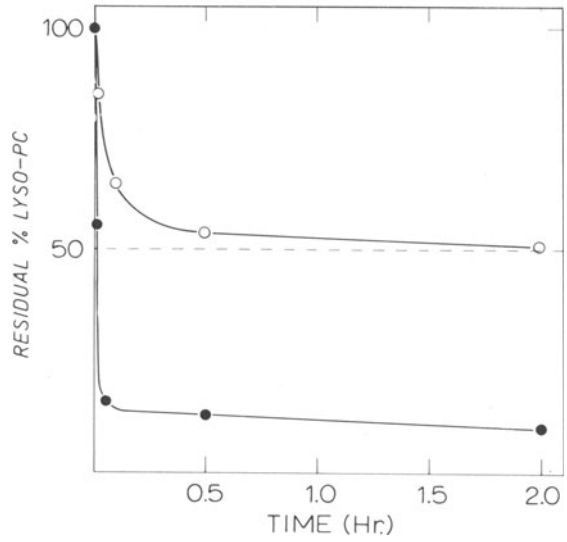

Figure 37. Time course of lyso-PC hydrolysis by lysophospholipase in glycophorin-containing large unilamellar vesicles of 18 : 1_c/18 : 1_c-PC (●—●) and total erythrocyte lipids (○—○). The vesicles contained 5 mole% of lyso-PC. For further details see Van der Steen *et al.* (1981).

shapes are present), are considerably less leaky (Van der Steen *et al.*, 1982). The presence of defects induced by the protein in the 18 : 1_c/18 : 1_c-PC bilayer is also apparent from the fast transbilayer movement of lipids in these vesicles. Lyso-PC incorporated into these vesicles is nearly completely degraded by externally added lysophospholipase, demonstrating that lyso-PC molecules rapidly move from the inner to the outer monolayer of these vesicles (Figure 37). In contrast, for the total lipid extract from the erythrocyte membrane, only 50% of lyso-PC located in the outer monolayer of the vesicles can be degraded (Van der Steen *et al.*, 1981).

Regulation of lipid composition apparently based on a balance of molecular shapes instead of "fluidity" has recently been observed for *A. laidlawii* cell membranes (Wieslander *et al.*, 1980, 1981a; Christiansson *et al.*, 1981). Growing this micro-organism in the presence of cholesterol or anaesthetics greatly influences the monoglucosyl–diglucosyldiglyceride ratio in the membrane. Similar types of adaptation were noticed when the growth temperature was varied. When the perturbation induced an increase in the cone character of the lipids, this ratio was decreased, and vice versa. Although at present these shape-conservation ideas are still speculative, the initial results are most promising and could lead to a better understanding of lipid diversity in membranes.

VIII. FUNCTIONAL ASPECTS OF LIPID POLYMORPHISM

In addition to those functional properties related to the barrier function of the membrane including the proper sealing at lipid–protein interfaces by lipids with appropriate shapes, the observation that nonlamellar lipid structures can occur in model membranes under "physiological" conditions suggests new possibilities for the functional roles of lipids in biological membranes. We discuss some of these possibilities here.

A. Fusion

Membrane fusion is an ubiquitous event in cell biology. Virtually every membrane will fuse with another membrane at some stage, and this process is very fast (in the order of milliseconds) and strictly regulated. Complexities associated with different types of fusion (for instance endocytosis, exocytosis, and cell division) and large variations in membrane composition have hampered the formulation of a universal mechanism for this process. Although in recent years it has become apparent that membrane proteins play essential roles [such as in the case of receptor-mediated endocytosis of viruses (Helenius *et al.*, 1980)], it is obvious that membrane lipids must be actively involved in the actual fusion event. In particular, it is difficult to imagine that bilayer structure of lipids at the fusion interface is continuously preserved during this step. At some stage, the lipids will have to locally adopt a (transiently) nonbilayer lipid configuration. This forms the basis for the hypothesis that H_{II}-preferring lipids, by virtue of their ability to adopt nonlamellar lipid structures, actively participate in the fusion process.

This hypothesis is now supported by a large number of observations made in

model systems. Addition of fusogenic lipids to erythrocytes results in membrane fusion, which process is accompanied by the formation of the hexagonal H_{II} phase (Cullis and Hope, 1978; Hope and Cullis, 1981). The fusion process and H_{II} phase formation appear to be related as is shown in Figure 38 for the glyceromonooleate-induced fusion of human erythrocytes. Chemically related compounds such as glyceromonostearate which do not fuse erythrocytes also do not cause the H_{II} phase (Hope and Cullis, 1981). Unilamellar vesicles made of a mixture of bilayer and H_{II}-preferring lipids will fuse when they are brought under conditions in which the tendency to form the H_{II} phase is increased (Verkleij *et al.*, 1979b, 1980; Hope *et al.*, 1983). In the fused vesicles, lipidic particles are observed which often appear to be localized at the side of fusion. Figure 39 shows this for unilamellar PC/CL (1 : 1) vesicles which were fused upon the addition of Ca^{2+}. Recent rapid-freezing experiments performed on systems in which vesicle fusion could be kinetically monitored (via an assay in which mixing of aqueous compartments was measured) reveal that at the earliest moments of vesicle fusion, no lipidic particles can be observed (Verkleij *et al*, 1983; Baerer *et al.*, 1982). They only appear after several rounds of fusion. This suggests that the lipidic particles are intermediates which are stabilized by as yet unknown structural parameters. In view of the very high rate of vesicle fusion (half-times less than 1 sec), the possibility cannot be excluded that transient formation of inverted micelles during vesicle fusion might escape detection by freeze-fracture-electron microscopy. It also

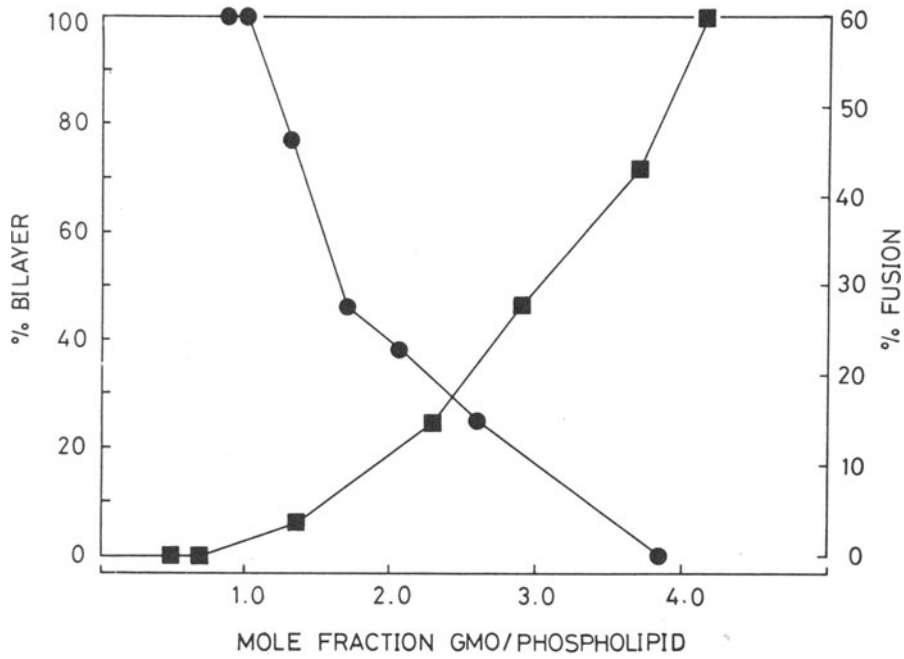

Figure 38. A comparison of the extent of fusion between erythrocytes and the amount of phospholipid remaining in the bilayer phase in erythrocyte membranes at various membrane concentrations of glyceromonooleate (GMO). Reproduced with permission from Hope and Cullis (1981).

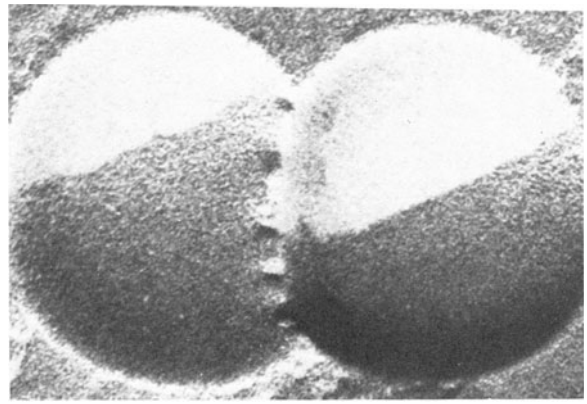

Figure 39. Lipidic particles at the site of fusion of PC-CL vesicles induced by Ca^{2+}. Final magnification $\times$ 100,000. Reproduced with permission from Verkleij *et al.* (1979a). See this reference for further details.

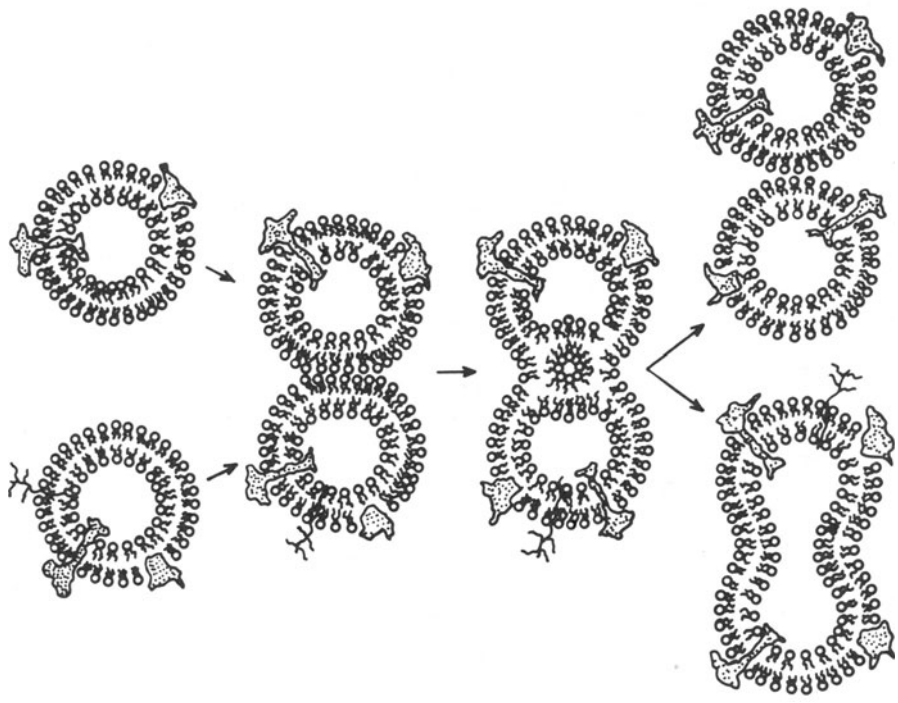

Figure 40. Proposed mechanism of membrane fusion proceeding via an inverted cylinder or inverted micellar intermediate. The process whereby the membranes come into close apposition is possibly protein-mediated, whereas the fusion event itself is proposed to involve formation of an "inverted" fusion intermediate. Reproduced with permission from Cullis *et al.* (1982).

should be noted that the presence of nonbilayer lipids is not a prerequisite for vesicle fusion. For instance, 14 : 0/14 : 0-PC vesicles fuse upon repeatedly passing the gel → liquid-crystalline phase transition (Van Dyck *et al.*, 1978) as well as PS vesicles upon the addition of Ca^{2+} (Papahadjopoulos *et al.*, 1978). However, in view of the fact that in every biological membrane, H_{II} type of lipids occur (which is not the case for PS, for instance), this suggests more general possibilities for the involvement of nonlamellar lipids in fusion events.

Prior to fusion, two membranes must move into close contact, an energetically unfavorable event because of strong intermembrane repulsion forces. The energy barrier for fusion includes head group dehydration. It is interesting that the H_{II} type of lipids have low head group hydration which will facilitate the fusion event. A model taking into account the involvement of inverted structures as fusion intermediates is shown in Figure 40.

B. Transport

Dynamic formation of inverted structures in bilayers can provide an attractive pathway for the transbilayer transport of lipids and polar solutes (Figure 41). Local compositional fluctuations or agents which trigger H_{II} structure could cause bilayer invagination, possibly followed by inverted micelle formation. When the inverted micelle subsequently "dissolves" in the opposite monolayer, transbilayer transport of lipids and polar molecules in the aqueous compartment has occurred.

The transbilayer movement of lipids is indeed increased under conditions in which lipidic particles can form as indicated by several studies (Noordam *et al.*, 1981; Gerritsen *et al.*, 1980). Egg-PE/18 : 1_c/18 : 1_c-PC/cholesterol/18 : 1_c/18 : 1_c-PA (50 : 16 : 30 : 4) dispersions undergo a temperature-dependent transition from extended bilayers to structures characterized by isotropic ^{31}P NMR signals and associated lipidic particles. This transition is accompanied by a threefold increase in the PC pool which can be exchanged by phospholipid exchange protein (Figure 42) suggesting a direct relationship between the occurrence of nonbilayer lipid structures and an increased transbilayer movement of PC (Noordam *et al.*, 1981).

Similar behavior is observed for PC/CL liposomes in which Ca^{2+} addition (which induced lipidic particle formation) is accompanied by a large increase in the exchangeable PC pool (Gerritsen *et al.*, 1980). In general, in order to act as an ionophore for a particular component, the lipid molecules must be able to form a complex with the solute to be transported which is "soluble" in a hydrophobic environment. Using a simple two-phase system it is observed that phospholipids can indeed dissolve polar solutes into an organic phase (Cullis *et al.*, 1980a; Tyson *et al.*, 1976). Table 4 shows that CL and PA effectively sequester Ca^{2+} into a chloroform phase. The neutral lipid PC is ineffective as is another negatively charged phospholipid, PS. As both CL and PA can form the H_{II} phase upon addition of Ca^{2+} this suggests a correlation between the ability of a lipid to dissolve a divalent cation into an organic phase and the formation of the H_{II} phase in aqueous dispersions. This correlation can be extended to the effect of ruthenium red, the classical inhibitor of Ca^{2+} transport in mitochondria. This molecule inhibits the uptake of Ca^{2+} by CL into the chloroform phase (Table 4) as

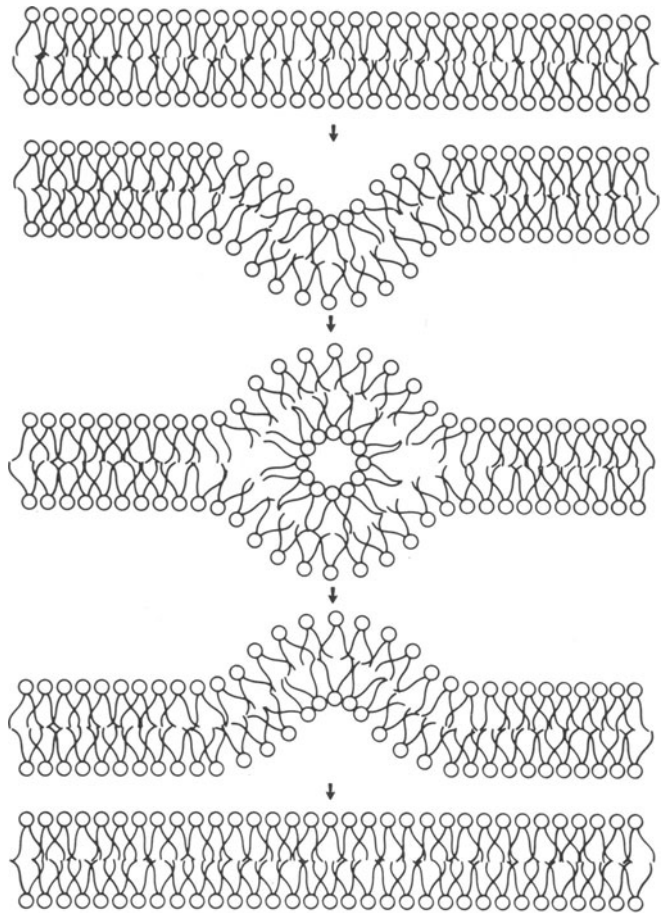

Figure 41. Dynamic formation of inverted micelles provides a permeation pathway for lipids and polar solutes.

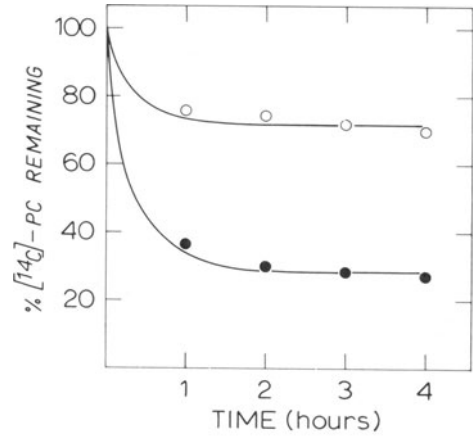

Figure 42. Phospholipid exchange protein catalyzed transfer at 20°C of [^{14}C]-18 : 1_c/18 : 1_c-PC from aqueous dispersions of PE/PC/PA/cholesterol liposomes (○———○). The exchangeable pool greatly increases upon briefly heating the liposomes to 60°C followed by cooling to 20°C (●———●). Under this condition, an isotropic ^{31}P NMR signal and lipidic particles are induced in the liposomes. See Noordam *et al.* (1981) for further details.

Table 4. Amount of Ca^{2+} Taken up into Chloroform in the Presence of Various Phospholipid Species[a]

Lipid species	Amount of Ca^{2+} taken up (μmoles)
CL	4.7
CL + ruthenium red (1 : 1)	0.5
PA	10.7
PS	1.1
PE	0.0
PC	0.4
No lipid	0.0

[a]Amount of phospholipid present in all cases corresponds to 6.0 μmoles phosphorus. For details see Cullis *et al.* (1980a).

well as the formation by Ca^{2+} of the H_{II} phase of CL (Figure 43; Cullis *et al.*, 1980a). Together with Ca^{2+} and Mn^{2+} permeability studies in liposomal systems (De Kruijff *et al.*, 1979; Gerritsen *et al.*, 1980; Serhan *et al.*, 1981), this suggests that CL and PA might act as ionophores for divalent cations. It is tempting to speculate that the receptor-mediated increase in cytosolic Ca^{2+} levels may result from an ionophoric

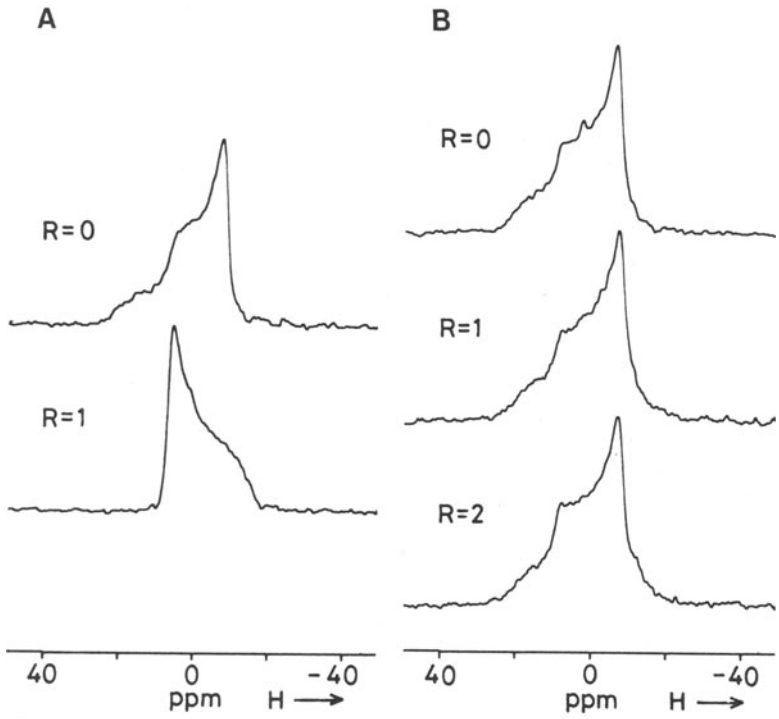

Figure 43. 81.0 MHz ^{31}P NMR spectra at 30°C of CL liposomes in the (A) absence and (B) presence of equimolar ruthenium red. The ratio *R* refers to the molar ratio of Ca^{2+} to CL. Reproduced with permission from Cullis *et al.* (1980a). See this reference for further details.

effect of PA which is formed during the receptor-mediated breakdown of PI (Salmon and Honeyman, 1980; Putney *et al.*, 1980).

C. Protein Insertion and Transport

Many proteins synthesized on free or bound ribosomes have to be inserted or transported across membranes. In recent years, considerable progress has been made in understanding the molecular details of these processes. This applies particularly to recognition and processing of the proteins. However, no universal picture has yet emerged on the exact way in which the polypeptide chain is inserted in and moved across the membrane. Some models postulate protein channels (Blobel and Dobberstein, 1975) as transport pathways, whereas in others, "spontaneous" insertion into the lipid bilayer is proposed (Wickner, 1980; Engelman and Steitz, 1981). For bacterial secretory proteins, the involvement of acidic phospholipids has recently been suggested (Nesmeyanova, 1982). In this model, the transport is thought to be coupled to transbilayer movements of negatively charged lipids linked to metabolic interconversion of CL to PG. In view of the phase properties of the bacterial lipids, hydrophilic channels (inverted short cylinders) were proposed as the structures through which the major hydrophilic part of a secretory protein is linearly translocated. An alternative but related model for protein insertion and translocation is presented in Figure 44. The partially charged leader sequence interacts with negatively charged lipids in the outer monolayer which, by adopting a nonbilayer configuration, are cotranslocated with the leader peptide. When the opposite membrane–water interface is reached, these negatively charged phospholipids are released in the inner monolayer. During translocation of hydrophobic stretches of peptide, no cotranslocation of negatively charged lipids occurs. The nonbilayer intermediate in Figure 44 resembles the semi-inverted micelle proposed recently (Fromherz, 1983).

These models are supported by the observations that proteins and peptides such as cytochrome *c* and gramicidin can induce nonlamellar lipid structures in model membranes. In particular, the notion that a mismatch in length of the hydrophobic part of a peptide and the bilayer thickness can trigger such transitions (van Echteld *et al.*, 1982) opens the possibility that during polypeptide chain synthesis and transport, the lipid structure around the polypeptide chain is responding to the progress of the insertion process.

IX. LIPID STRUCTURE IN BIOLOGICAL MEMBRANES

The model membrane studies described so far have provided a framework relating structural and functional abilities of nonbilayer lipids. We now turn to the biological membrane with two important questions in mind. First, is there any evidence for nonbilayer lipid structure in biological membranes, and second, which processes occurring in biological membranes can be better understood given these new insights regarding membrane lipid behavior? To answer these questions we analyze the structural and functional properties of selected membrane systems.

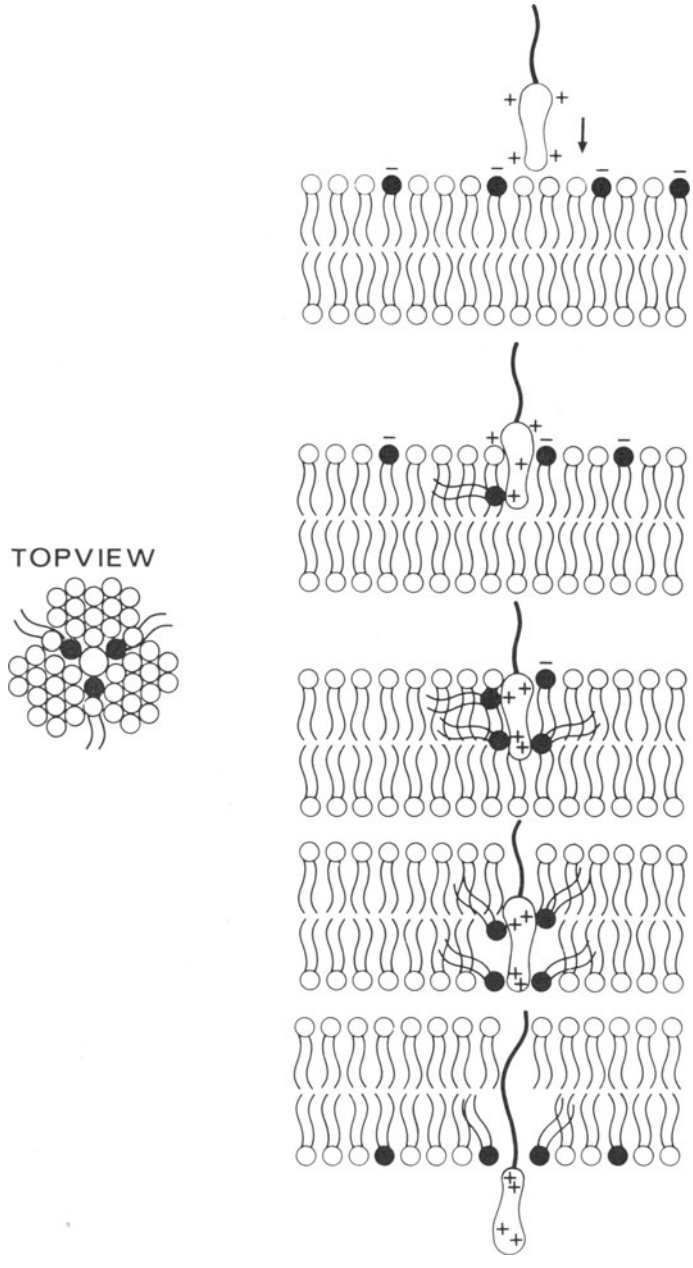

Figure 44. Model of translocation of the positively charged signal peptide of a protein across a membrane. Upon electrostatic interaction between the signal peptide and negatively charged lipids, nonlamellar lipid structures are formed, which enable the signal peptide to cross the membrane. The translocation is driven among other factors by the protein synthesis or the membrane potential. The dark line represents a hydrophobic stretch of the polypeptide chain. In the top view, it is shown that the acyl chains of the phospholipids adopting this nonlamellar configuration are shielded from aqueous medium by the head groups of the lipids in the bilayer (see also Nesmeyanova, 1982; Fromherz, 1983).

A. Erythrocyte Membrane

Within the detection limits (which are in the order of a few percent), it can be concluded that the phospholipids in the erythrocyte membrane are organized in a bilayer configuration (Cullis and Grathwohl, 1977). With the exception of the induction of the H_{II} phase by chemical fusogens (Cullis and Hope, 1978; Hope and Cullis, 1981), no other structural organization of the lipids in the erythrocyte has been observed even after such harsh treatments as extensive phospholipase degradation (Van Meer *et al.*, 1980) or proteolytic digestion of the membrane proteins (Cullis and Grathwohl, 1977). Figure 45 illustrates by ^{31}P NMR that the bilayer structure is an inherent property of the total lipid extract of that membrane. The stability of the bilayer structure might be related to the low metabolic activity of that membrane or to the large mechanical stresses the red cell has to overcome in narrow capillaries during its long lifespan in the circulation.

Special consideration has to be given to the well-known lipid asymmetry (Op den Kamp, 1979) of the erythrocyte, which appears to be typical for plasma membranes. The preferential localization of PS and the H_{II}-preferring PE in the inner monolayer suggests that the structural stability of that layer might be particularly sensitive to electrostatic interaction between PS and other polar molecules. For instance, Ca^{2+} addition to a lipid extract mimicking the inner monolayer will trigger a bilayer $\rightarrow H_{II}$ phase transition (Hope and Cullis, 1979). Phospholipid flip-flop and vesicle release might be two areas related to a possible inner monolayer instability. Whereas phospholipid flip-flop in erythrocytes normally is very slow, chemical modification of the underlying spectrin network increases the transbilayer movement of PS and PC (for review see Haest, 1982). From model membrane studies it is known that spectrin will bind to PS (Mombers *et al.*, 1979) and this binding inhibits Ca^{2+}–PS interactions. It therefore can be speculated that, upon chemical modification of spectrin, the interaction with PS is altered thereby affecting the structural properties of the inner monolayer,

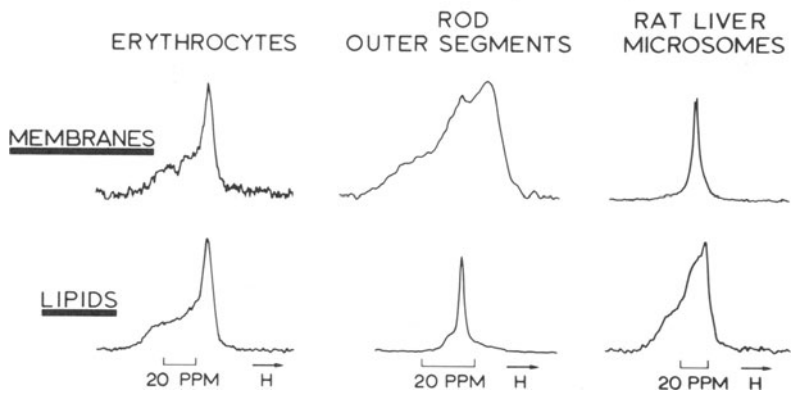

Figure 45. 36.4 MHz ^{31}P NMR spectra of intact biological membranes and derived aqueous extracts of the total membrane lipids at 37°C. For details see Cullis and de Kruiff (1979), De Grip *et al.* (1979), and De Kruijff *et al.* (1978).

possibly resulting in transbilayer transport via transiently formed inverted micellar structures. Protocols which result in an increased intracellular Ca^{2+} concentration lead to blebbing off of small vesicles from the erythrocyte membrane (Lutz *et al.*, 1977). A model for this process which takes into account the structural preferences of the inner monolayer lipids has been proposed (Cullis and De Kruijff, 1979; Cullis *et al.*, 1982). Finally, although the erythrocyte can hardly be considered as a general model for a plasma membrane, the notion that in model membranes WGA–glycophorin interactions can modulate bilayer–nonbilayer transitions (Section V-C.3) could well be relevant to lipid–receptor interactions and structural changes (for instance endocytosis of the clustered receptor–effector complex) occurring in plasma membranes.

B. Endoplasmic Reticulum (Microsomes)

The endoplasmic reticulum of eukaryotic cells is a complex intracellular membrane network and can be considered to be the main metabolic factory of the cell. It is the organelle in which the synthesis and processing of the cellular proteins, lipids, and carbohydrates occurs, and can be isolated after rupturing the cells in the form of small 1000–2000 Å diameter vesicles, which are called microsomes.

^{31}P NMR techniques have revealed that at 37°C a considerable proportion of the endogenous phospholipids of rat (De Kruijff *et al.*, 1978, 1980b), rabbit (Stier *et al.*, 1978), and beef liver (De Kruijff *et al.*, 1978) microsomes undergo rapid isotropic motion (Figure 45). It is interesting that at lower nonphysiological temperatures, ^{31}P NMR spectra typical of the lamellar organization were observed (De Kruijff *et al.*, 1978; 1980b; Stier *et al.*, 1978). The isolated lipids were found to be organized in a bilayer. Similar results were obtained from ^{13}C NMR experiments (De Kruijff *et al.*, 1980c).

In the intact perfused rat liver, a temperature-dependent bilayer → "isotropic" transition of some 15% of the endogenous rat liver membrane phospholipids can also be observed by ^{31}P NMR (De Kruijff *et al.*, 1980b). Unfortunately, NMR techniques cannot give information on the exact nature of the structure giving rise to the isotropic signal. Although lateral diffusion of lipids around highly curved bilayer surfaces could be partially responsible for this signal, the possibility that (transiently forming) inverted micelles arising from the influence of proteins such as cytochrome P450 (Stier *et al.*, 1978) give rise to such a signal cannot be excluded. This is particularly possible given the hexagonal H_{II} phase-preferring character of the PE component (De Kruijff *et al.*, 1980b), the observations that upon dehydration H_{II} phase lipid is found in microsomes (Crowe and Crowe, 1982), the network structure of the endoplasmic reticulum (Morré *et al.*, 1979), and the various metabolic processes occurring in this membrane. For instance, the phospholipid-synthesizing enzymes are asymmetrically oriented into the microsomal membrane. However, the products are found on both sides implying rapid transbilayer movements of the lipids. At 37°C, such rapid flip-flop of PC and PE has been observed (Zilversmit and Hughes, 1977; Van den Besselaar *et al.*, 1978; Hutsen and Higgins, 1982). At lower temperatures, under conditions where ^{31}P NMR indicates bilayer structure, the rate of this process is greatly decreased (Van den Besselaar *et*

al., 1978). As discussed in Section VIII-C, protein insertion and transport, which occurs on a massive scale in the rough endoplasmic reticulum, might involve dynamic structural reorganization of the lipid. For the processing and sorting of newly synthesized proteins in the endoplasmic reticulum and Golgi apparatus, membrane fusion events appear to play vital roles (Rothman, 1981). Although studies on fusion of isolated microsomes are limited (Paiement *et al.*, 1980), the fact that upon mild homogenization of the tissue of the various membranes it is only the endoplasmic reticulum network which readily breaks up into small vesicles demonstrates that this membrane has the ability to vesiculate readily.

C. The Inner Mitochondrial Membrane

For a review of the possible involvement of nonbilayer lipid structures in inner mitochondrial membrane functioning see De Kruijff *et al.* (1981). Freeze-fracturing (Van Venetië and Verkleij, 1982; Hackenbrock *et al.*, 1976) and ^{31}P NMR studies (Hackenbrock *et al.*, 1976) have indicated that the endogenous phospholipids in the inner membrane of functionally intact rat liver mitochondria at 37°C are predominantly (>95%) organized in a liquid-crystalline lamellar phase. This characteristic is maintained during oxidative phosphorylation processes (De Kruijff *et al.*, 1982a). Several observations suggest that the bilayer stability of the inner mitochondrial membrane is limited. ^{31}P NMR of isolated inner mitochondrial membrane ghosts showed a tem-

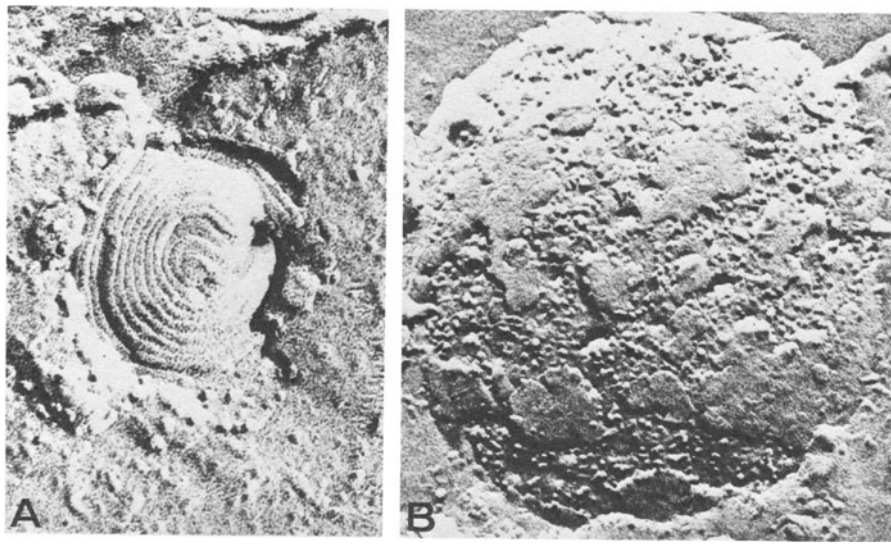

Figure 46. Freeze-fracture electron microscopic pictures of rat liver mitochondria incubated with (A) 5 mM Mn^{2+} or (B) 5 mM Ca^{2+}. The patchwork-like structure on the fracture face of the mitochondrion is indicative of the contact sites proposed in Ogawa *et al.* (1975). Reproduced with permission from Van Venetië and Verkleij (1982).

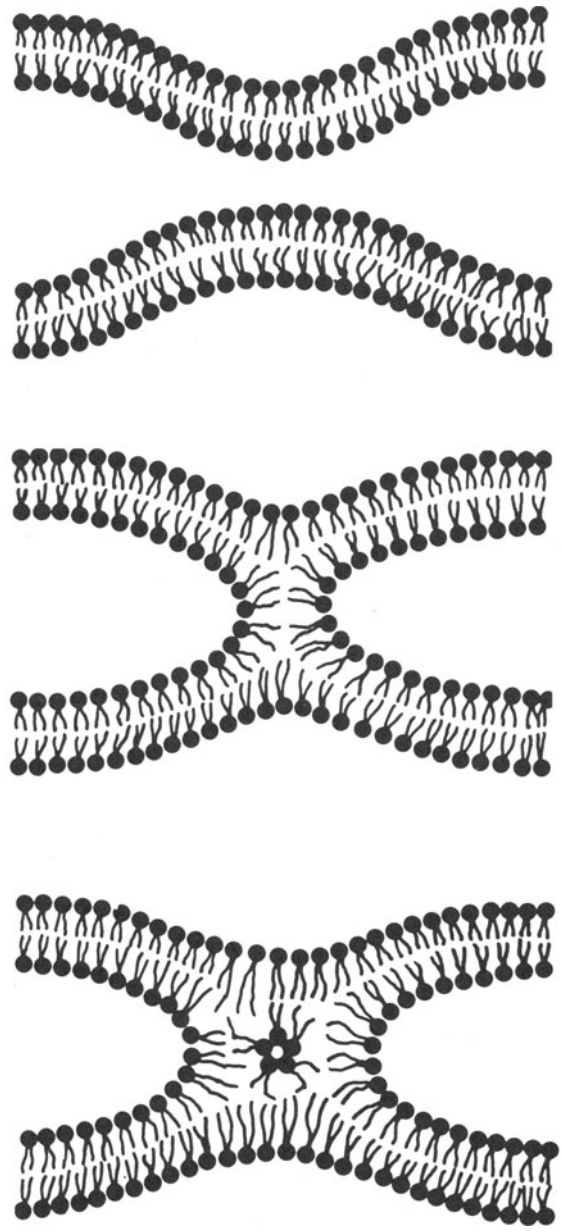

Figure 47. Structural possibilities of the lipid part at the contact site of the outer and inner mitochondrial membrane.

perature-dependent bilayer-isotropic transition such that at 37°C part of the phospholipids undergo isotropic motion (Cullis *et al.*, 1980b). This fraction is greatly increased upon the addition of Ca^{2+}. Incubation of mitochondria in divalent cation solutions has revealed some interesting freeze-fracture results (Van Venetië and Verkleij, 1982). In the presence of low concentrations of Mn^{2+} membrane, lipids segregate and form small areas of H_{II} phase in the mitochondria (Figure 46). This result is of interest because of the relatively high Mn^{2+} concentrations occurring in mitochondria (Ogawa *et al.*, 1975), and is compatible with the finding that this cation has the highest affinity for negatively charged phospholipids and promotes H_{II} phase formation in CL liposomes (Van Venetië and Verkleij, 1981). With other divalent cations, contact sites between the outer and inner membrane are induced (Figure 46). These contact sites between the inner and outer membrane were observed under all conditions which favor H_{II} phase formation of the lipids (Van Venetië and Verkleij, 1982). Figure 47 illustrates the various structural possibilities for these contact sites. Biochemical evidence supporting the existence of such sites comes from studies on the ATP-ADP translocation (Brdiczka and Kolb, 1978) and the transport of PC from the outer to the inner membrane in the presence of Ca^{2+} (Ruygrok *et al.*, 1972). These experiments make model A of Figure 47 unlikely, however. No evidence is available to discriminate between the other models. The suggestion that transitory inverted micellar structures are involved in divalent cation transport is supported by these structural changes induced by relatively high concentrations of the divalent cations. Furthermore, the finding that apocytochrome *c* which is the precursor of cytochrome *c* induces such structures (Van Venetië and Verkleij, 1982), suggests the involvement of contact sites in the import of mitochondrial proteins as has also been suggested from *in vitro* protein translocation studies (Gasser *et al.*, 1982).

D. Bacterial Membranes

In common with other membranes, spectroscopic studies have revealed that the majority of the lipids in bacterial membranes are organized in a lamellar phase. Nevertheless, several studies again indicate that local departures from bilayer structure are required for proper membrane functioning. In analogy with the mitochondrial system, the contact sites (sites of adhesion) between outer and inner membrane of gram-negative bacteria could well be formed via nonbilayer lipid structures (Lugtenberg and Van Alphen, 1983; Bayer, 1979). The fact that the isolated lipids readily adopt nonbilayer lipid structures (Burnell *et al.*, 1980b) and the rapid flip-flop of newly synthesized phospholipids (Rothman and Lenard, 1977) suggest a transport pathway via inverted micellar systems. The evidence favoring a model of translocation of secretory bacterial proteins involving nonbilayer lipid structures (Nesmeyanova, 1982) is discussed in Section VIII-C (see also Figure 44). For *A. laidlawii* it has been shown that the membrane lipid composition is regulated in such a way as to maintain a proper balance of molecular shapes (Wieslander *et al.*, 1980, 1981a; Christiansson *et al.*, 1981) suggesting the importance of both bilayer and nonbilayer lipids for membrane function.

E. Rod Outer Segment (ROS)

Evidence for existence of the hexagonal H_{II} phase in biological systems comes from freeze-fracture studies on disk membranes of frog retinal rod outer segments (Corless and Costello, 1981). Embedded within the lamellar array of ROS disk membranes, a series of H_{II} type lipid inclusions were observed. Although the functional significance of these inclusions is unknown, they may have relevance to the enhanced rates of lipid redistribution and turnover (Corless and Costello, 1981). ^{31}P NMR on bovine ROS disk membranes revealed only lamellar structures, which suggests a profound bilayer-stabilizing role of rhodopsin as, in the total lipid extract at 37°C, nonbilayer lipid structures are preferred (Figure 45; De Grip *et al.*, 1979). That the lipid composition in retinal cells is such that the membrane is very close to a transition to a hexagonal H_{II} type of organization is indicated by experiments in which liposidosis is induced chemically in rats. Under these conditions, hexagonal H_{II} phase lipid inclusions were observed in the adrenal cells and the retinal ganglia cells (Buchheim *et al.*, 1979). Possible functional roles of the H_{II}-preferring PE in this membrane might be its involvement in the sites of high bilayer curvature at the end of the disks or by allowing close proximates of the disks due to the low hydration of the PE head group.

F. Chloroplast and Prolamellar Body

The etioplasts which are found in leaves of plants grown from seed in the dark, represent an arrested stage in the normal development of proplastids into chloroplasts. In the etioplast which transforms into chloroplasts after exposure to light, a highly organized membrane structure, the prolamellar body, can be observed by electron microscopy (Simpson, 1978). Figure 48 shows the fine structure of such a structure. The prolamellar body is considered to consist of interconnected tetrahedrally-branched tubes, which greatly resembles the structure of some of the cubic phases observed in lipid–water systems (Larsson *et al.*, 1980). The functional significance of this structural organization is unknown. Of the various factors which might be responsible for the unique structure of the prolamellar body, the H_{II} phase-preferring monogalactosyldiglyceride (Shipley, 1973; Sen *et al.*, 1982) is the most likely. Since, in the chloroplast, the thykaloid membranes are organized in the form of stacked lamellae, the etioplast → chloroplast transformation resembles the cubic → lamellar transition. For further details on the relationship of nonbilayer lipids and chloroplast structure see Murphy (1982).

Another example of an interwoven bilayer network is the tubular myelin of the developing rat lung (Chi and Lagunoff, 1978). At the contact sites of the bilayers string-wise organized particles and pits have been observed by freeze-fracturing. Whether these structures are related to the lipidic particles is at yet unknown.

G. Tight Junction

Tight junctions are structures by which cells can be joined and which separate the apical and basolateral plasma membrane domains of polarized cells. Since the protein (Boulan and Sabatini, 1978) and lipid (Van Meer and Simons, 1982) com-

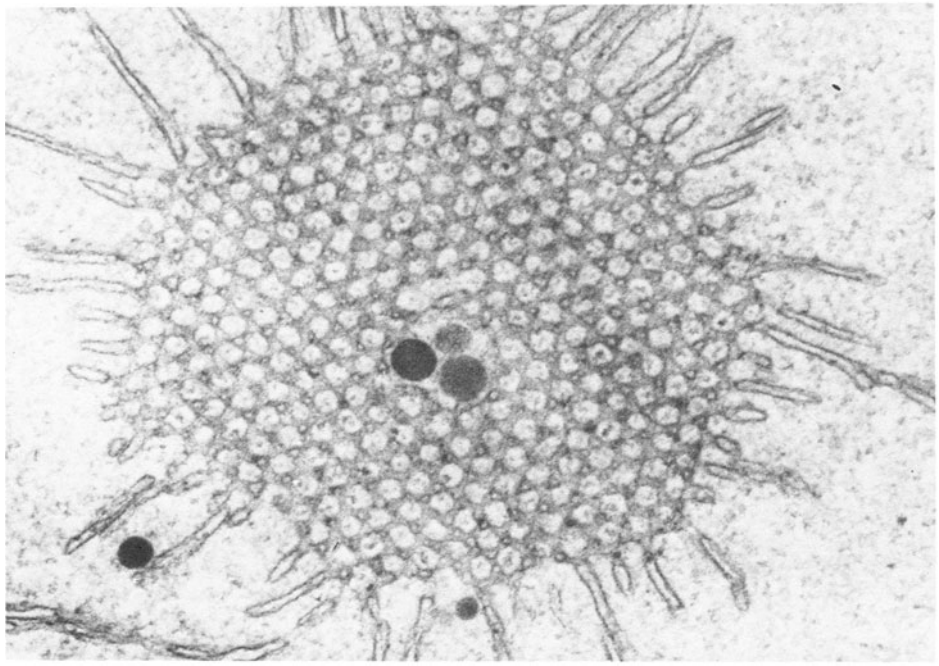

Figure 48. High-magnification micrographs of the prolamellar body of etiolated seedlings of barley. Reproduced with permission from Simpson (1978).

positions of these domains are different, it appears that the tight junction acts as a barrier to lateral diffusion. From experiments using fluorescent-labeled lipids, it could be demonstrated that lipids introduced in the outer layer of the apical part of the cell membrane remains in that layer, whereas lipids which can move to the inner layer of that part of the plasma membrane will subsequently redistribute over the entire cell membrane (Dragsten *et al.*, 1981).

Morphologically, the tight junction can be visualized in the form of tubes. From freeze-fracture results, it has been suggested (Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982; Verkleij, 1980) that the tight junction is formed by lipids which are organized in intrabilayer H_{II} type of tubes (Figure 49). In this model, the cells are in a state of arrested fusion and the tubes can be considered to be stable fusion intermediates, which possibly are stabilized by membrane proteins in conjunction with membrane–cytoskeleton interactions. Evidence for similar arrested fusion intermediates has been obtained for adipose tissue of rats (Blanchette-Mackie and Scow, 1981a,b).

X. CONCLUDING REMARKS

The general picture on the structure of membranes which has emerged over the last decade is that the lipid bilayer forms the backbone of the membranes and maintains

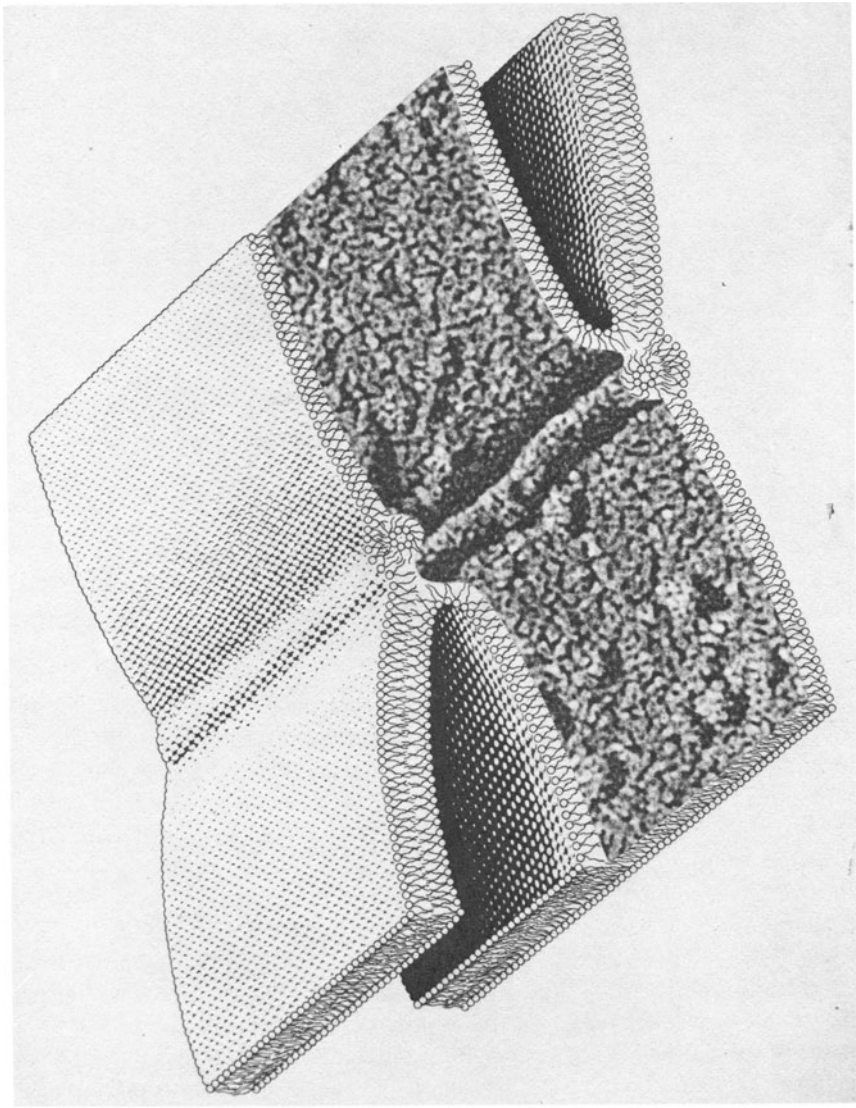

Figure 49. Diagram of a cross section of a tight junction strand combined with freeze-fracture micrographs. Reproduced with permission from Kachar and Reese (1982).

the most important permeability barrier function. However, for specific functions, local and long or shortened departures from bilayer structure must occur. The recognition of the various structural preferences of membrane lipids and the underlying molecular shape concept has opened new avenues toward a molecular understanding of these processes. Observation in model membranes of the stable structural intermediates has given insight regarding the possible molecular structure of nonbilayer lipid structures in membranes.

In the immediate future, these concepts and models will have to be tested. Due to greatly improved techniques and increased sophistication of protein and peptide chemistry, our understanding of lipid polymorphism and lipid–protein interactions should soon result in a better understanding of the vital interplay between these membrane components.

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