LIPID POLYMORPHISM, LIPID ASYMMETRY AND MEMBRANE FUSION

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INTRODUCTION

In this review we describe the intimate relation between lipid polymorphism and membrane fusion, and briefly introduce recent work on factors leading to asymmetric transbilayer distributions of lipid. Such asymmetric distributions may be expected to play a major regulatory role in the ability of membranes to fuse.

LIPID POLYMORPHISM AND FUSION

Two distinct processes involved in membrane fusion are first those giving rise to close apposition of membranes, and second the interbilayer process giving rise to the completed fusion event. Here we focus on the second event, observing that it is topologically impossible for fusion to proceed without some local departure from lipid bilayer organization at the fusion interface. This has naturally led to an examination of the abilities of lipids to adopt non-bilayer structure (lipid polymorphism) to determine whether such structures may play an intermediate role in the fusion event.

In order for lipid polymorphism to be related to membrane fusion, four conditions must be satisfied. First, lipids which can adopt non-bilayer organization must be present in membranes. Second, factors which regulate or stimulate fusion in vivo must regulate the structural properties of appropriate lipid dispersions in a consistent manner. Third, the structural transitions demonstrated by lipids must provide logical intermediate structures. Finally, the structural intermediate available must be compatible with a non-leaky fusion event. In the next section we review studies supporting these conditions and subsequently relate these observations to fusion of selected model and biological membranes.

Lipid Polymorphism and Factors Regulating the Structural Preferences of Lipids

The ability of aqueous dispersions of lipids to assume a variety of structures on hydration is well-characterized and has been extensively reviewed elsewhere (Cullis & de Kruijff, 1979, Cullis et al., 1985). Four major points can be made. First, individual species of lipids found in

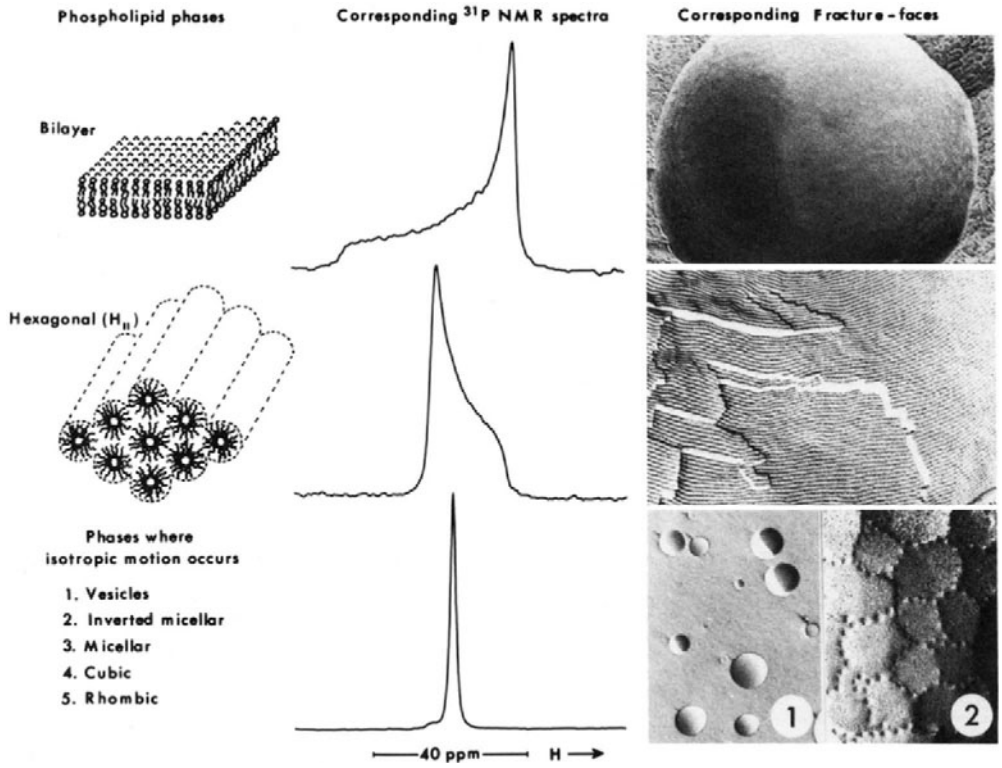

Figure 1. ^{31}P NMR and freeze-fracture characteristics of phospholipids in various phases. The bilayer ^{31}P NMR spectrum was obtained from aqueous dispersions of egg yolk phosphatidylcholine and the hexagonal (H_{II}) phase spectrum from phosphatidylethanolamine (prepared from soybean phosphatidylcholine). The ^{31}P NMR spectrum representing isotropic motion was obtained from a mixture of 70 mol% soya phosphatidylethanolamine and 30% egg yolk phosphatidylcholine after heating to 90°C for 15 min. All preparations were hydrated in 10 mM Tris-acetic acid (pH 7.0) containing 100 mM NaCl and the ^{31}P NMR spectra were recorded at 30°C in the presence of proton decoupling. The freeze-fracture micrographs represent typical fracture faces obtained from bilayer and H_{II} phase systems as well as structures giving rise to isotropic motional averaging. The bilayer configuration (total erythrocyte lipids) gives rise to a smooth fracture face, whereas the hexagonal (H_{II}) configuration is characterized by ridges displaying a periodicity of 6 to 15 mm. Common conformations that give rise to isotropic motion are represented in the bottom micrograph: (1) bilayer vesicles (∿100 nm diameter) of egg phosphatidylcholine prepared by extrusion techniques and (2) large lipid structures containing lipidic particles. This latter system was generated by fusing SUVs composed of egg phosphatidylethanolamine and 20 mol% egg phosphatidylserine which were prepared at pH 7 and then incubated at pH 4 for 15 min to induce fusion.

membranes can adopt either the bilayer or hexagonal H_{II} phase indicated in Fig. 1. As indicated in Table 1, a large proportion of membrane lipids either adopt the hexagonal (H_{II}) phase on hydration at physiological temperatures or induce hexagonal structure in mixed lipid systems. The most notable examples are (eukaryotic) phosphatidylethanolamines (PE's) as well as cholesterol. Cholesterol has a remarkable ability to induce H_{II} phase organization in mixed lipid systems. Non-bilayer lipids such as PE can of course be stabilized in a bilayer structure by the presence of bilayer-preferring lipids such as phosphatidylcholine (PC), phosphatidylserine (PS) or sphingomyelin (SPM). It is usually found that between 20 and 50 mol% of the bilayer-preferring lipids is required to maintain a net bilayer organization when mixed with H_{II}-preferring lipids such as PE.

The second point is that the structural preferences of these pure and mixed lipid systems are very sensitive to a variety of factors known to regulate fusion events in vivo. Perhaps the clearest example is Ca^{2+}, which can trigger bilayer to H_{II} transitions in multilamellar vesicle (MLV) systems composed of pure cardiolipin (CL), and as shown in Fig. 2, in mixtures of PE with the negatively charged lipids PS, phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol (PI) or CL (Cullis et al., 1985) as well as PE-PS-cholesterol and PE-PS-cholesterol systems (Tilcock & Cullis, 1981; Bally et al., 1983; Tilcock et al., 1984; Cullis et al., 1985). This ability extends to MLV systems with lipid compositions corresponding to the erythrocyte inner monolayer (Hope & Cullis, 1979). Other examples of factors which can trigger bilayer to H_{II} transitions include low pH as observed for systems containing PS (Hope & Cullis, 1980), PA (Farren et al., 1983) and PS-PE (Tilcock & Cullis, 1981); high ionic strength as observed for PE-PS-cholesterol systems (Bally et al., 1983); and proteins such as cytochrome c (de Kruijff & Cullis, 1980) and gramicidin (van Echteld et al., 1982). A particularly illustrative example of the correlation between in vitro fusion processes and the ability of endogenous lipids to adopt H_{II} phase organization is given by the action of lipid soluble "fusogens" (Ahkong et al., 1973) on the structural preferences of MLV's composed of erythrocyte membrane lipids. As shown in Fig. 3, fusogens induce H_{II} phase structure in such dispersions, whereas structurally related non-fusogens do not. A strong correlation therefore exists between factors known to promote fusion in vivo and in vitro and their ability to promote H_{II} phase organization in various model MLV systems.

A third point concerns the relation between the bilayer to hexagonal H_{II} transition and membrane fusion processes. Unsaturated PE dispersions undergo a bilayer to H_{II} transition as the temperature is raised, which usually occurs in the region of 10°C for PE of eukaryotic origin. ^{31}P-NMR results (Cullis et al., 1980) indicate that the inverted cylinders characteristic of the H_{II} phase form from closely apposed bilayers which fuse together, trapping some of the interbilayer water into the inverted lipid cylinders. The analogy to fusion is clear.

The fourth point concerns the fact that the hexagonal phase itself is clearly not an attractive fusion intermediate, in that formation of such a macroscopic structure would be expected to lead to extensive membrane disruption and leakage. However, mixtures of bilayer and H_{II}-preferring lipids often give rise to 'lipidic particle' structure visualized by freeze-fracture electron microscopy (see Fig. 4). Available evidence (Verkleij, 1984) indicates that these particles correspond to inverted lipid micelles formed between intersecting bilayers, and that these particles are intermediates between bilayer and H_{II} phase structure. This is indicated in freeze-fracture studies (Verkleij et al., 1980) where the particles are observed to line up in rows as apparent precursors to the

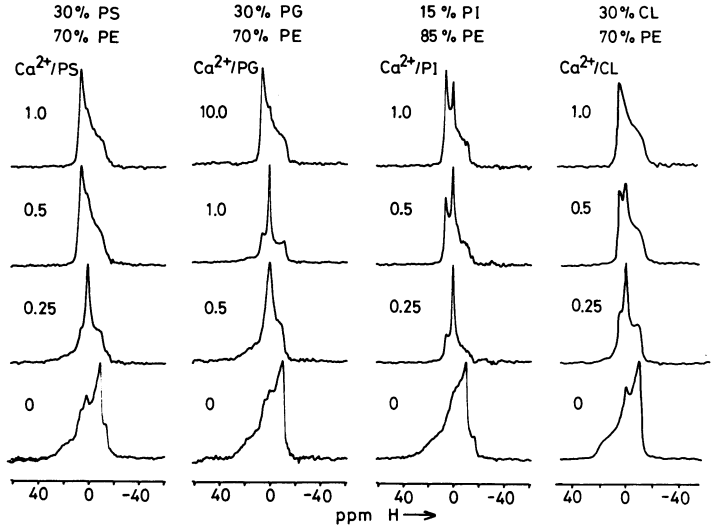

Figure 2. ^{31}P NMR spectra arising from mixtures of acidic phospholipids with soya phosphatidylethanolamine (a polyunsaturated PE derived from soya PC) in the presence of various molar ratios of Ca^{2+}. All samples were prepared from 50 µmol total phospholipid hydrated in MLV form by vortex mixing. The Ca^{2+} was added as aliquots from a 100 mM stock solution.

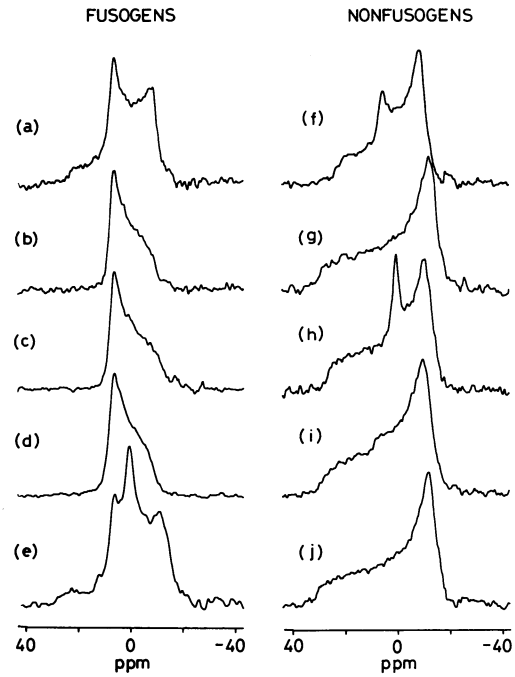

Figure 3. 81.0 MHz ^{31}P NMR spectra arising from reconstituted human erythrocyte lipids at 37°C in the presence of equimolar (with respect to phospholipid) fusogens (a-e) and non-fusogens (f-j). (a) Myristic acid, (b) palmitoleic acid, (c) retinol, (d) oleic acid, (e) glycerol monooleate, (f) palmitic acid, (g) triolein, (h) retinol palmitate, (i) stearic acid and (j) glycerol monostearate. Each sample consisted of 30 µmol of phospholipid, dispersed in 0.6 ml of buffer B (see Methods) and 10% $^{2}H_2O$, 20 µmol of Ca^{2+} were added, following lipid dispersion. Accumulated free induction decays were obtained as described in the legend to Fig. 1.

Table 1. Polymorphic Phase Preferences of Liquid Crystalline, Unsaturated Lipids

Lipid	Phase Preferences	
	Physiological Conditions	Other conditions
Phosphatidylcholine	L	H_{II}, low hydration and high temp
Sphingomyelin	L	
Phosphatidylethanolamine	H_{II}	L, pH $\geq$8.5, low temp
Phosphatidylserine	L	H_{II}, pH$\leq$3.5
Phosphatidylglycerol	L	H_{II}, high temp, high salt conc.
Phosphatidylinositol	L	
Cardiolipin	L	H_{II}, divalent cations, pH$\leq$3 high salt
Phosphatidic acid	L	H_{II}, divalent cations, pH$\leq$3.5 high salt
Monoglucosyldiglyceride	H_{II}	
Diglucosyldiglyceride	L	
Monogalactosyldiglyceride	H_{II}	
Digalactosyldiglyceride	L	
Cerebroside	L	
Cerebroside sulfate	L	
Ganglioside	M	
Lysophosphatidylcholine	M	
Cholesterol		Induces H_{II} phase in mixed lipid system
Unsaturated fatty acids		Induce H_{II} phase

Note: L = Lamellar, H_{II} = Hexagonal, M = Micellar

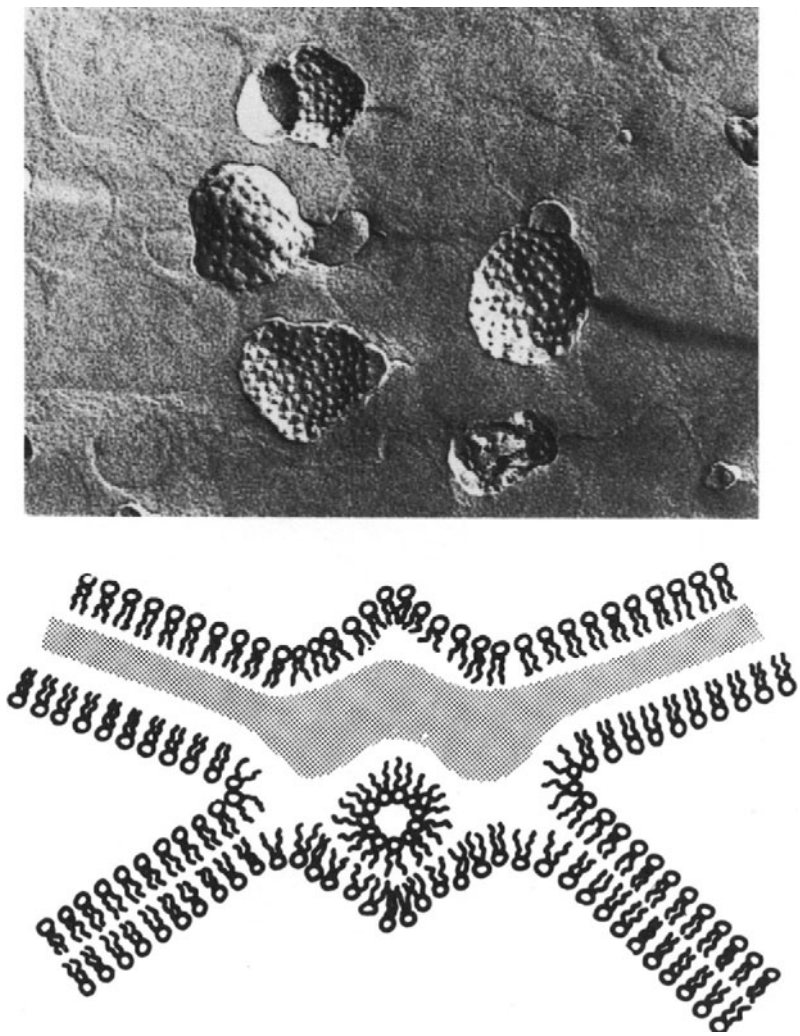

Figure 4. Freeze-fracture micrograph of lipidic particles induced by Ca^{2+} in a lipid system consisting of cardiolipin and soya phosphatidylethanolamine in the molar ratio of 1:4 (magnification X80,000). A model of the lipidic particle as an inverted micelle is depicted below the micrograph. The shaded area represents the fracture region.

inverted cylinders of the H_{II} phase. These inverted micelles are also attractive fusion intermediates, as they represent local, discrete departures from bilayer structure which are promoted by the same factors as promote bilayer to H_{II} transitions and which would not be expected to result in undue membrane disruption.

Fusion of Model and Biological Membranes

The results summarized above reveal a strong correlation between the ability of factors to promote membrane fusion and their ability to induce H_{II} phase or inverted micellar structure in MLV systems, as well as similarities between bilayer to non-bilayer transitions in model systems and membrane fusion events. This suggests that factors promoting H_{II} or inverted micellar lipid structure should promote fusion between model and biological membranes. Here we examine the validity of this prediction for unilamellar model vesicles, cell fusion induced by lipid-soluble fusogens and the fusion process involved in exocytosis.

As indicated in the previous section, a variety of MLV systems can be induced to undergo bilayer to H_{II} transitions by addition of Ca^{2+} or incubation at lower pH values. It would therefore be predicted that unilamellar vesicles with similar lipid compositions should first fuse to form larger vesicle systems before the large-scale aggregation which eventually gives rise to H_{II} phase formation. This prediction has been verified for small (sonicated) vesicle systems composed of PE-PS, PE-PA, PE-PG, PE-PI and PE-CL systems (Hope et al., 1983) as well as PC-CL systems (Verkleij et al., 1979) and an example for PE-PS systems is shown in Fig. 5. Similarly, it has been shown that incubation at pH <5 results in fusion of PE-PS and PE-PA vesicles (Hope et al., 1983). Fusion of large unilamellar vesicles (LUV's) composed of erythrocyte inner monolayer lipids (PC-PE-PS-cholesterol) in response to Ca^{2+} is also illustrated in Fig. 5.

An interesting feature of the Ca^{2+}-induced fusion of small unilamellar vesicles (SUV's) of PE-PS concerns the appearance of lipidic particles, often localized to regions corresponding to the fusion interface. Similar features are observed for other fusion systems (Verkleij, 1984; Hope et al., 1983) and it was originally proposed (Verkleij et al., 1979) that these particles correspond directly to inverted micellar fusion intermediates. However, further studies (Duzgunes et al., 1982) indicate that these lipidic particles are either formed subsequent to the actual fusion event or not observed at all, as in the case for the fusion of 'inner monolayer' LUV's (Fig. 5). It has been suggested (Siegel, 1984) that the actual fusion intermediates have a very short lifetime, which could preclude detection by freeze-fracture techniques.

With regard to lipid-soluble fusogens, the theory that fusion proceeds via a non-bilayer 'inverted' intermediate would predict that such agents should promote fusion at membrane concentrations sufficient to promote H_{II} phase formation. As previously indicated a variety of lipid-soluble fusogens do induce H_{II} phase formation in MLV systems composed of total erythrocyte lipids (as well as erythrocyte 'ghost' membranes) (Hope & Cullis, 1981), whereas chemically related non-fusogens do not. Further, the membrane concentrations of oleic acid (Cullis & Hope, 1978) and glycerol monooleate (Hope & Cullis, 1978) required to induce detectable H_{II} organization (approximately equimolar with respect to phospholipid) also correspond closely to the membrane concentrations required to induce erythrocyte fusion (Hope & Cullis, 1981).

The possible role of non-bilayer intermediates in fusion events involved in exocytosis has been investigated (Nayar et al., 1982) for the chromaffin granule system, which undergoes Ca^{2+}-dependent fusion with

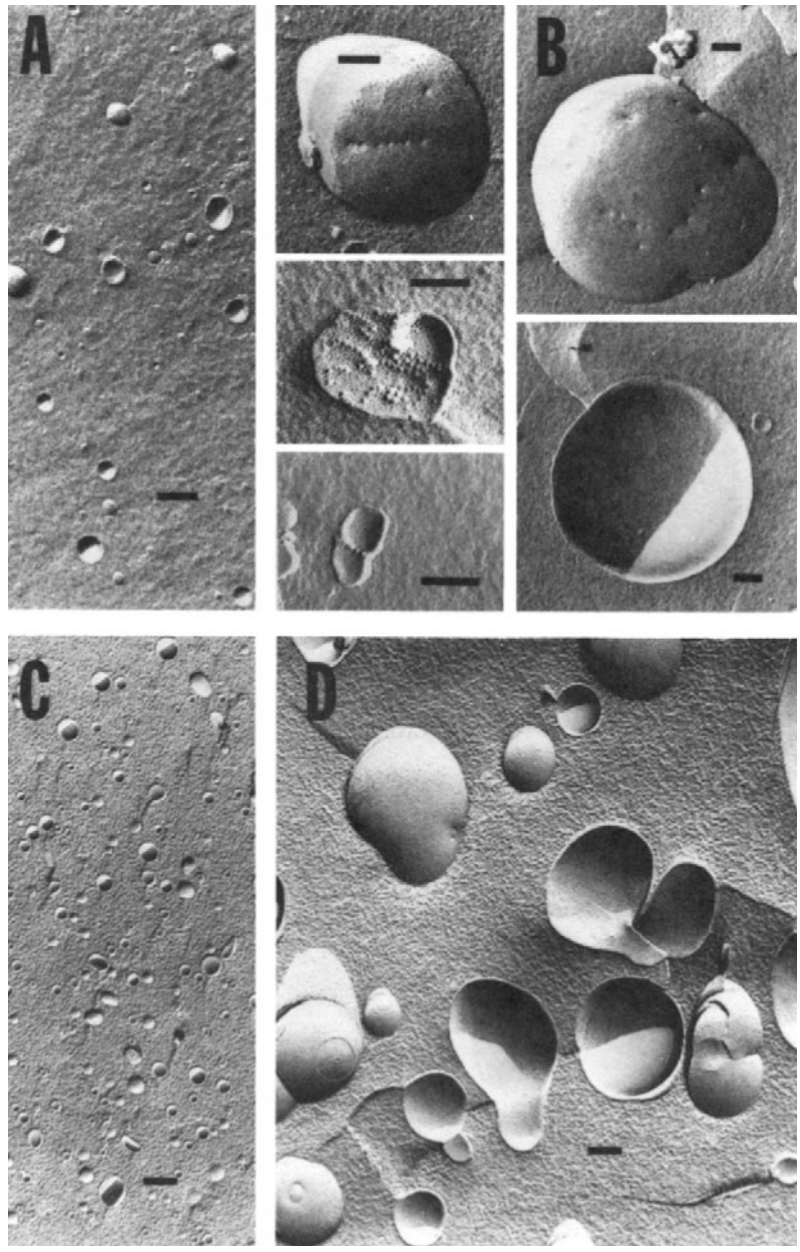

Figure 5. Freeze fracture micrographs of sonicated vesicle composed of PE-PS (A) were dialysed against Ca^{2+} to induce fusion (B). In (C) sonicated vesicles of extracted erythrocyte lipids, reconstituted in proportions corresponding to the inner monolayer lipid composition (PC/PE/PS/SPM cholesterol in the molar ratio 0.15:0.47:0.28:0.10:1), were dialysed against Ca^{2+} to induce fusion (D).

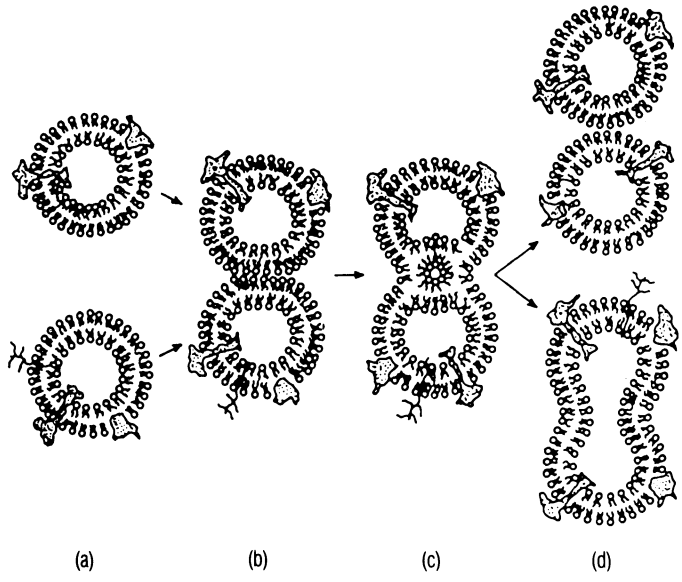

Figure 6. Membrane fusion proceeding via an inverted micellar intermediate.

the plasma membrane of chromaffin cells (Trifaro, 1977). This fusion event must initially involve granule fusion with the plasma membrane inner monolayer. By analogy with the erythrocyte membrane, it is likely that the inner monolayer is composed primarily of PE and PS. It may therefore be suggested that the presence of Ca^{2+} could trigger fusion by enhancing the proclivity of the inner monolayer for inverted micellar or H_{II} phase structure, resulting in fusion with closely apposed granule membranes. If this is the case, Ca^{2+}-stimulated fusion between granules and PE-PS liposomes should proceed readily. This has been observed for PE-PS SUV's incubated with chromaffin granules (Nayar et al., 1982), where levels of Ca^{2+} well below those required to induce granule-granule fusion induce massive PE-PS-granule fusion, with associated release of contents.

These and related (Cullis et al., 1985) observations provide strong evidence for a role of an inverted non-bilayer lipid organization in membrane fusion. The resulting fusion model we have proposed (Cullis & Hope, 1978) is illustrated in Fig. 6, where two membrane-bound systems are brought into close apposition (possibly a protein-mediated event), and an interbilayer inverted micelle (or short inverted cylinder) formed. Reversion of the intrabilayer inverted structure to bilayer structure could then either result in reformation of separate vesicles or a completed fusion event. It may be noted that the situation of Fig. 6(c), representing partially fused membranes, can apparently be a relatively stable situation, and may correspond to situations of arrested fusion noted for tight junctions (Kachar & Reese, 1982).

LIPID ASYMMETRY

Recently, our interest in fusion phenomena has been centred around the properties of lipid systems which exhibit asymmetric transbilayer distributions of lipids such as are observed in biological membranes. Such asymmetry poses interesting problems for fusion. For example, in the erythrocyte membrane and, apparently, other plasma membranes (Op den Kamp, 1979) the predominance of PC and sphingomyelin in the outer monolayer and PE and PS in the inner monolayer would suggest that the inner monolayer is more "fusogenic" than the outer. The question then arises as to whether the presence of bilayer lipids in one monolayer can influence the ability of the other to undergo fusion. While a system exhibiting asymmetric transbilayer distributions of phospholipids has not yet been achieved, LUV systems exhibiting asymmetric distributions of lipid which exhibit weak acid (e.g. fatty acids) and weak base (e.g. stearylamine) characteristics have been achieved (Hope and Cullis, 1987). These systems arose from initial speculations that lipid asymmetry may be related to ion gradients present across membranes, giving rise to membrane potential ($\Delta\psi$) and pH gradients (ΔpH). Here we summarize the methods involved in generating asymmetry due to transmembrane pH gradients and the characteristics of the resulting systems.

Influence of ΔpH (acidic inside) on the Transbilayer Distribution of Amino Lipids

Experiments were aimed at examining the influence of pH gradients on the transbilayer distributions of the amino-containing lipids, stearylamine and sphingosine. The reasoning behind these experiments is based on the abilities of water-soluble weak bases such as methylamine to permeate vesicle membranes (in the neutral form) to achieve transmembrane (aqueous) concentrations obeying the Henderson-Hasselbach relation.

$$\frac{[AH^+]_i}{[AH^+]_o} = \frac{[H^+]_i}{[H^+]_o} \qquad (1)$$

where AH^+ refers to the protonated amine and the subscripts i and o refer to the interior and exterior of the vesicle, respectively. In the case of amino lipids such as stearylamine and sphingosine, their localization to the membrane should result in similar redistributions where the transbilayer location of the protonated amine reflects the interior and exterior proton concentrations at the membrane interfaces.

These predictions were tested employing DOPC LUVs containing 5 mol% stearylamine. As shown in Fig. 7A when no transmembrane ΔpH is present (pH = 8.5 inside and out), the TNBS labeling of stearylamine proceeded to completion as assayed spectrophotometrically. This is consistent with a rapid redistribution of inner monolayer stearylamine to the outer monolayer as the outer monolayer stearylamine is depleted on reaction with TNBS. However, as shown in Fig. 7B, when a pH gradient is present (interior pH 5.0, exterior pH 8.5) no labeling of stearylamine can be detected. The subsequent addition of detergent to solubilize the vesicles resulted in complete labeling within 20 s. These results are clearly consistent with a localization of stearylamine to the inner monolayer in response to the pH gradient (interior acidic).

Influence of ΔpH (inside basic) on the Transbilayer Distribution of Fatty Acids

The ability of pH gradients to markedly influence the transmembrane distributions of these amine-containing lipids would imply that the transmembrane distributions of lipids which are weak acids, such as fatty acids, should also be strongly dependent on transmembrane pH gradients. Assuming that the neutral (protonated) form can permeate the membrane, this will result in transmembrane gradients according to

$$\frac{[RCOO^-]_i}{[RCOO^-]_o} = \frac{[H^+]_o}{[H^+]_i} \qquad (2)$$

where $RCOO^-$ refers to the unprotonated fatty acid. In vesicles exhibiting pH gradients where the interior is basic, fatty acids should therefore move to the interior monolayer. In order to determine whether this is the case, we employed an assay based on the well-documented ability of fatty acid-free BSA to deplete membranes of free fatty acid.

As shown in Fig. 8A for DOPC vesicles containing 10 mol% oleic acid, only a small fraction of the oleic acid can be removed by BSA when a transmembrane pH gradient (interior pH = 10.0, exterior pH = 7.0) is present. Alternatively, when the interior and exterior pH values are the same (Fig. 8B), 95% of the oleic acid elutes with BSA rather than the LUVs. This is consistent with a localization of oleic acid to the inner monolayer in the presence of a proton gradient (interior basic). Similar results were observed for stearic acid (results not shown).

These systems clearly offer attractive models for the influence of lipid asymmetry on fusion. For example, preliminary studies show that oleic acid can be localized to the outer monolayer when the vesicle interior is acidic. In vesicles with suitable lipid compositions the presence of this fusogen in the outer monolayer would be expected to promote fusion. Alternatively, systems with basic interiors would be

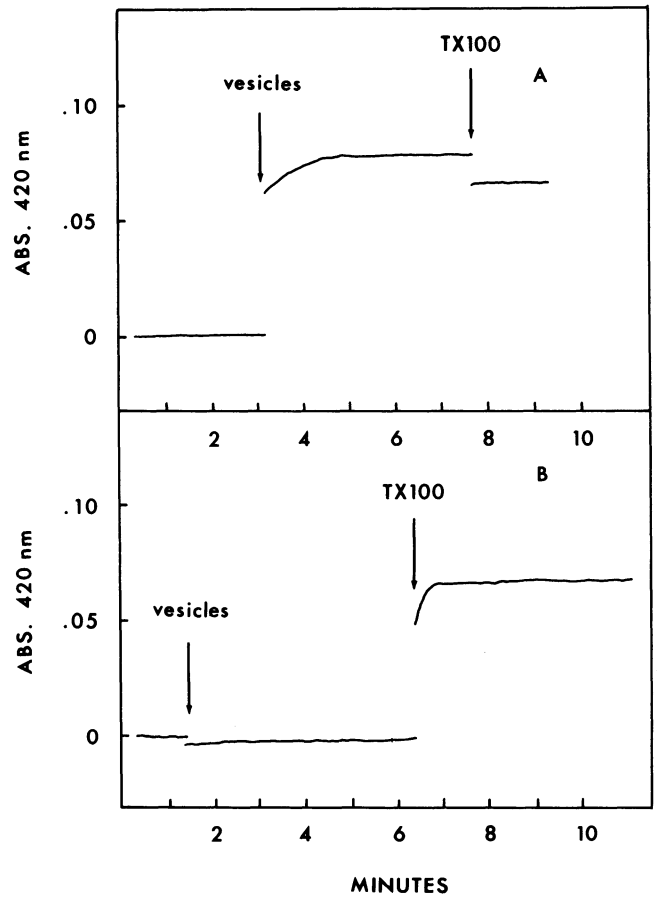

Figure 7. Influence of proton gradients (inside acidic) on the availability of stearylamine to TNBS in DOPC vesicles. (A) DOPE vesicles containing 5 mol% stearylamine. These LUVs do not exhibit a proton gradient (pH = 8.5) at the vesicle interior and exterior. Absorbance at 420 nm is monitored throughout the incubation. Following the addition of vesicles, Triton X-100 (TX100) is employed to solubilize the membranes and expose all available amines. A slight decrease in absorbance is observed due to dilution and solubilization effects. (B) DOPC LUVs exhibit a pH gradient (pH 5.0 inside and pH 8.5 outside).

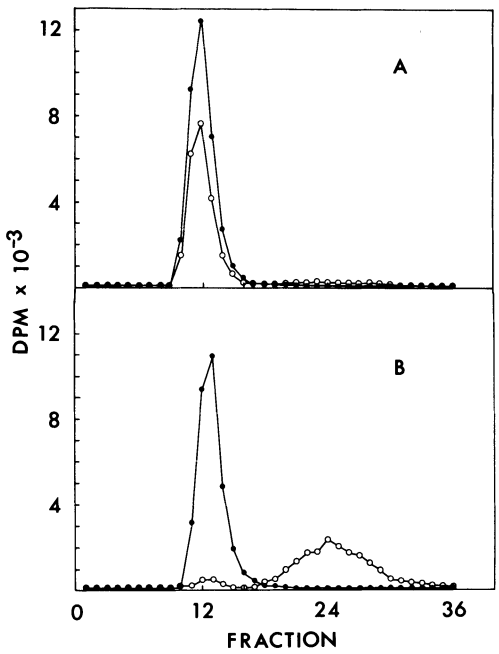

Figure 8. The effect of a membrane proton gradient on the ability of BSA to extract [^{14}C]oleic acid from a gel filtration column. (A) Vesicles contain 10 mol% oleic acid and exhibit an internal pH of 10.0. The external buffer is at pH 7.0. The peak represents vesicles (•) and oleic acid (o). BSA elutes between fractions 20 and 30. (B) Elution profile of vesicles with no proton gradient (pH 7.0 inside and outside). The majority of the oleic acid (o) now elutes with BSA, whereas the position of the vesicle peak (•) is unchanged.

rendered less susceptible to fusion events. These hypotheses are currently being investigated.

CONCLUDING REMARKS

In this article we have summarized evidence supporting a role of non-bilayer lipid structures in membrane fusion, and outlined initial studies leading to vesicle systems with asymmetric transbilayer distributions of lipid which more accurately reflect the biological situation. With regard to polymorphism, it may be concluded that lipid which can adopt non-bilayer structures are present in high proportions in membranes, that factors which initiate fusion in vivo induce non-bilayer (H_{II} phase) structure in appropriate model membrane systems, that the structures of H_{II} phase and inverted micellar organizations are consistent with an intermediary role in fusion and that the inverted micelle would provide an intermediate structure which may not give rise to induce membrane disruption. Some theoretical support for such a role of non-bilayer lipid structures is now available (see Siegel, D; this volume).

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