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Structural and Functional Aspects of Nonbilayer Lipids

B. de Kruijff, P. R. Cullis, and A. J. Verkleij

1. INTRODUCTION

The bilayer model of the lipid part of biological membranes has been very popular over the past few decades owing to the fact that it provided a rationale for many observations made in the field of membrane biology. In recent years, however, it has become increasingly clear that this bilayer model is incomplete as it does not explain two basic properties of membranes. The first is that next to bilayer-forming lipids there is an abundant occurrence of membrane lipids that after isolation and dispersion at physiological temperatures in aqueous buffers do not adopt a bilayer organization (nonbilayer lipids). Second, during many membrane processes like fusion and lipid flip-flop, (part of) the lipids will (temporarily) leave the bilayer organization (nonbilayer processes). The hypothesis that nonbilayer lipids and the structures they form are actively involved in nonbilayer processes had lead to the proposal of an alternative model of biological membranes (for reviews see Cullis and de Kruijff, 1979; de Kruijff *et al.*, 1980a). In this article we will summarize the present knowledge of the structural and functional properties of these nonbilayer lipids.

2. PROPERTIES OF NONBILAYER LIPIDS

2.1. Lipid Polymorphism

The ability of membrane lipids to adopt a variety of phases on hydration has been known for some time (Luzzatti *et al.*, 1968). X-ray analysis (for review see Shipley, 1973)

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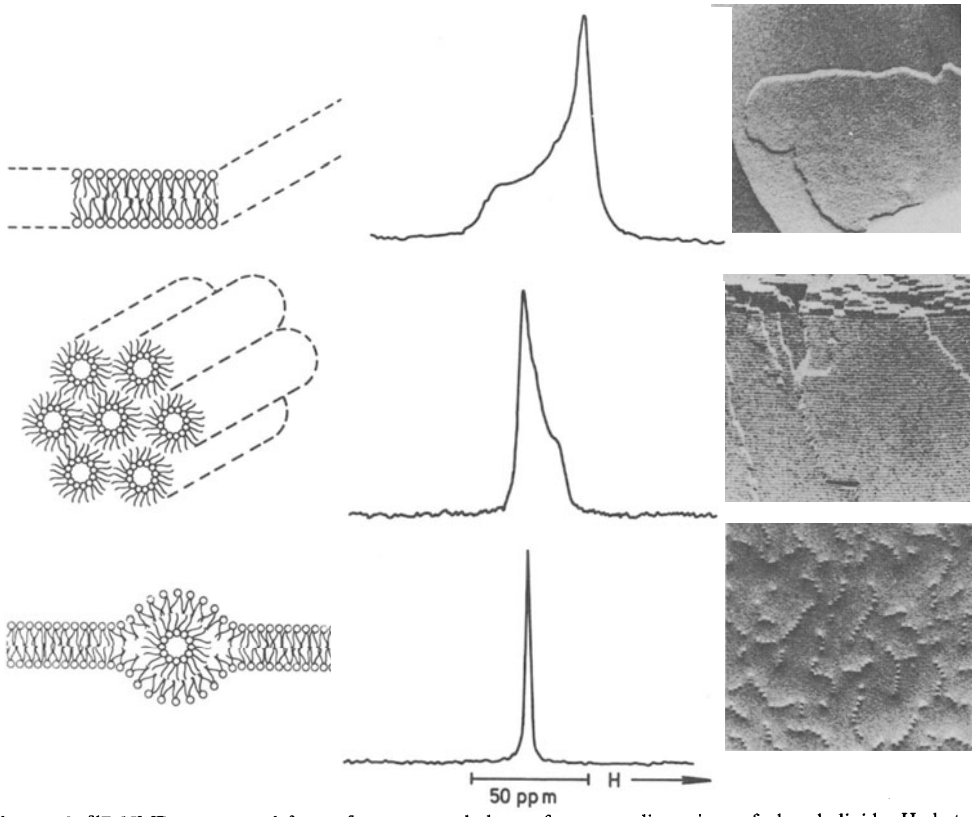

Figure 1. ^{31}P NMR spectra and freeze-fracture morphology of aqueous dispersions of phospholipids. Hydrated erythrocyte PE adopts the bilayer phase below 8°C and the H_{II} phase above 8°C (Cullis and de Kruijff, 1978). As an example of an "isotropic" phase, the ^{31}P NMR spectrum and freeze-fracture morphology (lipidic particles) of a PC/CL (Ca^{2+}) dispersion (Verkleij *et al.*, 1979b; de Kruijff *et al.*, 1979) are shown. As a model of this phase, the intrabilayer inverted micelle is shown.

and in more recent years ^{31}P NMR and freeze-fracture studies on fully hydrated preparations of the major membrane lipids have shown that as a rule either the bilayer or the hexagonal (H_{II}) phase is preferred. In the case of ^{31}P NMR the lineshape obtained from model systems as well as biological membranes is a sensitive indicator of the phase(s) adopted by the phospholipids (Cullis and de Kruijff, 1979). As is shown in Fig. 1, an asymmetrical lineshape with a low-field shoulder is characteristic of bilayer phospholipids, whereas H_{II}-phase phospholipids exhibit a narrower lineshape with reversed asymmetry. Alternatively, lipids in structures that allow isotropic motion ($\tau_C < 10^{-4}$) give rise to narrow symmetric signals. Typical bilayer-forming lipids are phosphatidylcholines (PC), sphingomyelin, diglucosyl- and digalactosyldiglycerides. The main H_{II} lipids are unsaturated phosphatidylethanolamines (PE), monoglucosyl- and monogalactosyl diglycerides, and cardiolipin (CL) in the presence of Ca^{1+} (for references see Cullis and de Kruijff, 1979). Phosphatidylserines (PS) and phosphatidylglycerols prefer the bilayer phase at neutral pH and ambient temperatures. At lower pH (Hope and Cullis, 1980) or at elevated temperatures (Harlos and Eibl, 1980), a hexagonal phase can be adopted. Cholesterol can induce formation of hexagonal phases from bilayer systems.

The phase preferences of a lipid can be understood in terms of the dynamic molecular shape of the molecule (Cullis and de Kruijff, 1979; Israelachvili *et al.*, 1977). H_{II} lipids

will exhibit a "cone" shape where the polar head group is at the smaller end of the cone. Lipids that have an inverted cone shape (e.g., lysophospholipids) organize themselves in micelles, whereas lipids with a more cylindrical shape pack optimally into a bilayer. The observation that an equimolar mixture of lysophosphatidylholine (inverted cone shape) and cholesterol (cone shape) is organized in a bilayer (Rand *et al.*, 1975) nicely illustrates this molecular shape concept.

2.2. Modulation of Bilayer–Nonbilayer Transitions

An important characteristic of the phase preferences of membrane lipids is that transitions between the H_{II} and the bilayer phase can occur. For example, in the case of unsaturated PEs, bilayer $\rightarrow$ H_{II} transitions occur as the temperature is increased through a characteristic value, which is dependent on the fatty acid composition. This transition temperature is about 8°C for human erythrocyte PE (Fig. 1). These transitions can also be induced isothermally by changes in divalent cation concentration. For example, Ca^{2+} can induce a bilayer $\rightarrow$ H_{II} phase transition for beef heart cardiolipin (Fig. 2) (Cullis *et al.*, 1978) and for PE/PS (Cullis and Verkleij, 1979) or PE/CL (de Kruijff and Cullis, 1980a) mixed systems. Most interestingly, lipid–protein interactions also can modulate the phase behavior of the lipids. Cytochrome *c* specifically induces the H_{II} and an isotropic phase in CL-containing systems (Fig. 2) (de Kruijff and Cullis, 1980a), whereas poly-L-lysine causes this effect on mixed PE/CL bilayers (de Kruijff and Cullis, 1980b). That integral mem-

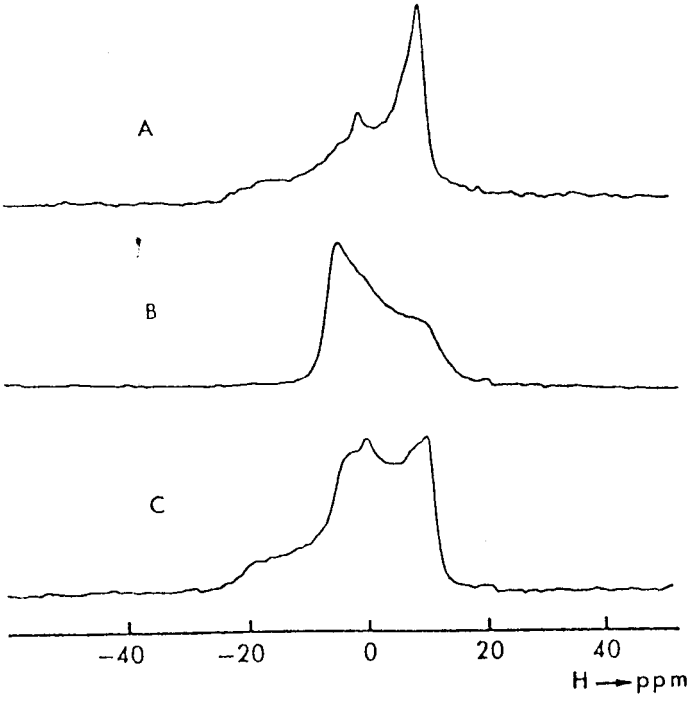

Figure 2. 81-MHz ^{31}P NMR spectra at 30°C of an aqueous dispersion of cardiolipin (A) in the presence of Ca^{2+} (B) and cytochrome *c* (C). Beef heart cardiolipin (50 μmoles) was dispersed in 1.0 ml 100 mM NaCl, 10 mM Tris–HCl, 0.2 mM EDTA, pH 7.0. In (B) 0.1 ml 1 M $CaCl_2$ and in (C) 0.2 ml buffer containing 36 mg oxidized cytochrome *c* was added.

brane proteins stabilize bilayer structures may be inferred from the observation that in total lipid extracts of the rod outer segment (de Grip *et al.*, 1979) and *E. coli* cytoplasmic membrane (Burnell *et al.*, 1979), the H_{II} and an isotropic phase are observed whereas the lipids in the intact membranes are mainly organized in bilayers.

2.3. Lipidic Particles

Because biological membranes contain a mixture of bilayer and H_{II} lipids, the phase behavior of such mixtures is of obvious interest. ^{31}P NMR studies have demonstrated that instead of gradually going from an H_{II} phase to a bilayer structure when a bilayer lipid is titrated into an H_{II} type of lipid structure, a new spectral component is observed indicating fast isotropic motion of the lipid molecules (Fig. 1). This behavior was observed for many different lipid mixtures including the hydrated total lipid extracts from the rod outer segment (de Grip *et al.*, 1979), *E. coli* (Burnell *et al.*, 1979) and inner mitochondrial membranes (Cullis *et al.*, 1980a). The organization of this intermediate phase is therefore of particular interest. However, a variety of lipid structures can give rise to narrow symmetric ^{31}P NMR spectra characteristic of isotropic averaging, including small bilayer vesicles, micelles, inverted micelles, or phases like the cubic ones. Freeze-fracture analysis of these preparations has shown that on the fracture face of these pure lipid systems numerous particles and pits are present that often are organized in a stringlike fashion (Verkleij *et al.*, 1979a; de Kruijff *et al.*, 1979). All present evidence suggests that these lipidic particles represent intrabilayer inverted micelles that are located either inside one bilayer or at the nexus of intersecting bilayers. This tendency of nonbilayer lipids to prefer an inverted micellar structure instead of the H_{II} phase in mixtures with bilayer lipids opens intriguing functional possibilities.

2.4. Biological Membranes

From the previous data it is clear that nonbilayer structures, in particular inverted micellar ones, might occur in biological membranes. ^{31}P NMR investigations of human red cell membranes have shown that the lipids are organized in extended bilayers (Fig. 3). This organization is extremely stable, being unaffected by extensive treatment with phospholipases and proteolytic enzymes (Cullis and de Kruijff, 1979). This stability might be related to the large mechanical stress that the erythrocyte encounters in the circulation or alternatively might reflect the relatively low metabolic activity of this plasma membrane. A very different situation is observed in metabolically more active membranes such as the endoplasmic reticulum of the rat liver (Fig. 3). At physiological temperatures a lineshape is observed that demonstrates isotropic motion for a large fraction of the endogenous phospholipids. At lower temperatures the spectrum indicates bilayer structure. This temperature-dependence behavior has been observed for isolated rat (de Kruijff *et al.*, 1980c), rabbit (Stier *et al.*, 1978), and beef (de Kruijff *et al.*, 1978) liver microsomes, as well as for the rat liver inner mitochondrial membrane (Cullis *et al.*, 1980b) and the *E. coli* inner membrane (Burnell *et al.*, 1979). ^{31}P (de Kruijff *et al.*, 1980b) and ^{13}C (de Kruijff *et al.*, 1980c) NMR studies on intact rat liver also demonstrated isotropic motion of part of the membrane phospholipids at 37°C. Although the isotropic motion could originate from rapid lateral diffusion of the lipids over curved bilayer surfaces, the data would be consistent with the transient occurrence of nonbilayer lipid structures, such as inverted micelles.

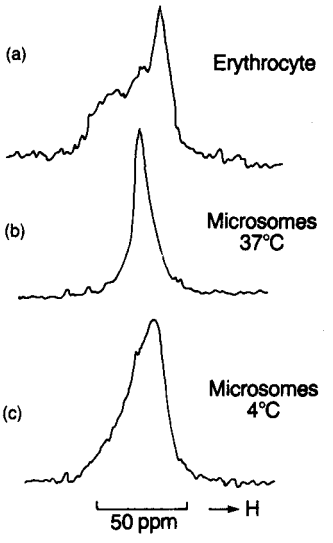

Figure 3. ^{31}P NMR spectra of various biological membranes. Reproduced with permission from *TIBS* (1980) **5**, 79–81.

2.5. Functional Aspects

Fusion and lipid flip-flop are clear examples of nonbilayer processes in which nonbilayer lipids have been shown to play an active role. For example, chemical fusogens that fuse erythrocytes also induce the H_{II} phase in the erythrocyte membrane (Cullis and Hope, 1978), the extent of fusion being proportional to the amount of H_{II} phase formed (Hope and Cullis, 1981). Furthermore, in different lipid systems vesicle fusion is accompanied by the appearance of lipidic particles at the site of fusion (Verkleij *et al.*, 1979b, 1980). Similarly, phospholipid flip-flop in model membrane systems is greatly enhanced by the presence of nonbilayer lipid structures (Gerritsen *et al.*, 1980). The fast flip-flop of lipids in the endoplasmic reticulum membrane at 37°C (Zilversmit and Hughes, 1977; van den Besselaar *et al.*, 1978) and in bacterial membranes (Rothman and Leonard, 1977) also is fully consistent with the transitory formation of inverted micelles.

Nonbilayer lipids and the structures they form appear to be especially important for the inner mitochondrial membrane, as this membrane is particularly rich in nonbilayer lipids. At 37°C in the presence of Ca^{2+}, both CL and PE, which amount to 60% of the total lipids, prefer the H_{II} phase. That inverted micelles of the cardiolipin–Ca^{2+} complex play a role as ionophore in Ca^{2+} transport across the inner mitochondrial membrane is indicated by the following observations: (1) Ca^{2+} induces the formation of lipidic particles in CL-containing membranes (Verkleij *et al.*, 1979a; de Kruijff *et al.*, 1979); (2) these particles facilitate divalent cation transport (de Kruijff *et al.*, 1979; Gerritsen *et al.*, 1980); (3) ruthenium red, a potent inhibitor of Ca^{2+} transport in mitochondria, blocks the formation of nonbilayer phases by Ca^{2+} in CL-containing membranes and also blocks the uptake of Ca^{2+} into an organic phase by CL (Cullis *et al.*, 1980b).

Literature data as well as our own studies on cytochrome *c*–CL systems (for review see de Kruijff *et al.*, 1981) suggest that nonbilayer lipids may play an important role in posttranslational protein insertion, as the formation of nonbilayer lipid structures can provide a low-energy pathway for the translocation of polar head groups into or across a lipid bilayer. Finally, the observations that cytochrome *c* specifically induces nonbilayer structures in CL-containing bilayers (de Kruijff and Cullis, 1980b) and that CL is absolutely

required for the enzymatic activity of cytochrome oxidase (Fry and Green, 1980) suggest that an intramembrane cytochrome *c*–CL complex is important for electron transport between cytochrome *c* and cytochrome oxidase.

3. CONCLUDING REMARKS

Although our understanding of the structural and functional aspects of nonbilayer membrane lipids is still at a primitive level, we feel that the results obtained so far are exciting and appear to be leading to a new appreciation of the role of lipids in membranes. Further research will have to be directed toward a characterization of the molecular structure of the "isotropic" phases observed in biological membranes. New areas of research will include the chloroplast membranes, which like the mitochondrion are particularly rich in nonbilayer lipids. Furthermore, it can be expected, and to some extent has already been documented, that a detailed understanding of the phase characteristics of functionally very important nonbilayer lipids such as phosphatidic acids, diglycerides, and phosphatidylinositol (phosphates) may lead to a molecular interpretation of biological signal transmission.

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