CHAPTER 9

Liposome Fusion

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1. INTRODUCTION

Fusion of biological membranes is a fundamental process in many cellular functions, including the intracellular delivery of lipids, proteins, and metabolites by transport vesicles, endocytosis, exocytosis, cell division, cell-cell fusion, fertilization, and the invasion of cells by enveloped viruses. These complex fusion processes involve proteins that bring the fusing membranes into close proximity and promote fusion through membrane destabilization. While a number of fusion-promoting proteins have been characterized (White, 1992), the exact mechanism of the fusion events remain poorly defined. The use of liposomes as model biological membranes permits the study of membrane fusion as it relates to the lipid bilayer in relatively simple systems.

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The essence of membrane fusion is the formation of a continuous lipid bilayer from two closely apposed but separate lipid bilayers, or vice versa. This encompasses both the formation of a single membrane compartment from two previously distinct membrane-bound vesicles and the formation of a second vesicular compartment internal or external to a larger progenitor membrane. Intrinsic to these processes is the requirement for an intermediate nonbilayer lipid structure, in the sense that two bilayers cannot combine to form one bilayer without some local intermediate departure from bilayer structure. Early studies demonstrating the ability of fusogens to induce fusion in biological membranes and to detect associated nonbilayer structures (Cullis and Hope, 1978) have led to a number of proposed structural intermediates.

Here we present a brief review of the polymorphic properties of lipids and the ways in which these structural preferences may be modulated. It is then emphasized that factors that induce "inverted" nonbilayer structures, such as the hexagonal H_{II} phase, in previously bilayer structures invariably induce fusion between LUVs of the same lipid composition. This is consistent with an inverted micellar or other inverted intermediate as a general feature of membrane fusion. Further, the transbilayer distribution of fusogenic, potentially nonbilayer lipids can play a regulatory role in membrane fusion.

II. LIPID POLYMORPHISM

Biological membranes are complex mixtures of a variety of lipid species. Their composition is highly differentiated, not only among organisms and among tissues of a given organism, but also among the membranes of cells and organelles of a given tissue and between monolayers of a given membrane. The lipid composition is clearly important to the functional requirements of the membrane. The phase preference of a particular lipid influences the properties of a membrane in which it is included. Of particular interest here is the relationship of lipid composition to stability of the bilayer phase and the propensity for membrane fusion.

As detailed elsewhere in this volume, many of the lipid species that comprise biological membranes can also exist in nonbilayer phases when dispersed as a single species in aqueous media. The phase preference of a given lipid depends on the chemical structure of the lipid, the temperature, and a variety of extrinsic chemical influences. The polymorphic properties of lipids have been extensively reviewed (Cullis and de Kruiff, 1979; Gruner *et al.*, 1985; Lindblom and Rilfors, 1989; Seddon, 1990). Among the nonbilayer phases available to natural lipids are the micellar phase, the H_{II}

phase, and various cubic phases. For comparison, the arrangement of lipid molecules in the bilayer, micellar, and hexagonal phases are depicted in Fig. 1.

Bilayer-preferring lipids form liposomes that can exist as MLVs, composed of many concentric lipid bilayers, or can be transformed into unilamellar vesicles by extrusion or sonication (Szoka and Papahadjopoulos, 1980). LUVs are useful as models of biological membranes where a single bilayer surrounds a cell or organelle. Dispersions of hexagonal-forming lipids exist in close-packed cylinders with hydrophilic cores and hydrophobic interstices. Lipid mixtures composed of bilayer and hexagonal phase-preferring lipids can also form "lipidic particle" structures as visualized by freeze-fracture electron microscopy (Verkleij, 1984). These particles may consist of intrabilayer inverted micelles with hydrophilic centers and hydrophobic surfaces, or may reflect interbilayer stalks or other structures related to the cubic phase.

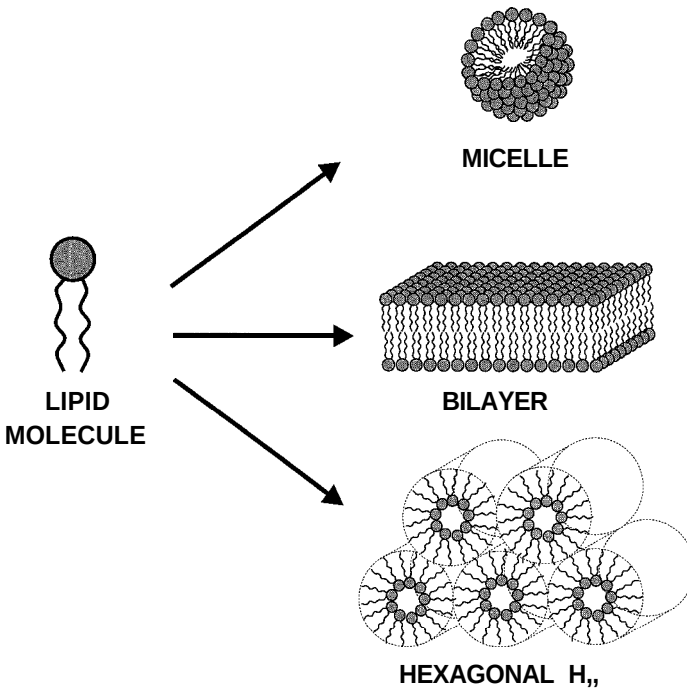

FIGURE 1 Organization of lipid molecules in micelles, bilayer, and hexagonal (H_{II}) phases.

A. Pure Lipids

Among the lipids that are found in biological membranes, those that will in isolation form micelles upon hydration exist only as minor components. However, lipids which can adopt the hexagonal phase are present at substantial levels, usually at least 30 mol% of total lipids. The examples of the phase preferences of pure lipids given in Table I demonstrate the effect of chemical environment on the phase preference of some lipids. Aqueous dispersions of unsaturated PE adopt the hexagonal structure, while unsaturated PA or phosphatidylserine (PS) will exist in bilayers unless the headgroup charge is neutralized either by low pH or the presence of multivalent cations, such as Ca^{2+}.

The morphologies of pure lipids have been related to their time-averaged molecular geometries, or "shape," and in particular to the relative surface areas occupied by the hydrophilic headgroup and the hydrophobic hydrocarbon chains. A model describing this relationship has been detailed previously (Israelachvilli *et al.,* 1980; Cullis *et al.,* 1986). Lipid molecules for which the headgroup and hydrocarbon structures subtend equal areas in a membrane (e.g., PC) approximate cylinders and pack into stable bilayers. Those lipids for which the headgroup area is large relative to the hydrocarbon (e.g., lysolipids, detergents) have inverted cone geometries and form micelles. Conversely, those with relatively small headgroups have conical geometries and form inverted micelles or hexagonal structures (e.g., PE). Accordingly, increasing the effective area subtended by the hydrocarbon chains by increasing chain length, increasing unsaturation, or increasing temperature promotes the formation of the hexagonal phase. While this shape model is a simplification of a complex combination of molecular forces, it offers excellent correlation between experimentally observed phase preferences and the molecular geometries of lipids.

TABLE I
Polymorphic Phase Preferences of Membrane Lipids

Bilayer	Hexagonal H_{II}
PC	PE
PS	PS (pH < 3)
PA	PA (+ Ca^{2+})
PG	PA (pH < 3)
CL	CL (+ Ca^{2+})
PI	Fatty acids

B. Lipid Mixtures

The morphologies of mixed lipid systems offer greater insight into the role of lipid diversity in biological membranes. The properties of a wide variety of binary and tertiary lipid mixtures have been documented (Seddon, 1990; Cullis *et al.*, 1990). Generally, it has been found that hexagonal-preferring lipids, such as unsaturated PE, form stable bilayers upon the addition of 20-50 mol% of a bilayer-forming lipid, including PC, PS, or SPM. The addition of cholesterol to membrane-stabilized PE mixtures containing PC or SPM induces the hexagonal phase to reform. These observations are consistent with the concept of cholesterol exhibiting a "cone" shape, compatible with H_{II} phase structure. Alternatively, micelle-forming lipids, such as detergents, can form bilayers in mixtures with hexagonal-forming lipids, as is the case with detergent-PE systems (Madden and Cullis, 1982).

The polymorphic phase behavior of lipid mixtures leads to the possibility that nonbilayer lipid structures can occur in biological membranes. In particular, there are lipid species and mixtures present in membranes that can adopt the hexagonal phase under physiological conditions. Thus local phase transitions induced under physiological conditions could disrupt the bilayer structure and, given the close apposition of a second membrane, lead to membrane fusion.

C. Factors Modulating Lipid Polymorphism

As described earlier, the physical and chemical environment of lipid dispersions can have profound effects on lipid polymorphism and, by extension, lipid shape or morphology. The neutralization of headgroup charge by variation of pH or the presence of polyvalent ions (e.g., Ca^{2+}) can lead to destabilization of the lamellar phase and formation of the hexagonal phase (Tilcock ***et al.,*** 1988). A similar effect is seen in systems containing insufficient water to fully hydrate lipids that form bilayers under water-saturated conditions. Under such conditions, the headgroup size is effectively smaller, leading to a preference for the H_{II} phase (Cullis *et al.*, 1986a). The hexagonal phase can also be promoted by increasing the temperature, which results in increased hydrocarbon chain motion. Dioleoyl-PE, for example, adopts bilayer structures below 10°C and the hexagonal phase at higher temperatures (Tilcock ***et al.,*** 1982). Increasing hydrocarbon chain unsaturation also favors H_{II} formation (Tilcock and Cullis, 1982b). The factors that modulate the phase behavior of membrane lipids are summarized in Fig. 2.

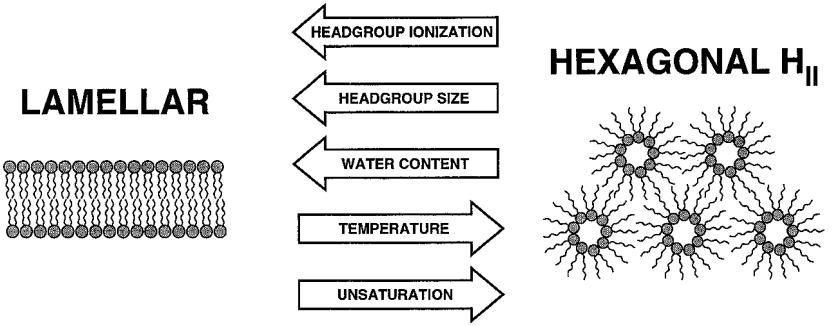

FIGURE 2 Factors influencing the bilayer-to-hexagonal (H_{II}) phase transition in membrane lipids.

III. LIPID POLYMORPHISM AND FUSION

A. Fusion Promoters and Nonbilayer Structures

The formation of nonbilayer lipid structures is very clearly associated with membrane fusion. The factors that promote the hexagonal phase in lipid dispersions have all been shown capable of inducing fusion in LUVs. Evidence for this association comes largely from phosphorus-31 NMR spectroscopy of MLV systems (Cullis and Hope, 1978; Cullis and de Kruiff, 1979) lipid and contents mixing assays using fluorescent probes for unilamellar vesicles (Ellens et al., 1984; Struck et al., 1981), and freeze-fracture electron microscopy for both multi- and unilamellar lipid systems (Verkleij *et al.*, 1980). Conversely, stabilization of the bilayer phase, for example, by the addition of lyso-PC, can inhibit fusion in liposomal systems (Martin and Ruysschaert, 1995).

The ^{31}P-NMR spectra in Fig. 3 illustrate the effect of bilayer-destabilizing factors on lipid morphology. The addition of Ca^{2+} to DOPE-DOPC-DOPS-Chol vesicles results in a change in the ^{31}P-NMR lineshape from one that corresponds to bilayer structures to one that is associated with the hexagonal phase (Fig. 3a). The bilayer spectrum, which exhibits a high-field peak and low-field shoulder separated by approximately 40 ppm, corresponds to motional averaging due to rapid axial rotation of the phospholipid molecules. In the hexagonal H_{II} phase additional motional averaging due to lateral diffusion around the narrow tubes of this structure results in a ^{31}P-NMR lineshape with reversed asymmetry that is narrower by a factor of 2. Formation of the hexagonal phase is also observed upon the addition of fusion-promoting, or "fusogenic," lipids such as diacylglycerides

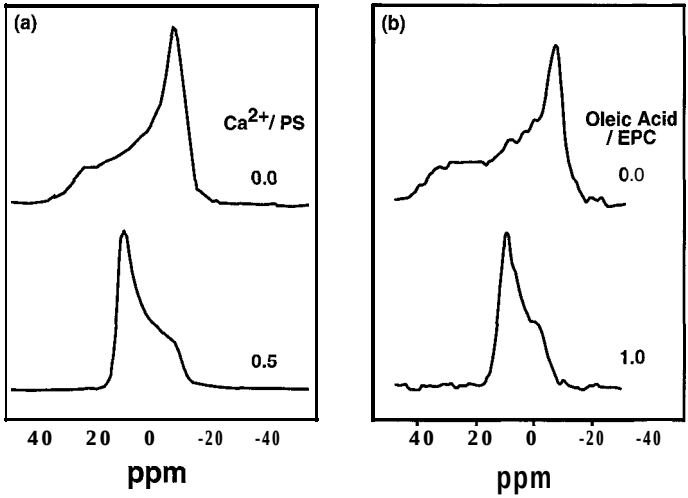

FIGURE 3 (a) An 81-MHz ^{31}P-NMR spectrum of a DOPE-DOPC-DOPS-Chol (1: **1: 1:** 3) mixture at 30°C before (upper) and after (lower) the addition of Ca^{2+} to a Ca^{2+}–DOPS ratio of 0.5 (Tilcock et al., 1988). The observed chemical shift anisotropies are typical of bilayer and hexagonal phases, respectively. (b) A 36.4-MHz ^{31}P-NMR spectrum of EPC at 30°C with (upper) and without (lower) equimolar oleic acid (Cullis *et al.*, 1981).

or fatty acids to stable bilayers (Hope and Cullis, 1981). Equimolar oleic acid added to EPC MLVs results in the characteristic change in the spectrum corresponding to induction of the H_{II} phase, shown in Fig. 3b. Promotion of the hexagonal phase by increasing temperature and decreasing hydration has also been demonstrated by ^{31}P-NMR studies (Tilcock et al., 1982; Tilcock and Cullis, 1987).

8. Nonbilayer Structures as Intermediates in Fusion

Further evidence for the role of nonbilayer structures in membrane fusion comes from the observation that fusion in model membranes is often accompanied by the appearance of "lipidic particles" in freeze-fracture micrographs, as shown in Fig. 4. These structures have been interpreted as inverted micelles sandwiched between two bilayers (Cullis and Hope, 1978; Verkleij ***et al.,*** 1980), although other interpretations, such as precursors to the cubic phase or interbilayer stalks, are possible. The main point is that these structures appear to represent connections between closely apposed bilayers, and that such connections are observed for lipid mixtures that support fusion. Disruption of the ***trans*** monolayers in an inverted micelle

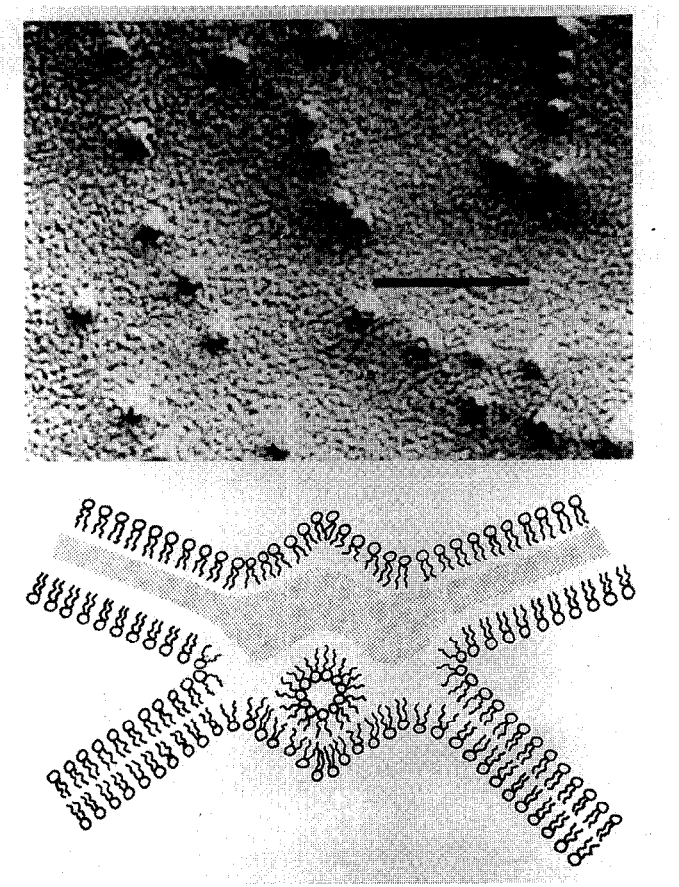

FIGURE 4 (Top) Freeze-fracture electron micrograph of 7.5 mol% soy phosphatidylinositol in soy phosphatidylethanolamine. Each particle is about 10 nm in diameter, and the bar represents 100 nm. (Bottom) The accompanying illustration indicates the proposed fracture plane around an inverted micelle formed at the contact point of two bilayers (Cullis and Hope, 1985).

intermediate would result in the formation of a fusion "pore," as illustrated in Fig. 5a. However, it should be noted that lipidic particles are not observed in all model systems that exhibit fusion, and, in systems where they are found, they are observed after at least some fusion events have already occurred.

Among other lipid structures that have been proposed as intermediates in membrane fusion events, of note is the "stalk" intermediate described by Chernomordik and co-workers (Markin *et al.,* 1984; Chernomordik et

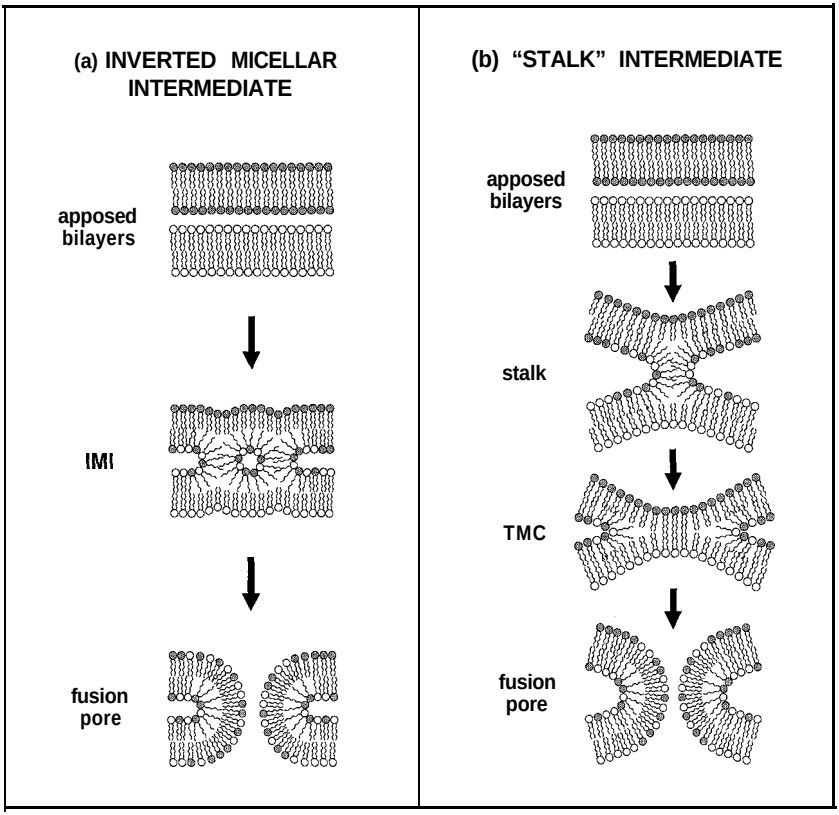

FIGURE 5 Proposed mechanisms of membrane fusion indicating key intermediate structures. (a) Two apposed membranes form an interlamellar micelle intermediate, or lipidic particle, which leads directly to the formation of an interlamellar attachment, or fusion pore. (b) The cis monolayers of the apposed membranes mix to form a stalk intermediate that expands radially to a TMC arrangement. Expansion of the TMC leads to rupture as a result of curvature and interstitial stresses and creates a fusion pore. (After Siegel, 1993.)

al., 1985). They proposed the existence of a semitoroidal lipid structure formed by coalescence of the outer monolayers of fusing membranes (Fig. 5b). The suggested mechanism for fusion involves an increase in the radius of the stalk to form a TMC, followed by rapid radial expansion of the TMC, giving rise to an area difference between the cis and trans monolayers. The resulting tension would lead to rupture of the TMC and membrane fusion.

A detailed theoretical comparison of the free energies associated with inverted micelle intermediates and stalks has been carried out by Siegel

(1993). It was shown that, for a wide variety of lipid compositions that exhibit fusion, the formation of a stalk intermediate is clearly energetically favored. However, Siegel showed that the expansion of the TMC as proposed would lead to energetically unfavorable structures due to the accompanying rapid increase in hydrophobic interstitial volume, and that rupture of a small-radius TMC to form a fusion pore is preferred. This is consistent with the observation that the addition of trace hydrocarbons greatly affects fusion rates by reducing the free energy associated with the hydrophobic interstices (Walter and Siegel, 1993; Walter et al., 1994). Based on these results, a modified mechanism for fusion involving a stalk intermediate leading rapidly to fusion pore formation has been proposed.

While these energy considerations are useful in comparing the lipid structures that have been proposed as fusion intermediates, there is an insufficient understanding of the actual energy-intensive events involved in the proposed fusion mechanisms, specifically, membrane rupture and pore formation. Other than the lipidic particles observed in some fusing systems, the lifetime of fusion intermediates are probably very short, making direct observation difficult or impossible. Furthermore, it is probable that fusion proteins affect the energies of these proposed structures or, possibly, give rise to different intermediate lipid structures in the fusion of biological membranes. As a result, it remains difficult to definitively assign the nonbilayer lipid structures that may be involved in membrane fusion.

IV. LIPID ASYMMETRY AND FUSION

The concentrations of various lipid species on the cytosolic and plasma or lumenal monolayers of biological membranes is highly regulated. Among the possible functional roles this transbilayer lipid asymmetry may have is to regulate membrane fusion through the influence of spontaneous monolayer curvature. For example, PE and PS are found mainly on the cytosolic monolayer of eukaryotic plasma membranes, and PC and SPM constitute the major lipids of the outer monolayer (Houslay and Stanley, 1982). Hope *et al.* (1983) demonstrated that liposomes approximating the lipid composition of the inner monolayer fused in the presence of Ca^{2+}, while liposomes modeled on the outer monolayer were resistant to fusion. In addition, erythrocytes that have lost this transbilayer lipid asymmetry are prone to fusion (Tullius *et al.,* 1989). Furthermore, myoblasts, which readily fuse to form myotubes, have higher concentrations of PE and PS on the outer monolayer of the plasma membrane than erythrocytes (Sessions and Horwitz, 1983), and the concentrations of these lipids increase prior to fusion (Santini *et al.*, 1990).

The influence of transbilayer lipid asymmetry on the propensity for membrane fusion can also be demonstrated in model systems. In lipid vesicles composed in part of lipids bearing weak acid or weak base headgroups, asymmetry can be induced by imposing a pH gradient across the membrane. Neutral lipid species can partition into the hydrophobic region of the bilayer much more readily than their charged counterparts, and accumulation of the charged form on one monolayer can be readily achieved (Hope and Cullis, 1987). If the lipid is one that promotes nonbilayer structures, its accumulation on the outer monolayer of LUVs will promote membrane fusion. Conversely, transport of such a lipid to the inner monolayer of vesicles will prevent fusion with other membranes.

A liposomal system exhibiting transbilayer lipid asymmetry-modulated membrane fusion was reported by Eastman *et al.* (1992). Vesicles composed of 10 mol% DOPA in DOPC-DOPE-PI (25 : 60 : 5) fused upon the addition of 8 mM Ca^{2+}. However, it was shown that, by lowering the external pH of these vesicles, DOPA was transported to the inner monolayer as illustrated in Fig. 6a, and that the rate of fusion upon the addition of Ca^{2+} was reduced as the DOPA was transported to the inner monolayer (Fig. 6b). A similar system using the protonatable aminolipid AL1, which rapidly redistributes across the bilayer in response to imposed pH gradients, has also been characterized (Bailey and Cullis, 1994). Vesicles composed of 5 mol% AL1 in EPC-DOPE-Chol (35 : 20 : 45) are resistant to fusion in the presence of a pH gradient (inside acidic) and give extensive fusion upon dissipation of the gradient in the absence of multivalent ions.

The ability to control the concentration of fusogenic lipids on the outer monolayers of model membrane vesicles has provided an additional means to explore the mechanics of membrane fusion. These systems also indicate a possible role for transbilayer lipid asymmetry in regulating fusion of biological membranes. The inhibition of viral glycoprotein-induced fusion upon addition of bilayer-stabilizing lipids, specifically lyso-PC, to the outer monolayers of biological membranes has already been demonstrated (Chernomordik *et* al., 1993). While it has been suggested that specific interactions between the exogenous lipid and fusion proteins may be responsible for these inhibitory effects (Gunther-Ausborn *et* al., 1995), the observance of similar effects in a variety of biological systems (Vogel *et* al., 1993; Yeagle *et al.,* 1994) indicates a more general role for the influence of asymmetrical monolayer curvature effects on the propensity for lipid bilayers to be transformed into nonbilayer intermediates leading to membrane fusion.

V. CONCLUSION

The study of lipid polymorphism and membrane fusion in liposomal systems has provided a useful approach to understanding the complex

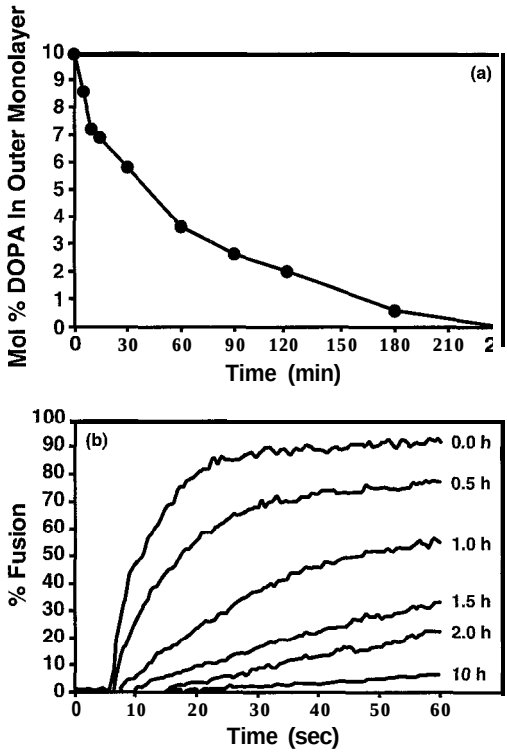

FIGURE 6 (a) Transbilayer transport of DOPA in large unilamellar vesicles composed of DOPC-DOPE-PI-DOPA (2.5:60:5: 10) by applying a pH gradient (inside pH = 7.5, outside pH = 4.0) at 37°(C. (b) Effect of transbilayer asymmetry of DOPA on fusion of the vesicles by the addition of 8 mM Ca^{2+}. (Reprinted with permission from Eastman, S. J., Hope, M. J., Wong, K. F., and Cullis, P. R. [1992]. Influence of phospholipid asymmetry on fusion between large unilamellar vesicles. Biochemistry 31, 4262-4268.)

process of fusion in biological systems. The obvious requirement for intermediate nonbilayer structures in the fusion of lipid bilayers, combined with an understanding of the phase behavior of lipid mixtures, has led to investigations that have clearly established that factors that promote nonbilayer lipid phases also promote fusion.

In our view, it is likely that membrane fusion in biological membranes also proceeds by the formation of transient nonbilayer intermediates. The role of membrane proteins can be postulated to provide the close apposition necessary for fusion to proceed and possibly also to directly induce the formation of the requisite nonbilayer lipid intermediates. However, there is no direct evidence as yet to support the hypothesis that biological membrane

fusion proceeds via the formation of a pore formed by nonbilayer lipids. Techniques that would detect the small fraction of membrane lipid likely to be involved in such intermediates are lacking, and the transient nature of such intermediates further mitigates against direct observation. It is possible that future studies using chemical cross-linking in biological or reconstituted "virosomal" systems containing fusion proteins may provide further information on the actual intermediates involved in membrane fusion.

List of Abbreviations

AL1	1,2-Dioleoyl-3-N,N-dimethylaminopropane
Chol	Cholesterol
CL	Cardiolipin
DOPA	Dioleoylphosphatidic acid
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DOPS	Dioleoylphosphatidylserine
EPC	Egg phosphatidylcholine
H_{II}	Inverted hexagonal phase
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
NMR	Nuclear magnetic resonance spectroscopy
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
SPM	Sphingomyelin
TMC	*Trans* monolayer contact

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