THE FUNCTIONAL ROLES OF LIPIDS IN BIOLOGICAL MEMBRANES

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Biomembranes
Volume 1, pages **1-28.**

ISBN: **1-55938-658-4**

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I. INTRODUCTION

Biological membranes surround ceils and organelles, divide the interior of eukaryotic cells into distinct compartments, and provide surfaces for the localization of metabolic enzymes, transport proteins, receptors, and various substrates. In addition, membranes are semipermeable barriers which regulate the transport of water, ions, and other metabolites, thereby providing a means of controlling thc internal environment. Our basic understanding of membrane structure has changed little since Singer and Nicholson (1972) first proposed the fluid-mosaic model over 20 years ago. Biological membranes are fluid (liquid–crystalline) lipid bilayers, into which proteins can insert or associate at the surface. Until recently, membrane research has focused primarily on the protein components, with the lipid portion viewed as a convenient barrier and environment for enzymes. However, biological membranes contain a wide diversity of lipids, far more than are needed to perform structural functions, and these lipids require elaborate metabolic pathways for their synthesis and transport. This suggests specific roles for the individual lipid components of membranes. Much is known concerning how different lipids affect the physical properties of membranes, and how individual lipids may function in such fundamental processes as fusion. On a more general level, understanding how individual lipids contribute to the overall electrostatic and hydrophobic properties of the membrane is basic to understanding the factors which regulate protein association and insertion, and which result in a sufficiently fluid matrix for the functioning of membrane enzymes.

Many comprehensive reviews have been written dealing with the physical properties and functional roles of lipids in membranes (Cullis and De Kruijff, 1979; Cullis et al., 1985; Gruner et al., 1985; Cullis et al., 1986a,b; Lindblom and Rilfors, 1989; Cullis et al., 1990; Seddon, 1990; Cullis and Hope, 1991; Bloometal., 1991). This review will serve to summarize and update earlier treatments, and thus some familiarity with the different structures and classes of lipids, the common types of model membrane systems and the techniques by which they are formed, and the basic physical properties of bilayers is assumed. This review will focus on lipid polymorphism and its relation to membrane fusion, and the role of membrane order in growth and protein function.

II. LIPID DIVERSITY

The main classes of lipids found in eukaryotic biological membranes include the glycerophospholipids, the sphingolipids, and cholesterol (Chol) (Cullis and Hope, 1991). Of the former group, phosphatidylcholine (PC) is the major lipid, but phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and cardiolipin (CL) are also major lipid species in biological membranes, A representative chemical structure of a common phospholipid, 1-palmitoyl-2-

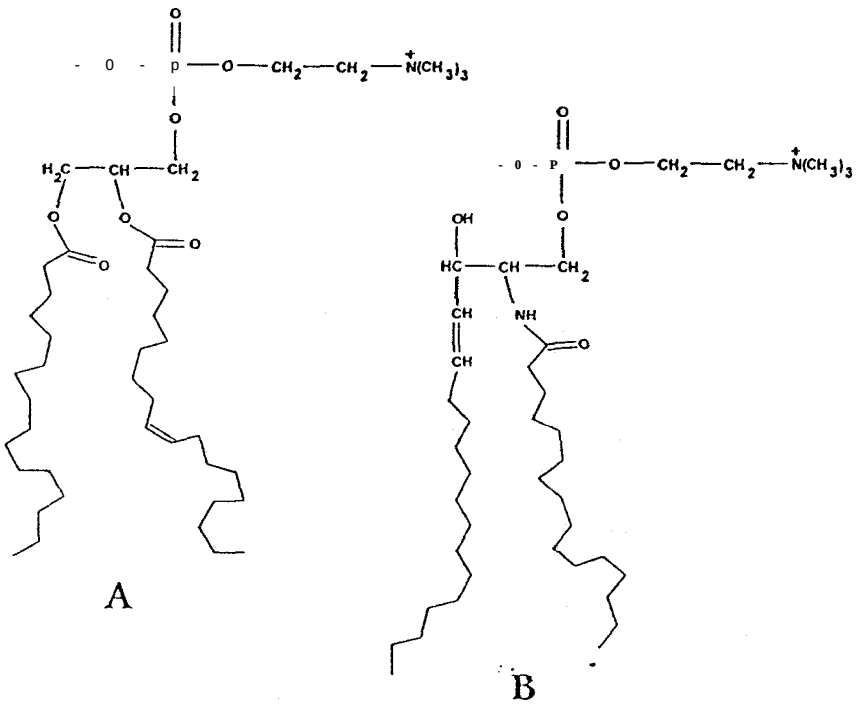

Figure 1. Chemical structures of representative phospholipids POPC (A) and SPM (B).

oleoyl-sn-glycero-3-phosphocholine (POPC), is shown in Figure 1A. The sphingolipids (CER), and glycosphingolipids (GSLs). The structure of SPM is shown in Figure 1B, where its similarity with PC is apparent. CER is a SPM molecule in which headgroup has been replaced with a hydroxyl group; the GSLs contain carbohydrate headgroups where the number of sugarresidues can range from one, in the glucosyl-

GM_1 (GM_1).

In procaryotic membranes, themajorphospholipids are PE, phosphatidylglycerol (PG), and CL; PC is not usually present (Gurr and Harwood, 199 1). Also present

diglucosyl-diacylglycerol

organelles

increased diversity is

unsaturation), gree of

membrane (e.g., that of the erythrocyte) can vary significantly between species (Lehninger, 1975). Furthermore, the inner and outer monolayers often contain asymmetric lipid distributions (Op den Kamp, 1979). This has been characterized in numerous membranes, where the same trends are observed (for a summary, see Cullis and Hope, 1991). PC and SPM are found primarily on the outer cell surface, and PE and PS are found primarily on the cytosolic side. The glycolipids are exclusively localized on the outer surface of ceils. These distributions appear to be maintained by several proteins, such as the aminotranslocase which is responsible for the transport of PE and PS (Devaux, 1991). The location of glycolipids on the outer cell surface is understood in terms of their receptor functions, but the role of the other lipids is not as clear. However, the presence of PS on the outer surface may be a signal of cell senescence (Tanaka and Schroit, 1983), and its localization on the inner surface with PE may be required to maintain a surfacecapableof fusion in processes such as endocytosis and organelle fusion (Dcvaux, 1991).

III. LIPID PHYSICAL PROPERTIES AND PHASE TRANSITIONS

Most biological membranes are 'fluid' at physiological temperatures, arequirement for proper function. The fluid-membrane phase usually refers to the liquid-crystalline bilayer phase, although membranes that contain large quantities of Chol can adopt a different fluid-phase known as liquid-ordered (Bloom et al., 1991; see below). How does the organism regulate membrane fluidity? Answers to this question have come from studies of the physical properties of model membranes. Model membranes composed of well-defined lipid species can exist in a number of different phases. The gel-state exists at low temperatures, where hydrophobic interactions between the acyl-chains are maximized. This results in the chains adopting the all-*trans* conformation where molecular motion is severely restricted; the chains are highly ordered (see Section VI for a discussion of order parameters). Lateral diffusion is extremely slow in the gel-state, with coefficients in the range of 10^{-9} to 10^{-11} cm^2/s (Wu et al., 1977; Vaz et al., 1982; Schneider et al., 1983; Kapitza et al., 1984). As the temperature is raised above T_m, *the* gel-to-liquid-crystalline phase transition temperature, the lipid chains melt, forming the fluid liquid–crystalline phase. In this state, the chains are less ordered due to rapid molecular motion (such as rotation about the long molecular axis and *trans–gauche* isomerization). Lateral diffusion is much more rapid, with coefficients in the range of 10^{-6} to 10^{-8} cm^2/s (Mackay et al., 1978; Vaz et al., 1982, 1985). Forsomelipids, a further increase in temperature results in the formation of nonbilayer phases, such as the hexagonal H_{II} (Cullis and De Kruijff, 1979; Seddon, 1990) and cubic (Lindblom and Rilfors, 1989) phases. This will be discussed more fully in Section-IV.

These transitions can be monitored by a number of techniques, including nuclear magnetic resonance (NMR), electron spin resonance (ESR), fluorescence, and

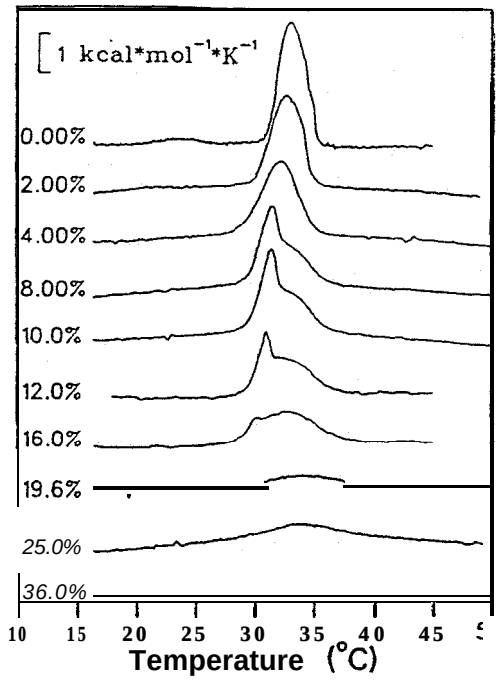

Figure 2. Effect **of Chol on the gel-to-liquid-crystalline phase transition of SEPC as measured by DSC. The Chol concentration is given** in mol %. (Reproduced from Linseisen et al., 1993, with permission.)

differential scanning calorimetry (DSC). All except the latter are spectroscopic techniques. Of the former, NMR is the most useful, and for the study of lipid-phase transitions the most powerful techniques are ^{2}H and ^{31}P NMR (Davis, 1979, 1983; Cullis and De Kruijff, 1979). Both of these are sensitive to the molecular motions present in the different phases, and thus give rise to characteristic spectra for each phase. A more complete discussion of ^{2}H NMR and its application to membranes and polymorphism will be given in Section VI. DSC measures enthalpy changes as a sample undergoes an endothermic or exothermic transition (Biltonen and Lichtenberg, 1993). Some representative DSC scans for 1-stearoyl-2-elaidoyl-sn-glycero-3-phosphocholine (SEPC) and SEPC:Chol mixtures are shown in Figure 2. Three parameters can be derived from such traces: the transition temperature T_m, characterized by the temperature showing the highest enthalpy; the transition enthalpy, which is proportional to the area under the curve; and the 'cooperativity' of the transition, which can be estimated from the width of the transition. Values of these parameters have been compiled for most common lipids (Biltonen and Freire, 1978; Mabrey and Sturtevant, 1978; McElhaney, 1982; van Osdol et al., 1989).

NMR and DSC studies of a wide variety of model systems shed light on the physical state of biological membranes. For instance, the high degree of unsaturation in most lipids is one mechanism whereby the temperature of transition to gel-state is reduced far below the physiological range (see Cullis et al., 1985). PCs with saturated hydrocarbon chains have T_ms that increase with chain length, reaching values greater than 40 °C for acyl-chains greater than 14 carbons in length (Biltonen and Lichtenberg, 1993). For lipids containing monounsaturated chains, the T_m values are reduced by 20–40 °C. Complete abolishment of the main transition in a saturated-lipid system can be accomplished by the presence of high quantities of Chol (Demel and De Kruijff, 1976). Similar behavior has also been observed in unsaturated systems. Figure 2 shows DSC endotherms of mixtures of Chol with the unusual lipid SEPC, which contains a single *trans* double bond at carbon 9 of the elaidoyl chain (Linseisen et al., 1993). Pure SEPC exhibits a single endothermic peak at 33.2 °C with an enthalpy of 8.5 kcal/mol. As the concentration of Chol is increased to about 16 mol %, a decrease is observed in both the main transition temperature and the transition enthalpy. Above this concentration the enthalpy continues to decrease, leaving only a broad endothermic feature of unknown origin. When the Chol content is greater than 25 mol %, the system generally undergoes a transition to the liquid-ordered phase, characterized by the existence of highly ordered fluid chains over a wide temperature range (Ipsen et al., 1987; Vist and Davis, 1990). Cholesterol therefore functions to keep the membrane in a fluid environment. An excellent discussion of the effect of Chol from a ^{2}H NMR viewpoint is given in the recent review by Bloom et al. (1991).

IV. LIPID POLYMORPHISM

Most biological membranes contain appreciable quantities of lipids which are capable in isolation of forming liquid–crystalline nonbilayer structures. This is referred to as lipid polymorphism. Although numerous nonbilayer phases exist, the predominant one formed by membrane lipids is the hexagonal H_{II} phase, which consists of lipid cylinders packed in a hexagonal array (Cullis and De Kruijff, 1979; Seddon, 1990). In the H_{II} phase, the lipids are arranged with the phospholipid headgroups pointing towards the center of the cylinder, forming an aqueous channel with a diameter of approximately 2 nm. Other nonbilayer phases include micellar structures and cubic phases (Lindblom and Rilfors, 1989).

Lipid polymorphism has been extensively studied using X-ray diffraction (Luzzati and Husson, 1962; Luzzati et al., 1966; 1968a,b,c; Caffrey, 1985; Gruner et al., 1988; Tate et al., 1992), ^{2}H and ^{31}P NMR (Cullis and De Kruijff, 1979; Sternin et al., 1988; Lafleur et al., 1990a,c; Fenske et al., 1990; 1992; Gawrisch et al., 1992), and freeze–fracture electron microscopy (EM) (Verkleij, 1984; Hope et al., 1989). X-ray diffraction, employed in the early pioneering studies, is perhaps the most powerful technique, allowing elucidation of the precise details of the phase struc-

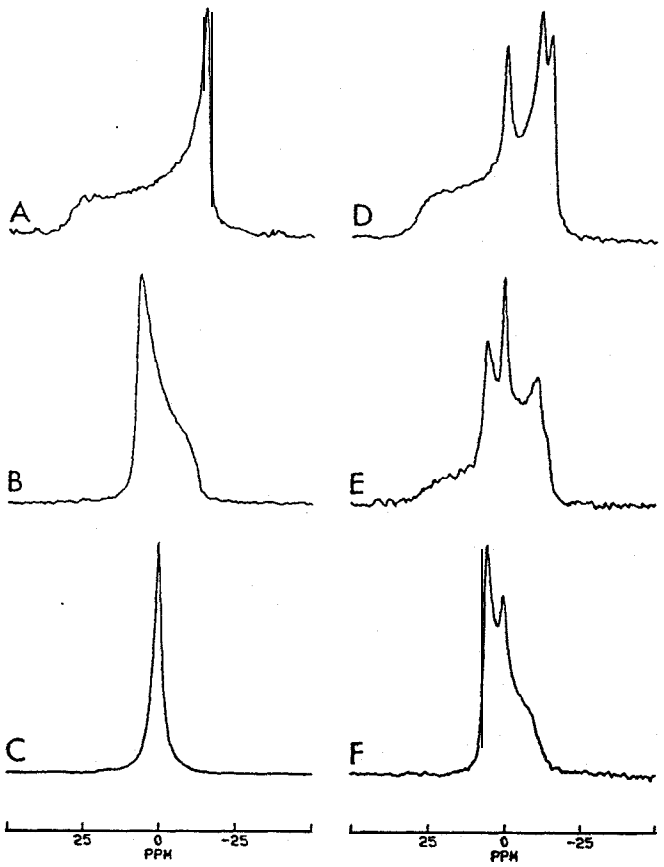

Figure 3. ^{31}P NMR as a tool for the characterization of lipid polymorphism. Liquid-crystalline lipid systems can exist as bilayer (Al, hexagonal H_{II} (B), or isotropic phase (C) assemblies, or as mixtures of these phases (D–F). (A) multilamellar dispersions of DOPC at 30 °C; (B) Hexagonal phase DOPE:Chol (1:1) at 20 °C; (C) LUVs of 20 mol % PA in DOPC at 20 °C; (D) DOPE:Chol:DOPC (1 :1:2) at 20 °C. The two upfield bilayer peaks originate from PC and PE, and are resolved due to the smaller shielding anisotropy of PE; (E) DOPE:Chol:DOPC (1:1:1) at 40 "C, where bilayer, H_{II}, and isotropic phases are resolved; (F) same as (E), but at 60 °C.

ture. However, NMR techniques have proven to be convenient and rapid for determining polymorphic phase tendencies of lipid mixtures. The most commonly utilized techniques have been ^{31}P NMR, and to a lesser extent ^{2}H NMR, both of which are sensitive to the different motional characteristics of the various lipid phases. Finally, freeze–fracture EM allows visualization of lipid phases, and can reveal irregular variation in local structure which may be unavailable from X-ray or NMR techniques.

The ^{31}P NMR lineshapes corresponding to bilayer, H_{II}, and "isotropic" phases (e.g., micelles, vesicles, or cubic phases) are well characterized (Cullis and De Kruijff, 1976, 1979; Seelig, 1978), and representativeexamples are shown in Figure 3. Large bilayer systems (on the order of microns) exhibit broad, asymmetric ^{31}P NMR spectra with a low-field shoulder and high-field peak, separated by about 40–50 ppm (Figure 3A). Hn systems exhibit a reverse asymmetry, and the width is reduced by a factor of two. This is due to additional motional averaging which results from diffusion of phospholipid molecules about the cylinder axis (Figure 3B). The term "isotropic" phase is applied to systems where the motions are sufficiently rapid to average the chemical-shift anisotropy tensor, giving rise to narrow-line, symmetrical NMR spectra. Examples of such systems include micelles, unilamellar vesicles with diameters <100 nm (Figure 3C), and cubic phases (Lindblom and Rilfors, 1989). ^{31}P NMR is also useful in characterizing complex lipid mixtures in which two or more phases coexist, such as occurs during phase-transitions. Examples are given in Figure 3D–F for mixtures of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC): 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE):Chol (see figure legend for details) which exhibit coexistence of bilayer and isotropic phases (Figure 3D); bilayer, H_{II}, and isotropic phases (Figure 3E); and H_{II} and isotropic (Figure 3F) phases.

Polymorphic phases can also be identified by freeze-fracture EM (Verkleij, 1984; Hope et al., 1989). Bilayers give rise to flat, featureless fracture-planes, whereas a regular corrugated pattern is observed with Hn phase. Some "isotropic phase" samples give evidence of small "lipidic particles," which may correspond to interbilayer attachment sites (this is discussed below in Section V).

A large variety of pure and mixed lipid systems have been investigated with regard to their polymorphic preferences. A comprehensive tabulation of this data can be found in Cullis et al. (1990). The results from eukaryotic cell phospholipids reveal that a large proportion of membrane lipids can adopt the H_{II} phase under appropriateconditions. Although PC and SPM form only bilayers in isolation, other lipids (PE, PS, PA, and CL) can form both lamellar and H_{II} phases depending on the conditions (pH, temperature, and acyl-chain unsaturation). Furthermore, Chol and long-chain unsaturated fatty acids can induce the H_{II} phase in some lipid mixtures. Of the nonbiiayer phase lipids, PE has been the most extensively studied. Depending on the level of unsaturation, many PEs form stablebilayers, undergoing lamellar-to-H_{II} transitions as the temperature is raised above some critical value. The tendency to form H_{II} phase is increased with increasing acyl-chain unsaturation. Other lipids, such as PS and phosphatidic acid (PA), will only form Hn phase at suffkiently low pH, where the negative charge on the headgroup is neutralized. Similarly, PA and CL will adopt the Hn phase in the presence of Ca^{2+}, which neutralizes the negative surface charge. The addition of Ca^{2+} to a lipid mixture isolated from human erythrocytes has been shown to trigger the formation of Hn phase (Hope and Cullis, 1979). The ability to regulate bilayer-nonbilayer transitions by controlling parameters such as pH and divalent cation concentration is

clearly important in an isothermal environment, and thus these observations may have biological relevance. Some of the glycolipids found in the membranes of plants and microorganisms also can be divided into bilayer and nonbilayer categories. Diglucosyl diacylglycerol (DGDG) is a bilayer-forming lipid, while monoglucosyl diacylglycerol (MGDG) favors the H_{II} phase.

Observations on pure lipid species, while informative in terms of the factors which modulate polymorphism, are not particularly relevant biologically. The nonbilayer lipids are always present in complex lipid mixtures, and therefore studies on mixed model systems are of interest. In general, increasing the proportion of bilayer-forming lipids results in a progressive stabilization of the bilayer phase, with complete stabilization usually achieved with 20–50% of the bilayer lipid (Cullis and De Kruijff, 1979; Hui et al., 1981; Tilcock et al., 1982; Boni and Hui, 1983; Cullis et al., 1986). The composition of theselipid mixtures remains the same during lamellar-to-nonlamellar transitions, suggesting that the bilayer andnonbilayer lipids are homogeneously dispersed in the two phases. An illustrative example involves lamellar and H_{II} phases in some PC:PE mixtures. ^{2}H NMR studies on selectively ^{2}H-labeled lipids reveals that the PC and PE are both evenly distributed in the two phases, when one might have predicted that the H_{II} phase would be enriched in PE and the lamellar phase enriched in PC (Tilcock et al., 1982). This has also been shown using ^{31}P NMR of oriented multibilayers of PC:PE and PC:PE:Chol mixtures, where the resonances originating from the two phospholipids are resolved in the bilayer phase (Fenske and Cullis, 1992). The proportion of PC:PE in the lamellar phase was that of the bulk ratio, and this ratio remained constant during the transition to H_{II} phase. Increasing the membrane complexity by introducing Chol as a third component is potentially important, due to the ubiquitous distribution of this lipid, and results in some interesting behavior. Cholesterol can induce the formation of H_{II} phase in PE-containing bilayer systems that have been stabilized by PC (lilcock et al., 1982).

Much effort has been directed at understanding the physical basis of lipid polymorphism, i.e., at defining the factors which determine the phase preference of a given lipid species. A qualitative yet successful approach, initially developed by Israelachvili and coworkers (1980), involves consideration of the molecular shapes of individual lipid molecules. This can be simply expressed by means of a shape parameter S:

$$S = V/A_o L_c \quad (1)$$

where V is the hydrocarbon chain volume, A_o is the hydrocarbon-water interfacial area, and L_c is the hydrocarbon chain length. $A_o L_c$ is the cylinder volume corresponding to the interfacial surface area and hydrocarbon chain length; if V is greater than this value, i.e., $S > 1$, then the chains must have a cone geometry, which would favor the H_{II} phase. If V is less than $A_o L_c$, then $S < 1$, and the acyl-chains have an inverted cone geometry. When $S = 1$, the acyl-chains occupy a cylinder,

which favors the bilayer phase. This can be easily understood by expressing the equation above as a function of the cross-sectional areas of the interface (A_o), and of the hydrophobic end of the lipid (Ah) (Cullis et al., 1990):

$$S = 1/3[1 + (A_h/A_o)^{1/2} + A_h/A_o] \quad (2)$$

When $A_I = A_,$, the lipids are cylindrical in shape, and $S = 1$. For $A_h > A_o$, the lipids are clearly cone shaped, and $S > 1$. For $Ah < A_,$, an inverted cone geometry gives $S < 1$. Bilayer phase lipids such as PC tend to have a cylindrical geometry, where the cross-sectional area of the headgroup is equivalent to that of the fatty acyl-chains. Lipids which prefer the H_{II} phase have a cone shape, where the lipid chains occupy a greater cross-sectional area than the headgroup, thereby forcing a curvature to the assembly which favors the hexagonal geometry. Detergent-like lipids, which form micellar structures, have an inverted cone geometry, where the headgroup cross-sectional area is greater than that of the chains. The 'shape' of the molecule is determined by all factors which will influence the sizes of the polar and nonpolar regions, and will include such terms as the hydration and charge of the headgroup, possible hydrogen-bonding interactions, the effect of counterions, and the extent of molecular motion. An example of this comes from a comparison of DOPC, which forms a bilayer phase at room temperature, and DOPE, which adopts the hexagonal Hn phase. The latter lipid, with it's smaller, less-hydrated headgroup, adopts a cone shape and favors the Hn phase. However, as the temperature is reduced to approximately 10 °C, the extent of *trans–gauche* isomerization is reduced, and the lipid acyl-chains become more ordered. This results in a reduced cross-sectional area being swept out by the acylchains, and the shape of the molecule becomes more cylindrical, allowing the aggregate to undergo a hexagonal-to-latnella transition, Although generally expressed in qualitative terms, at least one recent study provides quantitative support for the shape hypothesis. Lee et al. (1993) examined changes in the midpoint of the L_α-to-H_{II} transition of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolatnine (POPE) in response to the presence of 5 mol % of other lipids with varying headgroup size. This would result in changes in A_o but not A_h. The result was a linear relationship between the headgroup volume (calculated from covalent radii) and the midpoint-temperature of the transition. This adds further support to the idea that molecular shape is important *in* determining the structural properties of lipid assemblies.

A more quantitative approach to understanding the factors involved in the formation of nonbilayer phases has come from the "curvature" concept introduced by Gruner and coworkers (Kirk et al., 1984; Gruner, 1985). In the presence of nonbilayer lipids, a monolayer will tend to curl into cylinders with a spontaneous radius of curvature R_o, essentially executing a lamellar-to-hexagonal transition. However, certain constraints can prevent this transition. The stretching of acyl-chains is required to prevent the formation of intercylinder spaces in the hydrocarbon matrix of the H_{II} phase, but this process is energetically unfavorable. The

addition of hydrocarbon agents such as alkanes allows these spaces to be filled, thereby removing the packing constraints preventing the transition to H_{II} phase, and allowing the system to adopt its spontaneous radius of curvature, which can be measured by X-ray diffraction. A small radius of curvature indicates a strong tendency lo form H_{II} phase, whereas a larger radius of curvature, such as may occur in a PE/PC mixture, indicates considerably less tendency. The intrinsic radius of curvature thus provides a measure of the polymorphic tendencies of a lipid system. A fascinating example of this involves DOPE and its methylated derivatives (DOPE-Me and DOPC). As expected, the R_o values for DOPE and DOPC were small and large, respectively. However, monomethylated DOPE had an intermediate value, which translated into a propensity to form cubic phases (Gruner et al., 1988).

Further advances in modeling lameliar-to-nonlamellar transitions will require quantitative kinetic and thermodynamic data. The transition times for the L_α to H_{II} transition of several PEs have been measured using time-resolved X-ray diffraction (Caffrey, 1985; Tateet al., 1992). For large temperature jumps, the transitions were reversible, with time constants ranging from 0.1-3 s, in agreement with theoretical values calculated by Siegel (1986a). However, for small temperature jumps, the transition kinetics were much slower, in some cases occurring over several days (Tateet al., 1992). Theexchangeoflipid between coexisting lamellar and nonlamellar (H_{II} and cubic) phases has been examined using macroscopically oriented samples in conjunction with one- and two-dimensional ^{31}P NMR techniques (Fenske and Cullis, 1992). Although a wide variety of systems exhibited reversible L_α-to-H_{II} or h-to-cubic transitions, no exchange of lipid could be detected (on the seconds timescale) between coexisting phases at near-equilibrium conditions.

Despite a wealth of information on the polymorphic phase behavior of numemus lipid mixtures ***in vitro, there*** is no convincing evidence for the existence of nonbilayer phases ***in vivo,*** except perhaps in a transient manner or in certain pathological states (Buchheim et al., 1979). Since the nonbilayer lipids are not required to form a fluid bilayer matrix, and are not expressed as nonbilayer phases, they must be involved in other membrane processes. There are two areas where these lipids may play important roles. One possibility is that some of the intermediate structures of bilayer-to-nonbilayer transitions may function in membrane fusion, Another is that nonbilayer lipids can increase membrane order, which may be important in some situations. Both of these possibilities are discussed below.

V. LIPIDS AND MEMBRANE FUSION

The ability of membranes to fuse is a requirement for such processes as fertilization, cell division, exocytosis, endocytosis, viral infection, and intracellular membrane transport. Much evidence, both experimental and theoretical, suggests that the lipid components of membranes are involved in fusion processes. For example, vesicles

can be induced to fuse in the absence of protein with mixing of both the lipid and aqueous contents. Well-characterized systems include small unilamellar vesicles (SUVs) made of saturated PCs near the gel-to-liquid-crystalline phase transition (Lichtenberg et al., 1981), large unilamellar vesicles (LUVs) or SUVs containing PA or PS in the presence of Ca^{2+} (Prestegard and O'Brien, 1987), or LUVs containing a mixture of charged and nonbilayer lipids. Thus, LUVs made of certain PEPS mixtures will fuse upon addition of Ca^{2+} to form larger lamellar structures (which exhibit lipidic particle structure via freeze-fracture EM), followed by a transition to H_{II} phase (Hope et al., 1983). Fusion can alsooccurin systems in which the PS is replaced by another anionic lipid such as PA. Rapid fusion of LUVs composed of DOPC/DOPE/PI/1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) occurs in the presence of Ca^{2+} (Eastman et al., 1992). These same systems undergo a lamellar-to-hexagonal transition when studied as multilamellar vesicles (MLVs). Certain lipid-soluble fusogens, such as monoolein, are capable of inducing H_{II} or cubic phase structure in model and biological membranes (Hope and Cullis, 198 I; Lindblom and Rilfors, 1989). Topological considerations alone demonstrate that fusion cannot occur without local, transient departures from bilayer morphology at the fusion interface. These observations have led to the hypothesis that fusion may involve membrane lipids with polymorphic capabilities, proceeding via nonbilayer intermediates.

Although several models of bilayer-to-nonbilayer transitions have been proposed (Caffrey, 1985; Gruner, 1985; Siegel, 1984, 1986a,b,c, 1993), the most detailed theories have been developed by Siegel (1984, 1986a,b,c, 1993), who provides a unified description of both lamellar-to-hexagonal and lamellar-to-cubic transitions. Until recently, Siegel proposed that the first fusion intermediates are inverted micellar intermediates (IMI) (Figure 4A), which form between apposed bilayers at temperatures near T_H. The short-lived IMI rapidly assemble into either H_{II} phase precursors, or into interlamellar attachments (ILA), which are cubic phase precursors. The particular fate of a given lipid mixture depends on the ratio of the area per lipid headgroup in the lamellar and hexagonal phases, or on the spontaneous radius of curvature (Ellens et al., 1989). The transition to Hn phase does not lead to fusion, as leakage of the vesicle contents occurs. The ILA are thought to be fusion intermediates, and have recently been visualized using cryo-transmission EM (Siegel et al., 1989). It is now thought that ILAs give rise to the "lipidic particle" morphology observed with certain lipid systems using freeze-fracture EM (Ellens et al., 1989). Furthermore, the highly curved ILA structure explains the narrow "isotropic" resonances often observed in lipid mixtures by ^{31}P NMR. Isotropic resonances occur in lipid-dispersions containing nonbilayer lipids, and indicate the presence of a structure in which the lipid molecules are able to rapidly sample all possible orientations, such that averaging of the shielding anisotropy occurs (Lindblom and Rilfors, 1989). The only structures which fulfill these requirements are micelles, small vesicles, and cubic phases. Recently, it has been shown that liposome fusion occurs over the same narrow temperature range where isotropic

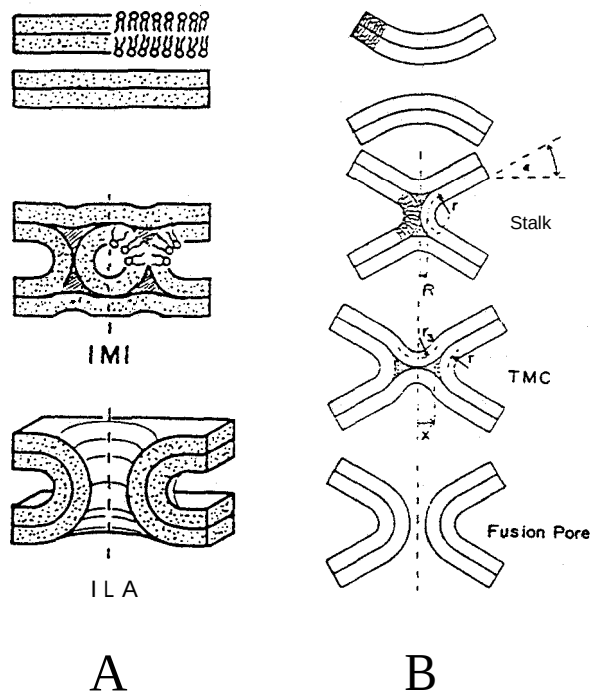

figure 4. Two possible mechanisms of membrane fusion, involving IMI (A) and stalk (B) intermediates, as proposed by Siegel (1993). The fusion pore and ILA are equivalent. See Siegel (1993) for details. (Reproduced from Siegel, 1993, with permission.)

^{31}P NMR resonances are observed (Ellens et al., 1989). Low concentrations of diacylglycerols (2 mol %) are able to lower T_H, the temperature at which isotropic resonances appear, and the temperature for fast membrane fusion by 15-20 °C (Siegel et al., 1989).

Siegel has recently proposed a modified theory of membrane fusion which involves the formation of stalk structures (Figure 4B) between apposed bilayers rather than the IMI mentioned above, and claims that this model is energetically more reasonable (Siegel, 1993). The putative stalk structures are transformed into ILA (or fusion pores) under certain conditions, and thus many elements of the two models overlap.

VI. ORIENTATIONAL ORDER IN LIPID BILAYERS

Many biological membranes contain significant quantities of bilayer and nonbilayer lipids. One possible role for the nonbilayer lipids, as discussed above, is in membrane fusion. Another role involves the regulation of membrane lipid order,

which may be of importance in modulating the activity of certain membrane-bound enzymes. The measurement of hydrocarbon orientational order provides a measure of the relative degree of flexibility of the hydrocarbon chain. As shown later on, this can be related empirically to membrane hydrophobic thickness or to the viability of a living organism. Orientational order can be measured using a variety of techniques, but the following discussion will focus on ^2H NMR spectroscopy, which has been widely used for this purpose (Seelig, 1977; Davis, 1983; Bloom et al., 1991).

The ^2H NMR spectrum of 1-[$^2H_{31}$]palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC-d_{31}) multilamellar dispersions is shown in Figure 5A. Thespectrum consists of 15 overlapping "Pake doublets," each of which corresponds to either a methylene group or the terminal methyl group of the fatty acyl-chain. The Pake doublet lineshape arises from random orientations of the C–^2H bond axis with respect to the bulk magnetic field direction. The two maxima in each doublet correspond to lipids located in regions of the bilayer where the angle between the bilayer normal and the external magnetic field is 90°. The separation between the maxima, known as the quadrupolar splitting ($\Delta\nu_Q$). is related to the C–^2H bond order parameter S_{CD} by the relation $\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD}$. $S_{CD} = (1/2)(3\langle\cos^2\beta\rangle - 1)$, where $\langle\cos^2\beta\rangle$ denotes the time average of the angular fluctuations of the C–^2H bond with respect to the director axis, defined as the normal to the bilayer surface. The order parameter is thus a measure of the angular excursions of the acyl-chains about the surface normal.

Over the past two decades, several techniques have been developed or enhanced which facilitate the measurement of S_{CD} in model and biological systems. The problems inherent in the acquisition of undistorted broad-line spectra were overcome by the development of quadrupolar echo pulse methods (Davis et al., 1976). Many experiments using specifically deuteriated systems were employed to determine order parameters at individual carbon atoms (Seelig, 1977); however, the time and effort required to produce a series of specifically acylchain-labeled lipids was enormous. The introduction of DePaking methods (Sternin et al., 1983) greatly simplified measurements of order parameters, particularly in systems labeled in many positions. Furthermore, the use of integration methods enabled one to derive a complete order profile from a dePaked spectrum of membrane lipids perdeuteriated in one of the lipid chains (Sternin et al., 1988; Lafleur et al., 1989). These techniques are illustrated in Figure 5, where the powder spectrum of POPC-d_{31} (Figure 5A, fop) has been dePaked to give the 0°-oriented spectrum (Figure 5A, *bottom*). Each half of the spectrum is integrated and divided into 14 equal areas corresponding to carbons 2-15 (the methyl group splitting is measured directly from the spectrum). The quadrupolar splittings are calculated from each unit area and are used to construct the smoothed or&r parameter profile (OPP), shown in Figure 5B. The shape of the OPP, which provides a signature of the lipid bilayer phase, consists of a plateau region (C2–C10) in which the order is relatively constant, followed by a rapid decrease in order towards the center of the bilayer.

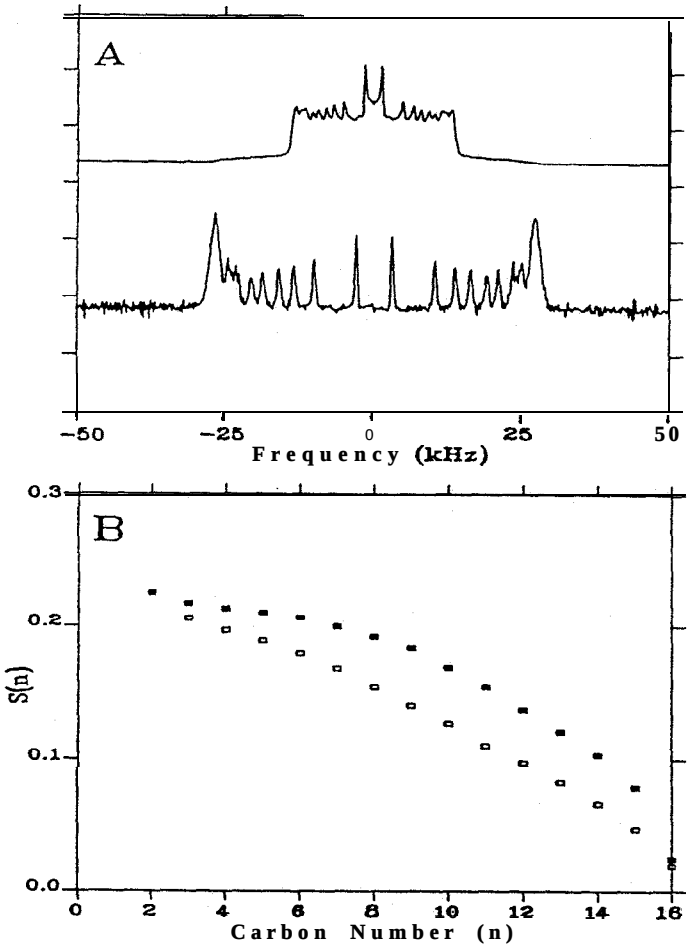

figure 5. (A) ^{2}H NMR spectrum of POPC-d_{31}, in the liquid–crystalline state (*top*). Enhanced resolution of the individual Pake doublets is obtained by dePaking the top spectrum, giving the spectrum below, which corresponds to the calculated oriented spectrum for an angle of 0° between the biiayer normal and the external magnetic field. The DePaked spectrum is integrated to give the smoothed order parameter profile shown in (B)(■). Also shown in (B) is the smoothed OPP for hexagonal phase POPE (□), obtained from the dePaked spectrum of POPE-d_{31} at 70 °C. The two profiles are normalized, but it should be remembered that the absolute order parameters of the H_{II} phase are approximately half those of the bilayer phase.

15

It has been noted that the shape of the order profile is conserved for a bilayer system. For example, Davis et al. (1980) observed that a more ordered bilayer exhibits a longer plateau region. In addition, temperature variation, a change in headgroup composition, or addition of Chol all affect the shape of the order profile in a predetermined manner. Lafleur et al. (1990b) were able to predict the shape of the profile from the arithmetic average of the order parameters $\langle S_{CD} \rangle$ determined from all positions. One finds that POPE-d_{31} bilayers at 60 °C, POPC-d_{31} bilayers at 20 °C, and POPC-d_{31}:Chol (8:2) bilayers at 40 °C all exhibit the same order profile. The conservation of the shape of the order profile was convincingly demonstrated by Morrow and Lu (1991) for a series of deuteriated PC bilayers when *one* adjusted for acyl-chain length at the same reduced temperature.

The variation of S_{CD} is quite different in the inverted-hexagonal phase (Sternin et al., 1988; Lafleur et al., 1990c). First, the magnitude of the order parameters is decreased by at least a factor of two due to increased motional averaging resulting from rotation about the long cylinder axis. Second, the plateau region is either very short or nonexistant, giving a relatively linear decrease in order down the length of the chain. To illustrate, the order profile of hexagonal phase POPE-d_{31} at 70 °C is shown in Figure 5B, normalized to the order profile of the lamellar phase. The lack of a plateau region occurs because of increased motional freedom (looser packing) of carbon atoms 2 through 8 relative to the bilayer phase.

It is of interest that Hn-forming lipids are more ordered when in the lamellar phase than are bilayer-forming lipids (Lafleur et al., 1990c; Monck et al., 1992). This observation may be explained by the increased lateral pressure on the lipid acyl-chains required to maintain a bilayer structure and is further supported by the observations that H_{II}-forming lipids confer an increase in $\langle S_{CD} \rangle$ in membrane lipid bilayers when mixed with lamellar-phase lipids (Cullis et al., 1986; Fenske et al., 1990; Lafleur et al., 1990b). In the mycoplasma *A. laidlawii*, this is a mechanism for modulating $\langle S_{CD} \rangle$ in the absence of other regulatory factors (Wieslander et al., 1980; Monck et al., 1992).

The application of ^{2}H NMR DePaking and integration methods to living systems is illustrated by studieson the microorganism *Acholeplasma laidlawii* strain B. This organism can be made fatty acid auxotrophic, which means that with suppression of *de novo* fatty acid biosynthesis, exogenously supplied fatty acids will be incorporated into the membrane lipids. Furthermore, binary mixtures of fatty acids will be incorporated in roughly a 50:50 molar ratio, with the more saturated chain occupying the sn-1 position of the glycerol backbone. Figure 6A shows a representative ^{2}H NMR spectrum for *A. laidlawii* grown on a fatty acid mixture containing deuteriated-palmitic acid. The spectrum displays the characteristic bilayer order profile. By varying the exogenous fatty acid composition of perdeuteriated-palmitic acid and oleic acid in the range between 80:20 and 20:80 (mol %), outside of which the organism grows poorly or not at all, it was possible to determine the range of order compatible with growth of the microorganism (Monck et al., 1992). This was found to be $0.14 < S_{CD} < 0.18$. It was suggested that the

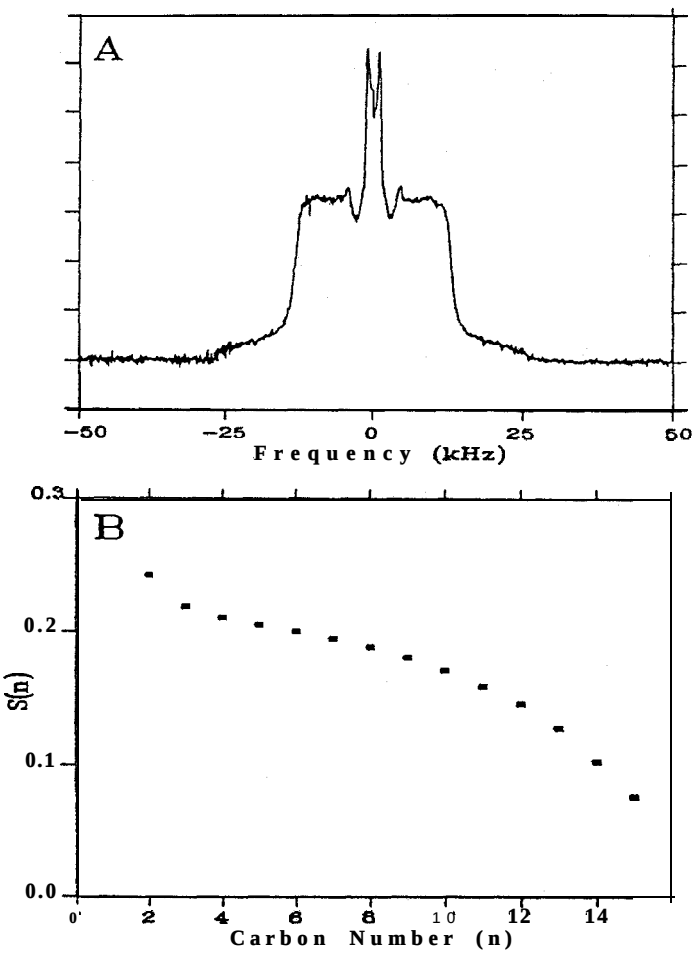

Figure 6. **(A) ^{2}H NMR spectrum of** A. *laidlawii* **grown on fatty acid** mixture containing **perdeuteriated palmitic acid. (B) Smoothed OPP obtained by** dePaking **and integration of (A).**

predominant lipid species, MGDG and DGDG, which in isolation prefer the H_{II} and bilayer phase, respectively, may play an important role in establishing the observed order profile. One may postulate that optimum membrane protein function can only occur within this range of order, perhaps due to a protein requirement for a certain membrane fluidity, or for a certain membrane thickness. A direct relationship between average membrane order $\langle S_{CD} \rangle$ and membrane thickness has been suggested (Seelig and **Seelig**, 1974; **Ipsen** et al., **1990),** which leads to the possibility of a correlation between optimal protein function and bilayer thickness.

The studies with *A. laidluwii* demonstrate that membrane order must be regulated within a certain range if growth is to occur. Other studies also demonstrate the necessity for regulating membrane order. Steady-state and time-resolved fluorescence anisotropy spectroscopy is often used to monitor changes in membrane order. A recent study by Behan-Martin et al. (1993) used both techniques to examine changes in order of a brain synaptic membrane fraction from a number of fish, mammalian, and avian species. Using two different fluorescent probes, they observed a striking relationship between membrane order and body temperature, with the membranes of cold-adapted species showing greater disorder than those of warm-adapted species. Similar values of membrane order were observed for all species when the comparison was made al their respective body temperatures, suggesting an optimum range of order compatible with proper brain function. These observations demonstrate conservation of membrane order over a wide range of thermal environments.

VII. PERMEABILITY AND THE OSMOTIC PROPERTIES OF MEMBRANES

Many of the physico-chemical properties of membranes are a function of the ensemble, and are not attributable to specific lipids or classes of lipids. Put another way, the sum total of interactions between lipids and between lipids and their aqueous environment determine such properties as membrane permeability and the effect of osmotic gradients on membrane structure. This is the most important aspect of membrane function as the ability to au as highly selective semipermeable barriers is essential for cell function and maintenance. The topic of membrane permeability has been reviewed elsewhere (Cullis and Hope, 1991; de Gier, 1993), and will not be discussed in detail here. Instead, we will focus on some recent work regarding the osmotic properties of vesicles, an area of research which may have significant biological relevance.

Liquid-crystalline membranes are highly permeable to water, with values of the permeability coefficient (P) in the range of 10^{-2} to 10^{-4} cm/s (Deamer and Bramhall, 1986). The permeability coefficients of other nonelectrolytes vary greatly, from 10^{-1} cm/s for ammonia to 10^{-6} cm/s for urea and glycerol to 10^{-11} cm/s for glucose. Lipid bilayers are highly impermeable to most ions, with P values ranging from 10^{-11} to 10^{-13} cm/s for ions such as Cl^- and Na^+, respectively (see de Gier, 1993). The exception is H^+, for which P is about 10^{-5} cm/s. These variations in permeability allow the establishment of osmotic gradients and electrochemical potentials across a lipid bilayer, as impermeable agents can be trapped within a vesicle, while semipermeable agents such as water or protons can still cross in response to osmotic or electrochemical potentials.

A number of different factors affect the permeability of water, nonelectrolytes, and electrolytes. For example, the surface potential at the membrane surface will

affect the ability of charged ions to cross. One factor which appears to affect all three classes of permeants mentioned above, is the order of the membrane. Generally, the more ordered the membrane, the less permeable it is. The increase in order can be established either by increasing the saturation of the fatty acyl chains, or by introducing Chol to the system. Gel-state lipids are particularly impermeable, but membranes in which gel and liquid-crystalline lipid coexist can be even more permeable than fluid membranes. This has been attributed to the presence ofdefects at the gel-liquid boundary.

Closed membrane systems containing an impermeable solute are thus susceptible to osmotic forces. For example, a unilamellar liposome encapsulating Na^+ can be made to swell or shrink by placing it in hypertonic (higher solute concentration) or hypotonic (lower solute concentration) solution, respectively. A few studies have examined the osmotic sensitivity of unilamellar vesicles of different sizes. Lichtenberg et al. (1981) examined vesicles prepared using a French pressure cell, with diameters ranging from 20-90 nm. By examining the fractional degree of self-quenchingoftrapped 6-carboxyfluorescein under conditions of osmotic stress, they found that vesicles with diameters greater than 40 nm were nonspherical under isoosmotic conditions, and could sustain an increase in the volume of the aqueous interior of almost twofold without leakage of the internal contents. Similar results have recently been obtained for much larger LUVs. Mui et al. (1993) characterized the osmotic properties of LUVs prepared by extrusion using cryo-transmission EM. LUVs prepared under isoosmotic conditions were found to be nonspherical, but could be made to "round-up" by placing them in an hypoosmotic medium. When LUVs were exposed to a sufficiently large osmotic differential, lysis was observed by the release of 6-carboxyfluorescein, with a residual osmotic differential remaining after lysis. A membrane tension of 40 dyn/cm was estimated (at 23 "C) from the maximum residual osmotic differentials obtained for LUVs varying in diameter from 90 to 340 nm.

Several studies have reported that SUVs are osmotically insensitive (Johnson and Buttress, 1973; Milon et al., 1986). Support for the oppositeview comes from recent work by Lerebours et al. (1993), who utilized freeze-fracture EM, ESR, and fluorescence spectroscopy to demonstrate that SUVs of diameter 20 nm are osmotically sensitive, swelling or shrinking in response to applied salt gradients of opposite direction. Unlike the results reported for LUVs (Mui et al., 1993), SUV swelling did not result in breakage of the membrane with release of entrapped material.

Whether the results of these studies are applicable to the behavior of biological membranes is not known. What they do indicate is that membrane shape is susceptible to osmotic forces, and that membrane curvature may influence the magnitude of the osmotic gradient that can be sustained while maintaining bilayer integrity. The possibility of osmotic forces playing a role in the establishment or maintenance of highly curved membranes of cellular organelles and intracellular membranes is intriguing.

VIII. THE ROLE OF LIPIDS IN PROTEIN FUNCTION

One of the more important roles of membrane lipids is the regulation of membrane protein activity. In some cases, it appears that specific lipids are involved in maintaining the activity of specific proteins; in other cases, protein activity appears to be sensitive to the overall membrane environment, which is modulated by all lipids present. The precise mechanisms involved are not all known, but could involve binding of lipids to protein regulatory sites, or the matching of membrane hydrophobic thickness with protein hydrophobic thickness, as delineated in the "mattress model" of protein-lipid interactions (Mouritsen and Bloom, 1984; Bloom et al., 1991; Nezil and Bloom, 1992, Mouritsen and Bloom, 1993). In this section, we will examine several recent studies in which protein activity is related to variation in membrane properties. The examples cited do not represent an exhaustive review of this field, but merely serve to illustrate an important role of lipids in biological membrane function.

Some proteins require specific lipids for maximum activity. Examples include protein kinase C (PKC) and many of the mitochondrial proteins. PKC, which is involved in transmembrane signaling, requires the presence of PS for its activity. Most of the electron transport complexes and several of the mitochondrial translocases require CL, a structurally unique phospholipid found in the inner mitochondrial membrane of eukaryotes, for maximum activity (see Robinson, 1993 and references therein). Although this suggests a direct CL-protein interaction, evidence for the presence of tightly bound CL exists for only two proteins, the ADP/ATP carrier, and the cytochrome c oxidase electron-transport complex. In the case of cytochrome c oxidase, much evidence suggests that two mol of CL binds to high-affinity sites of the bovine heart complex, thereby increasing the rate of electron-transport. The evidence for this view has recently been reviewed by Robinson (1993).

Numerous proteins appear sensitive to the overall physical state of the membrane. The activity of the sarcoplasmic reticulum Ca^{2+}-ATPase is modulated by Chol, apparently via changes in membrane fluidity or order (Madden et al., 1979; 198 1). PKC is also regulated in this manner. A large number of hydrophobic and amphipathic compounds can alter its activity. Numerous studies have shown that PKC enhancers and inhibitors tend to promote or inhibit the formation of hexagonal phase lipid, respectively, in appropriate model systems (Epand et al., 1991). Thus, PKC is sensitive to the physical state of the membrane, particularly with respect to its propensity to form nonbilayer phases. This suggests that PKC activity may be regulated by membrane order, which is increased by the presence of nonbilayer lipids (see Section VI).

Another protein whose activity seems to be influenced by membrane polymorphic characteristics is Gramicidin, a hydrophobic peptide which forms a membrane-spanning homodimer (linked at the N-terminals) that functions as a cation channel (Wallace, 1990). Depending on the solvent present when Gramicidin is added to

the membrane, either the channel conformation, or a nonchannel conformation in which the dimer is formed head-to-tail is obtained (Killian et al., 1988a,b; Bano et al., 1989, 1991). Conversion of the nonchannel form to the channel form can be accomplished by heating. Cox et al. (1992) found that the presence of PE in PC bilayers enhanced this conversion rate. In addition, a small amount of H_{II} phase lipid was detected by ^{31}P NMR in preparations containing the channel form. This led to the proposal that H_{II}-promoting lipids would induce the nonchannel to channel transition.

Other parameters which may influence the kinetics and thermodynamics of Gramicidin dimerization include hydrophobic mismatch between the hydrophobic length of the dimer and the hydrophobic portion of the lipid bilayer (Huang, 1986; Helfrich and Jacobson, 1990; Ring, 1992). As alluded to above, this may be an important factor in optimizing the function of many membrane proteins. It is interesting that some proteins, in the absence of specific (normally required) lipids, haveshowngoodactivitylevelsdependingon thethicknessofthemembranemilieu (Gut-r and Harwood, 1991). A specific example of this is the passive glucose transporter of the erythrocyte membrane, for which maximal activity is observed in the presence of PS, but which, in its absence, depends on the length of the lipid acyl-chains (Carruthers and Melchior, 1988).

Many other examples can be found in the recent literature of lipid modulation of membrane protein activities. A few examples include the photochemical protein rhodopsin (Gibson and Brown, 1993) and a Mg^{2+}-ATPase from human erythrocytes (Zimmerman and Daleke, 1993). Phospholipase A2 is also regulated by lipid physical properties; of particular interest is the suggestion that lipid lateral phase separation may play a role in modulating the lipase activity (Op den Kamp et al., 1974; Apitz-Castro et al., 1982; Jam et al., 1989; Burack et al., 1993).

ABBREVIATIONS

CER, ceramide.
Chol, cholesterol.
CL, cardiolipin.
$\Delta\nu_Q$, quadrupolar splitting.
DGDG, diglucosyl diacylglycerol.
DOPA, 1,2-dioleoyl-sn-glycero-3-phosphate.
DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine.
DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine.
DSC, differential scanning calorimetry.
EM, electron microscopy.
(e^2qQ/h), quadrupolar coupling constant.
ESR, electron spin resonance spectroscopy.
GM_1, ganglioside GM_1.

GSL, glycosphingoiipid.
H_{II}, hexagonal H_{II} phase.
ILA, interlamellar attachments.
IMI, inverted micellar intermediate.
L_α, lamellar liquid-crystalline phase.
LUV, large unilamellar vesicle.
MGDG, monoglucosyl diacylglycerol.
MLV, multilamellar vesicle.
NMR, nuclear magnetic resonance.
OPP, order parameter profile.
P, permeability coefficient.
PA, phosphatidic acid
PC, phosphatidylcholine.
PE, phosphatidylethanolamine.
PG, phosphatidylglycerol.
PI, phosphatidylinositol
PKC, protein kinase C.
PS, phosphatidylserine.
POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.
POPC-d_{31}, 1-[$^2H_{31}$]palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.
POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine.
POPE-d_{31}, 1-[$^2H_{31}$]palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine.
R_o, spontaneous radius of curvature.
S_{CD}, carbon-deuterium bond order parameter.
SEPC, 1-stearoyl-2-elaidoyl-sn-glycero-3-phosphocholine.
SPM, sphingomyelin.
S W small unilamellar vesicle.
T_H, lamellar to hexagonal phase transition temperature.
T_m, gel to liquid-crystalline phase transition temperature.
TMC, transmonolayer contact.

ACKNOWLEDGMENTS

The authors wish to thank Dr. John Holland and Dr. Thomas D. Madden for permission to publish some of their data prior to publication (the ^{31}P NMR spectra in Figure 3). We also acknowledge the assistance of Dr. Jenifer L. Thewalt, who helped with the preparation of some of the figures.

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