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# Interactions of Liposomes with Planar Bilayer Membranes

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**Summary.** The adhesion to horizontal, planar lipid membranes of lipid vesicles containing calcein in the aqueous compartment or fluorescent phospholipids in the membranes has been examined by phase contrast, differential interference contrast and fluorescence microscopy. With water-immersion lenses, it was possible to study the interactions of vesicles with planar bilayers at magnifications up to the useful limit of light microscopy. In the presence of 15 mM calcium chloride, vesicles composed of phosphatidylserine and either phosphatidylethanolamine or soybean lipids adhere to the torus, bilayer and lenses of planar bilayers of the same composition. Lenses of solvent appear at the site where vesicles attach to decane-based bilayers and lipid fluorophores move from the vesicles to the lenses. Because the calcein contained in such vesicles is not released, we interpret this as indicating fusion of only the outer monolayer (hemifusion) of the vesicles with the decane lenses. In the case of squalene-based black lipid membranes (BLMs), in contrast, vesicles do not nucleate lenses but they apparently do fuse with the torus at the bilayer boundary. Interactions leading to hemifusions between vesicles and planar membranes thus occur predominantly in regions where hydrocarbon solvent is present. Osmotic water flow, induced by addition of urea to the compartment containing vesicles, causes coalescence of lenses in decane-based BLMs as well as coalescence of the aqueous spaces of the vesicles that have undergone hemifusion with the lenses. We did not observe transfer of the aqueous phase of vesicles to the *trans* side of either decane- or squalene-based planar membranes; however, we cannot rule out the possibility particularly in the latter case, that rupture of the planar membrane may have been an immediate result of vesicle fusion and thus precluded its detection.

**Key Words** phospholipid · membrane fusion · BLM · membrane–membrane interactions · lipid vesicles

## Introduction

The mechanism of fusion of lipid vesicles with planar lipid membranes has been studied as a possible paradigm of the fusion of biological membranes [2, 4–6, 13, 15, 23–25]. Fusion has been monitored by the incorporation of protein channels from lipid vesicles into planar membranes [2, 4–6, 13, 15, 23, 25] and by detection of liposomal contents on the opposite side of the planar membrane [24]. Calcium or magnesium ion is required to cause adhesion between vesicles and planar bilayers [2, 4–6, 25] containing acidic lipids. This adhesion is stable upon removal of divalent ions [2]. Fusion does not occur with divalent ions in the absence of an osmotic stress [2, 4–6, 13, 15, 23–25]. If an osmotic stress is placed on adherent vesicles such that they swell, fusion (incorporation of channels, transfer of contents) occurs. This suggests a mechanism of fusion that occurs in two steps: an adhesion of membranes and an osmotically induced rupture at the site of adhesion.

To further investigate possible fusion mechanisms, we have examined the interactions of fluorescent vesicles with planar lipid membranes [14, 22] by light microscopy. In particular, we have used large unilamellar vesicles labeled with fluorescent lipids or loaded with partially self-quenched concentrations of the fluorescent dye, calcein [1, 11]. Distinct differences in the adhesion of lipid vesicles to solvent-containing and solvent-free planar bilayers were observed. Evidence for hemifusion, but not for fusion, was obtained.

## Materials and Methods

Brain phosphatidylserine, egg phosphatidylethanolamine, bacterial phosphatidylethanolamine and N-(lissamine) rhodamine B sulfonyldioleoylphosphatidylethanolamine were obtained from Avanti Polar Lipids. Dioctadecylindocarbocyanine was obtained from Eastman Kodak. Soybean lipid (Type IV from Sigma) was washed with acetone to remove neutral fats [10]. Calcein was obtained from Hach Chemical and was purified over lipophilic LH-20 Sephadex [19]. Decane (Eastman Kodak) and squalene (Sigma) were purified by silica gel column chromatography. Fluorescent latex beads were from Polysciences.

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Membranes were formed on a horizontal aperture separating a closed, lower compartment from a larger, upper compartment. The bilayer chamber, which was the size of a microscope slide, was mounted on the stage of a Nikon Optiphot microscope equipped for phase contrast, differential interference contrast and fluorescence microscopy. A diagram of the chamber and a complete description of the procedures will appear elsewhere [18].

Membranes were painted under a 10× dry objective and examined under a 40×, 0.65 NA (Nikon) or 100×, 1.2 NA (Leitz) water-immersion lens using a 22.5× eyepiece-body tube combination.

Large unilamellar vesicles (LUVs) were made by freeze-thawing and dialysis methods similar to those described previously [17]. Initially we used a 4:1 weight mixture of soybean lipid and phosphatidylserine, but a combination of phosphatidylserine/(egg or bacterial) phosphatidylethanolamine, 1:1, was used for most of the experiments described here. Calcein-loaded LUVs were formed by sonicating lipid in 200 mM calcein and saturated KCl to form small unilamellar vesicles. These were freeze-thawed three times in a dry ice-ethanol bath and then dialyzed against 50 mM calcein. External calcein was removed by diluting the resulting suspension approximately 50-fold with 150 mM KCl/10 mM MOPS, pH 7.4 (MbK) and centrifuging. The pellet was collected and resuspended in a solution of the same composition. LUVs containing lipid fluorophores were formed by sonicating lipid in saturated KCl followed by freeze-thawing and dialysis against 150 mM KCl/10 mM MOPS, pH 7.4. Sucrose-loaded LUVs were formed by slow hydration of lipid with 300 mM sucrose [20]. The concentration of LUV suspensions was adjusted to approximately 1–2 mg lipid/ml. Because of the presence of lipid fluorophore, the size of LUVs was easily determined by fluorescence microscopy. LUVs produced by freezing and thawing were larger than 0.5 $\mu$m in diameter. Smaller vesicles, had they been present, would have been visible under our conditions. Slow-hydration LUVs were usually larger than 5 $\mu$m in diameter.

Decane-based BLMs were painted from a 4–5% solution of lipid in decane. Phosphatidylserine(brain)/phosphatidylethanolamine(egg or bacterial), 1:1 in chloroform was freed of solvent under $N_2$ and placed under oil pump vacuum for 1 hr. Decane was added to the lipid, which dissolved completely after 1 hr under $N_2$. Squalene-based BLMs were painted from a 4% solution of lipid (phosphatidylserine/bacterial phosphatidylethanolamine, 1:1). Squalene was added to dried lipid, which dissolved completely after 3–4 hr under $N_2$. Membranes were painted in MbK and, after thinning, a solution of that composition containing 15 mM calcium chloride was perfused into the chamber. Vesicles (5–10 $\mu$l) at approximately 0.1–0.2 mg/ml were added above the membrane and allowed to settle onto it. Free vesicles and external dye were then removed by perfusion. This procedure produces a high density of vesicles adhering to the planar membrane.

Vesicles were osmotically stressed by perfusing an isotonic (300 mM) or hypertonic (450 mM) solution of urea into the *cis* (upper) chamber. In the latter case, vesicles bound to the membrane transiently shrink, but because urea is much more permeant than potassium, urea (with accompanying water) diffuses into the vesicles and in both cases the result is vesicle swelling. An alternative procedure, which had the same net effect and, which was sometimes used because of its simplicity, was addition of a small volume of urea solution (concentration ≥ isosmolar) to the upper chamber. Because the urea solution is denser than the membrane buffer, it sinks and displaces the latter from the upper surface of the membrane where the vesicles are attached.

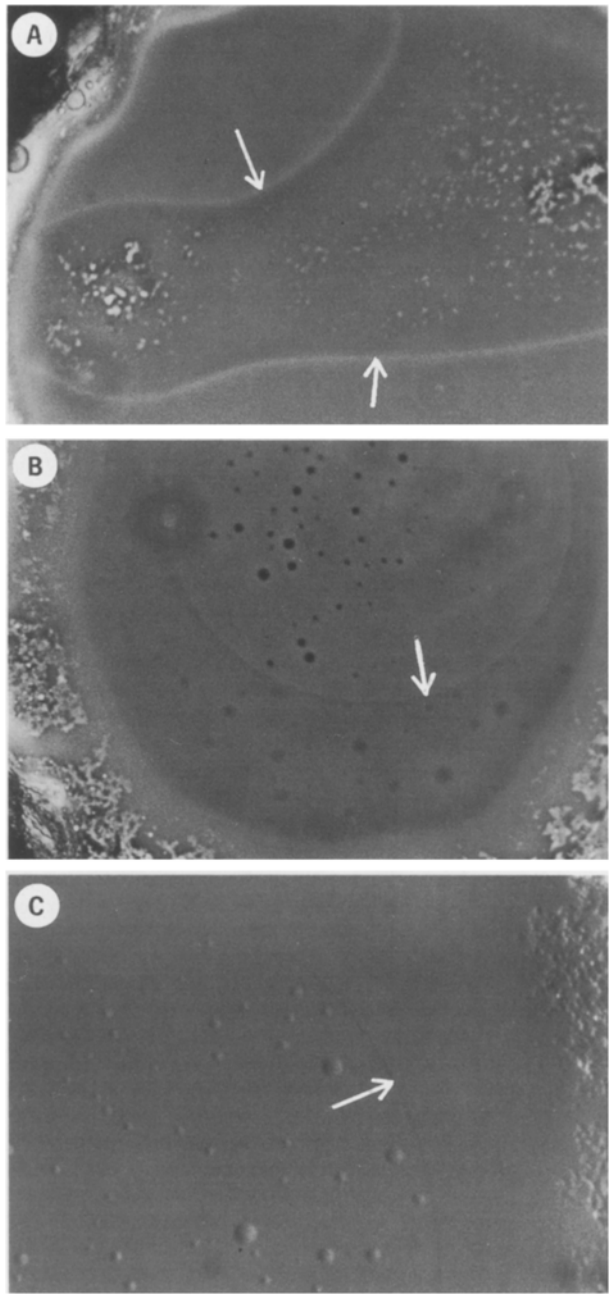


**Fig. 1.** Phase contrast and differential interference contrast (DIC) photomicrographs of decane-based BLMs. (*A*) Membrane thinning as observed with phase contrast optics. (*B*) Phase contrast photomicrograph of incompletely thinned BLM (BLM was painted in the presence of 15 mM calcium chloride). (*C*) Differential interference contrast photomicrograph of incompletely thinned BLM. Only completely thinned region contains lenses. The arrows indicate the edge of the bilayer. The body of the arrow has been placed in the bilayer region with the arrowhead at its boundary. BLMs were painted from a 4.5% solution of PS : soybean lipid 1 : 4 in decane. For (*A*), the BLM was painted and allowed to thin in 150 mM KCl, 10 mM MOPS, pH 7.4. In (*B*) and (*C*), the BLM was painted in 150 mM KCl, 10 mM MOPS, pH 7.4, 15 mM calcium chloride. The width of the phase contrast photomicrographs corresponds to 460 $\mu$m and that of the DIC photomicrograph to 230 $\mu$m

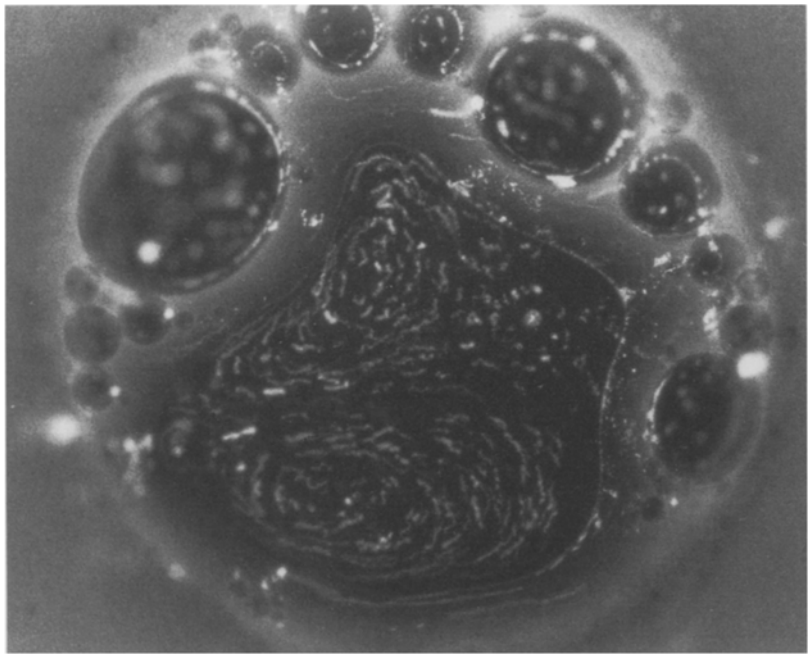

**Fig. 2.** Fluorescence photomicrograph of calcein-loaded, large unilamellar vesicles (LUVs) adhering to a decane-based BLM. LUVs bind to the bilayer and the torus and accumulate at the Plateau border. The bilayer region of the BLM is that with the whorls. Streaks are seen in this long exposure because of movement, due to perfusion, of adherent LUVs in the plane of the bilayer. The circular regions in the torus are bubbles of aqueous phase, which sometimes form during painting of the BLM. Both sides of such large bubbles thin, yielding a smaller version of the bilayer region of the BLM. The BLM was painted (4.5% soybean lipid : PS, 4 : 1 in decane) and allowed to thin in 150 mM KCl, 10 mM MOPS, pH 7.4. Then 150 mM KCl, 10 mM MOPS, pH 7.4, 15 mM calcium chloride was perfused into the upper chamber and LUVs were added above the membrane. After LUVs settled onto the membrane and adhered, unbound LUVs were perfused out. LUVs (soybean lipids : PS, 4 : 1) were prepared by freeze-thawing in 50 mM calcein and saturated KCl followed by dialysis against 50 mM calcein. The BLM diameter (including torus) is 500 $\mu$m

## Results

### Decane-Based BLMs

Figure 1*A* is a phase contrast photomicrograph of a typical BLM, painted from a 4–5% solution of lipid in decane, in the process of thinning. A mass of bulk decane is seen being drawn into the torus that will surround the bilayer. Incomplete thinning is easily seen by phase microscopy (Fig. 1*B*) as a darker area of the film and by differential interference contrast (DIC) microscopy as an area lacking lenses (Fig. 1*C*). Decane-based BLMs, spread in solutions lacking divalent cations, rarely showed evidence of incomplete thinning, which, however, was common when membranes thinned in the presence of millimolar concentrations of calcium or other divalent cations. Phase or DIC methods were essential to visualize details such as microlenses and differences between completely and incompletely thinned regions of the bilayer.

### Adhesion of LUVs to Decane-Based BLMs

The adhesion of LUVs to decane-based BLMs was investigated using calcein-loaded LUVs (soybean lipid/phosphatidylserine, 4 : 1, or phosphatidylserine/phosphatidylethanolamine, 1 : 1). BLMs were painted in MbK, allowed to thin and a solution of MbK containing 15 mM calcium chloride was perfused into the upper chamber. LUVs were added and allowed to settle onto and adhere to the BLM. Free LUVs and external dye were then perfused away. Figure 2 is a fluorescence micrograph of calcein-loaded LUVs bound to a BLM. LUVs bind to the torus as well as to the bilayer. After adhesion, LUVs remain bound and perfusion of EDTA solution (MbK with 10 mM EDTA) into the chamber to complex calcium ions does not release adherent LUVs. The circular streaks, which result from the long exposure, are due to LUVs moving on the bilayer as a consequence of the flow of perfusion solution across the surface of the bilayer. Vesicles are often carried to the Plateau border where they ad-

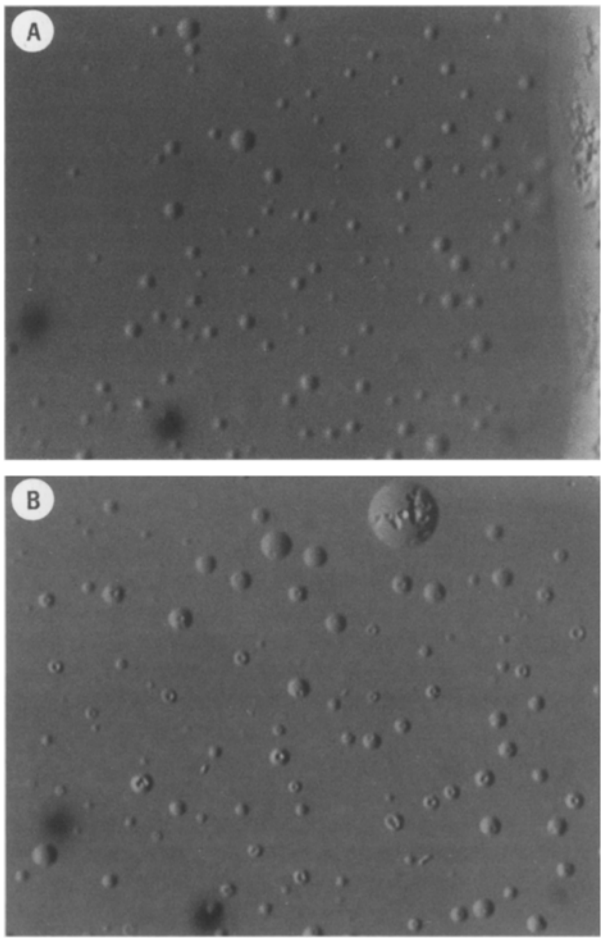


**Fig. 3.** Differential interference contrast photomicrographs of decane-based BLMs before and after adhesion of LUVs. (*A*) Bilayer in which microlenses are visible. (*B*) Bilayer with adherent LUVs. Adhesion of LUVs induces the formation of lenses under adhering LUVs. (*A*) and (*B*) do not represent the same BLM. Photomicrograph (*A*) was chosen for its multitude of lenses. Addition of LUVs greatly increases the number of lenses in typical BLMs. Bilayers were formed and manipulated as described in Fig. 2. The width of each photograph represents 230 $\mu$m

here and accumulate and, as a result, are slowly cleared from the bilayer. Vesicles were never observed to move from the torus into the bilayer region of BLMs.

To visualize the adhesion process in greater detail, we used DIC microscopy. Decane-based BLMs contain microlenses that are readily observed with DIC microscopy (Fig. 3*A*). Addition of LUVs results in appearance of dimpled lenses (Fig. 3*B*), the dimples of which are revealed by fluorescence illumination to be calcein-loaded LUVs. Lenses appear under adherent vesicles of all sizes from 0.5 to 30 $\mu$m in diameter. Addition of LUVs to BLMs containing few microlenses results in the formation of many dimpled lenses; therefore, vesicles do not simply bind to pre-existing lenses. Adhesion of negatively charged, fluorescent latex beads to decane-based BLMs did not elicit lenses.

Several observations suggested that vesicles did not remain intact after interactions with decane lenses. First, when present in large numbers, dimpled lenses coalesce to form larger vesicle-lenses and the vesicle structure becomes less distinct. Second, the fluorescence of calcein, initially in individual LUVs, becomes uniform over these lens-aggregates suggesting vesicle disintegration. Third, upon adhesion of LUVs (containing dioctadecylindocarbocyanine or rhodamine-PE) to decane-based BLMs, fluorescence instantly appears in the lens surrounding the bound vesicle. This is shown clearly for the LUV in the lower right corner of Fig. 4*A* and *B* (large LUV containing smaller LUVs) where fluorophore in the outer vesicle has partitioned into the lens. The other adherent vesicles in this figure show this less clearly because their diameters are nearly as large as the lenses to which they are adhering (*see* DIC photomicrograph, Fig. 4*B*). The transfer of fluorophore from vesicles to lenses is especially obvious after vesicle-lenses have coalesced. In such cases, the lenses are large and relatively uniformly fluorescent with little localization of lipid fluorophore in the vesicle membranes.

## Squalene-Based BLMs

Virtually solvent-free planar bilayers were spread from a squalene instead of decane solution of lipid. This technique has been described for glycerol monoolein membranes by White [22] who showed that a large molecule like squalene is essentially excluded from the intermonolayer region of the planar film. We found that squalene may also be used with a lipid composition of phosphatidylserine/phosphatidylethanolamine, 1 : 1. Such membranes thin, do not contain lenses and have a capacitance in the range reported for solvent-free membranes (approximately 0.7 $\mu$F/cm$^2$). Squalene-based BLMs are noticeably less dark under phase contrast than decane-based BLMs, presumably because they are thinner. The lipid does not always remain in solution in the torus after the membrane has been spread; often the lipid hydrates and extrudes liposomes over the BLM. Such membranes have unusually high conductances. Resistances of acceptable squalene-based BLMs were $3 \times 10^6$ to $3 \times 10^7$ $\Omega$-cm$^2$ and membranes with higher conductances or obvious structural defects were not used.

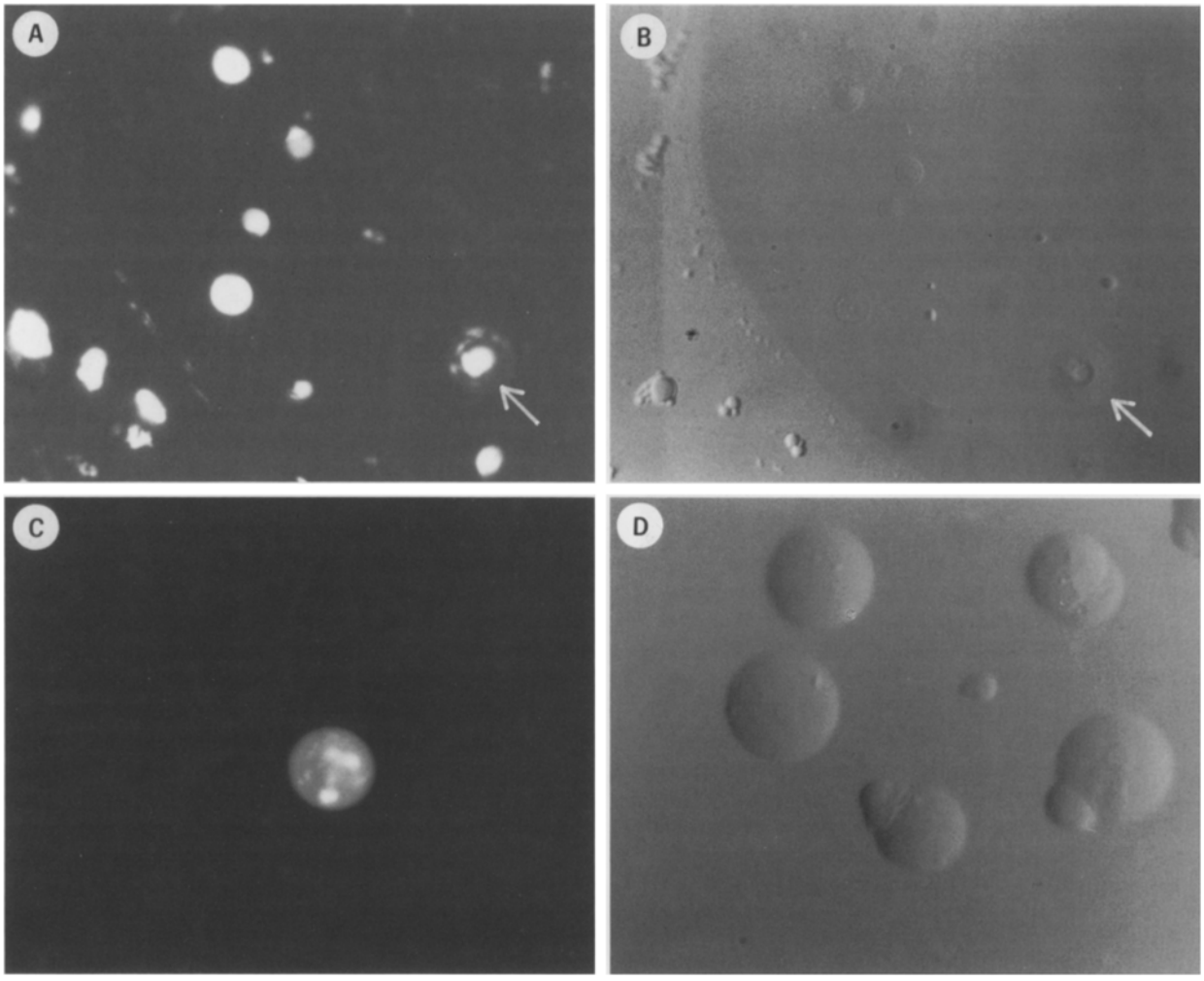


**Fig. 4.** Differential interference contrast and fluorescence photomicrographs of LUVs adhering to squalene-based and decane-based BLMs. (*A*) Fluorescence photomicrograph of LUVs (containing rhodamine PE) adhering to decane-based BLM. (*B*) DIC photomicrograph of same field of view as in (*A*). Lipid fluorophores move instantly from vesicles into lenses and the lenses become uniformly fluorescent as they coalesce. Adherent vesicles have moved slightly during the interval between the two exposures. The LUVs were hydrated in sucrose solution (without calcein) using lipids containing 2% rhodamine-PE. The width of the photographs represents 60 $\mu$m. (*C*) Fluorescence photomicrograph of LUVs (containing rhodamine-PE) adhering to squalene-based BLM. (*D*) DIC photomicrograph of same field of view as in (*C*). Upon adhesion, LUVs flatten onto the BLM but do not generate lenses and the lipid fluorophore does not migrate into the BLM. BLMs were made and manipulated as in previous figures. After the membrane had thinned, a solution of 150 mM KCl, 10 mM MOPS, pH 7.4, 15 mM calcium chloride was perfused into the top chamber. LUVs were added above the BLM and allowed to settle on and adhere to the BLM. Unbound LUVs were eliminated by perfusion. LUVs (PS : bacterial-PE, 1 : 1) were formed by hydration in 300 mM sucrose. The height of the photograph corresponds to 117 $\mu$m. The lipid composition of the BLM was the same as that of the vesicle

### Adhesion of LUVs to Squalene-Based BLMs

We investigated the adhesion of very large, sucrose containing uni- and paucilamellar PS/PE vesicles to squalene-based BLMs. Figure 4*C* is a DIC micrograph of such vesicles bound to a PS/PE BLM. As described above, a small volume of LUV suspension (2–5 $\mu$l) was added above the BLM from a microcapillary. The vesicles settle onto the BLM, and in the process of adhering, flatten on the bilayer, increasing in diameter by nearly 50%. A decrease in phase density accompanies the flattening of the vesicle. Adhesion of vesicles leads neither to the formation of lenses nor to the transfer of fluorophore from vesicle membrane to BLM as was the case with decane-based membranes. Figure 4*C* shows adherent vesicles under DIC, and Fig. 4*D* shows an adherent vesicle under fluorescence illumination. As may be seen in the photographs, there are no lenses surrounding the vesicles and the fluo-

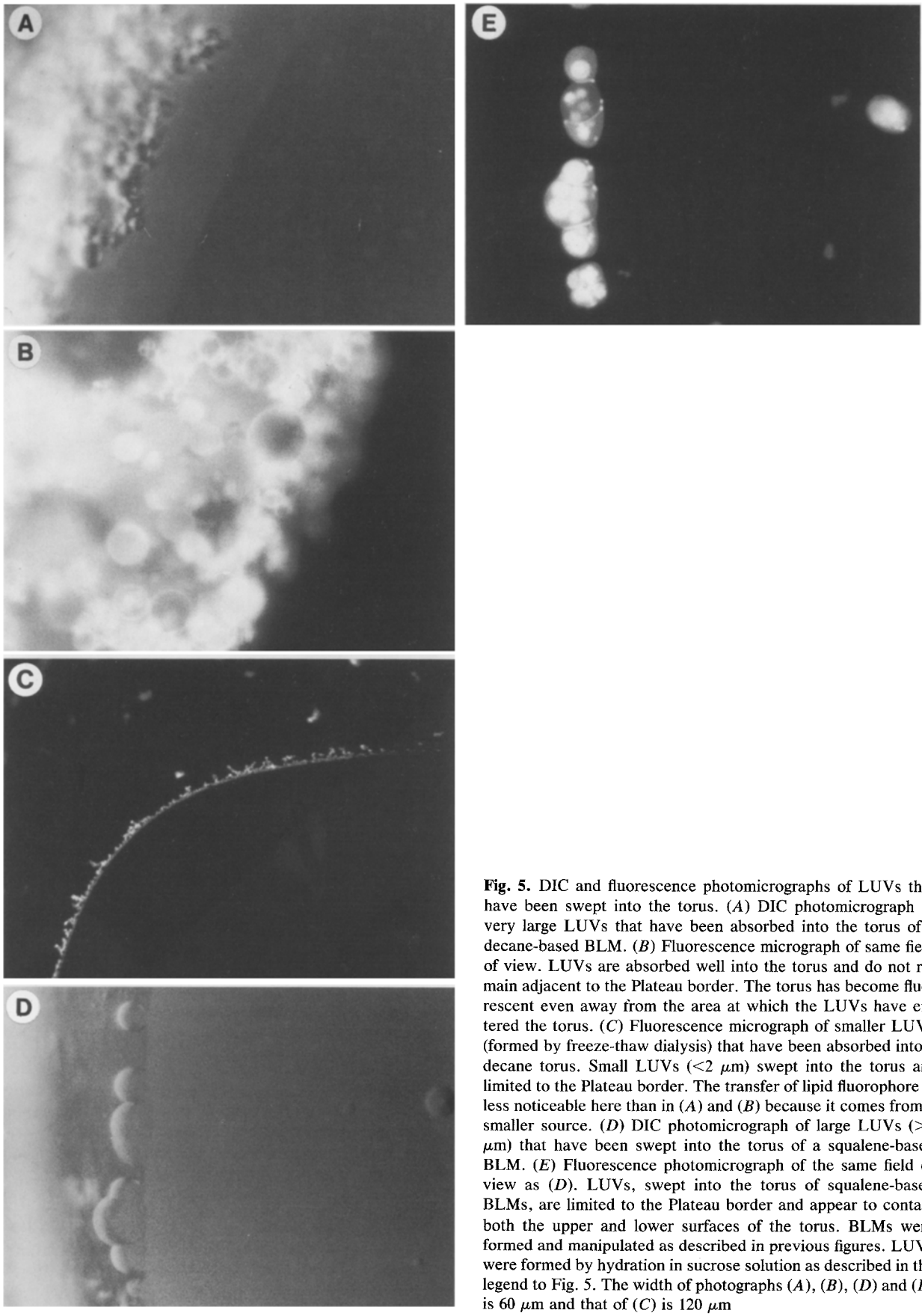


**Fig. 5.** DIC and fluorescence photomicrographs of LUVs that have been swept into the torus. (*A*) DIC photomicrograph of very large LUVs that have been absorbed into the torus of a decane-based BLM. (*B*) Fluorescence micrograph of same field of view. LUVs are absorbed well into the torus and do not remain adjacent to the Plateau border. The torus has become fluorescent even away from the area at which the LUVs have entered the torus. (*C*) Fluorescence micrograph of smaller LUVs (formed by freeze-thaw dialysis) that have been absorbed into a decane torus. Small LUVs (<2 $\mu$m) swept into the torus are limited to the Plateau border. The transfer of lipid fluorophore is less noticeable here than in (*A*) and (*B*) because it comes from a smaller source. (*D*) DIC photomicrograph of large LUVs (>5 $\mu$m) that have been swept into the torus of a squalene-based BLM. (*E*) Fluorescence photomicrograph of the same field of view as (*D*). LUVs, swept into the torus of squalene-based BLMs, are limited to the Plateau border and appear to contact both the upper and lower surfaces of the torus. BLMs were formed and manipulated as described in previous figures. LUVs were formed by hydration in sucrose solution as described in the legend to Fig. 5. The width of photographs (*A*), (*B*), (*D*) and (*E*) is 60 $\mu$m and that of (*C*) is 120 $\mu$m

rescence of the lipid label is confined to the outline of the vesicle. Adhesion of LUVs to squalene-based BLMs did not cause an increase in membrane conductance. Because the refractive index of MbK is less than that of an isosmotic sucrose solution, the sucrose-containing vesicles appear as dark spheres on a light background under the phase microscope. As with decane-based BLMs, removal of calcium from the bathing medium does not release vesicles from the BLM.

Adhering vesicles can move in the plane of the bilayer and, upon reaching the Plateau border, fuse with the torus, leaving the aqueous phase of the vesicles protruding into the torus (Fig. 5).

### Interaction of Vesicles with the Torus of Decane- and Squalene-Based Planar Bilayers

The difference between decane- and squalene-based membranes is also apparent in torus-vesicle interactions (Fig. 5). Large LUVs (several micrometers in diameter or larger) that are swept into a squalene torus become incorporated into the torus but remain at the Plateau border, as shown in Fig. 5*D* and *E*. In contrast, such large vesicles move well into the torus of decane membranes (Fig. 5*A* and *B*), although small vesicles are retained at the Plateau border (Fig. 5*C*). In both cases, fluorophore migrates from the vesicle membrane into the torus, but the extent and rate of transfer into decane is much greater than into squalene (*compare* Fig. 5*B* and *E*). It appears that partial fusion occurs in both cases, but that the rate of phospholipid dissolution is much greater in decane than in squalene.

### Interaction of LUVs with Decane-Based BLMs Under Conditions Leading to Osmotic Swelling of Vesicles

Possible fusion of liposomes to decane-based BLMs was investigated by observing the phase, differential interference contrast and fluorescence images of bound liposomes under conditions similar to those found to promote vesicle-BLM fusion [2, 4]. These conditions were produced either by adding a urea solution to the *cis* chamber or by perfusing the *cis* chamber with an isotonic or hypertonic solution of urea. Rhodamine-PE (or dioctadecylindocarbocyanine) served as a vesicle membrane marker and calcein (fluorescence) or sucrose (phase density) as markers for internal contents.

Addition of a small amount of concentrated (5 M) urea solution to the chamber caused immediate coalescence of liposome-lens structures and the formation of "bubbles" within the lenses. This phenomenon is shown in Fig. 6 in DIC (upper) and fluorescence (lower) photomicrographs. Perfusion with 300 mM urea produced a similar result. Individual vesicles were no longer distinguishable. Since urea is a permeant solute, it equilibrates with the vesicle aqueous interior. The latter, which in the lens would be covered by a single monolayer, expands with the influx of urea and water and presumably fuses to produce the bubbles seen in Fig. 6. Lipid fluorophore remained in the lens-complexes (Fig. 6, lower part). The use of vesicles containing self-quenched calcein provided a sensitive test for fusion, because release of the dye leads to its dilution, the visible result of which is a bright flash under fluorescence illumination. Simple lysis can also produce such a flash and although it is possible to distinguish between flashes resulting from fusion and those resulting from lysis, the total number of observed release events was too small for us to do so reliably. In any case, fusion, if it occurred, must have been extremely rare.

### Interaction of LUVs with Squalene-Based BLMs Under Conditions Leading to Osmotic Swelling of Vesicles

The possibility of LUV fusion with solvent-free (i.e., squalene-based) BLMs was also investigated. When we used very large unilamellar vesicles and perfused with a urea solution to impose an osmotic stress, we only rarely observed potential fusion events. When we used entrapped sucrose as a marker, we occasionally saw the disappearance of a vesicle and its phase-dense internal contents (sucrose); however, more common was the simple lysis of a LUV, the remnant of which was easily visible adhering to the BLM.

Vesicles did not always burst upon perfusion with a urea solution, but their swelling was evident from their decrease in diameter as they returned to the spherical from the oblate shape, as well as from their increased phase contrast. When vesicles were bound to squalene-based BLMs, the most common result of perfusion with a urea solution, either hyper- or isosmotic, was rupture of the planar membrane. BLMs without adherent vesicles were not susceptible to rupture under such conditions.

## Discussion

The application of phase, DIC and fluorescence microscopy to lipid vesicle-planar bilayer interactions under conditions allowing recording of images at high magnifications has revealed interactions more complex than recognized heretofore. The most un-

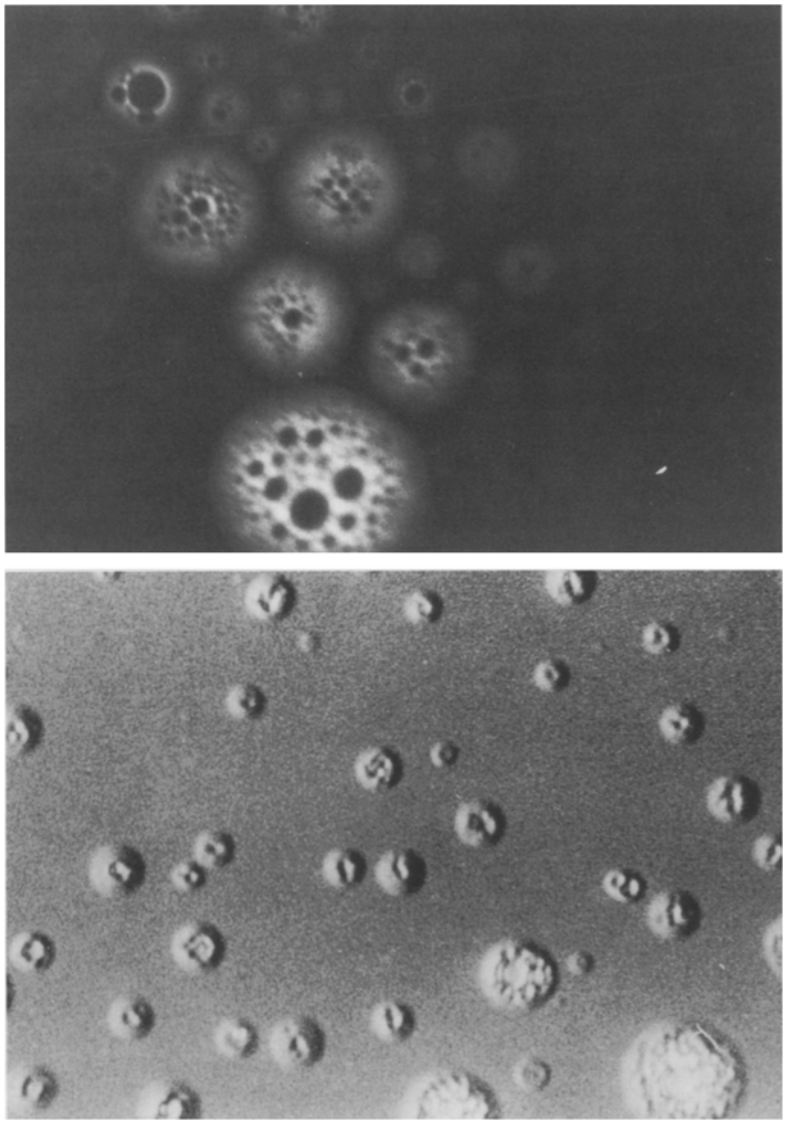

**Fig. 6.** Differential interference contrast and fluorescence photomicrographs of effects of osmotic stress on LUVs adherent to decane-based BLMs. Osmotic stress causes the formation of bubbles within large vesicle-lenses. The upper part of the figure shows the bubbles under DIC and the lower part shows a similar preparation under fluorescence illumination. After osmotic stress, lipid fluorophore remains confined to vesicle-lens structures (lower part). Decane-based BLMs were made and manipulated as in previous figures. LUVs (soybean lipid : PS, 4 : 1) were made as described in Fig. 2 by freeze-thaw dialysis but contained 1 mole% dioctadecylindocarbocyanine as a membrane-associated fluorophore. Adherent LUVs were osmotically stressed by addition of 20 $\mu$l of 5 M urea to the upper chamber. The urea solution was allowed to settle onto the membrane without agitation. The height of the photograph corresponds to 230 $\mu$m

usual behavior was found with decane-based planar bilayers, in which the solvent appeared to play a critical role in vesicle-membrane interaction. Unexpected properties of squalene-based membranes were also observed.

## Vesicle Interactions with Decane-Based Membranes

The formation of decane lenses at the area of contact between vesicles and BLM appears to be a consequence of the combination of the distortion induced between vesicles and one monolayer of the BLM combined with the availability of decane within the bilayer [8]. The binding between LUV and BLM mediated by $Ca^{2+}$ is strong enough to visibly flatten the vesicle. Since bilayers do not significantly stretch, this must involve extrusion of some of the internal water. It may also be that some solvent, especially in the case of decane-based membranes, may diffuse into the vesicles, which could easily add significantly to its area. The participating monolayer of the BLM must, to some extent, conform to the surface of the vesicle and the energy required to bend that monolayer must, therefore, come from the energy of adhesion. The monolayer on the *trans* side from the LUV would be forced to similarly conform to the curvature of the vesicle—thus incurring an additional bending energy—were there not solvent available. Flow of solvent into the area of contact would occur with little expenditure of energy and would relieve the *trans* monolayer of the necessity of conforming to the curvature im-

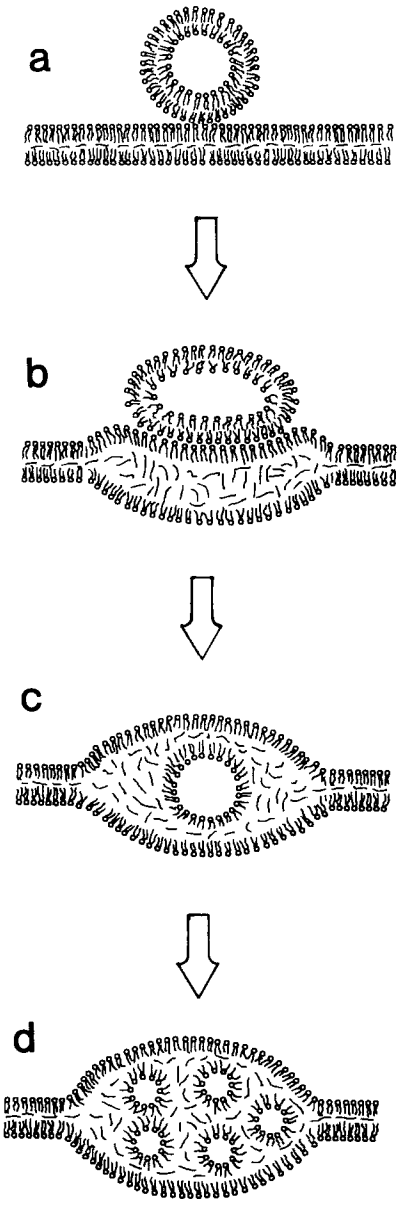


**Fig. 7.** Schematic representation of the interactions of LUVs with decane-based BLMs. (*a*) Adhesion. (*b*) Formation of lens in the BLM. (*c*) Fusion of the outer monolayer of the vesicle with the upper monolayer of the lens. After hemifusion, the two monolayers become continuous, causing the inner monolayer of the vesicle and the aqueous core, which it surrounds, to become immersed in the bulk decane of the lens. The hemifusion process is shown in more detail in Fig. 9. (*d*) Fragmentation of vesicle aqueous phase. Lipid dissolved in the lens partitions into the monolayer surrounding the vesicle core, increasing its area to volume ratio and leading to its fragmentation and dispersal throughout the lens

posed upon the *cis* monolayer by adhesion to the vesicle.

It appears likely that liposomes frequently fuse with the surface of decane lenses. Although the transfer of fluorophore from LUV to BLM could be simple migration from one structure to the other, its abrupt nature suggests fusion. Since it occurs preferentially at lenses, the fusion process must initially be limited to the outer monolayer of the vesicle and the monolayer covering the lens. The uniform distribution of calcein fluorescence over the large lenses that form by coalescence of smaller lenses would also seem to require fusion of the contacting monolayers of the BLM and LUV. The fact that the aqueous phase marker becomes distributed throughout the lens without being released to the external phase indicates that LUVs disintegrate within the lens, and hemifusion, which would inject the vesicle aqueous compartment into the lens, appears to be a prerequisite thereof.

The processes of adhesion, hemifusion and dispersion of vesicle contents, as we believe they occur in the case of decane-based membranes, are depicted in Fig. 7. The first step, adhesion, is self-explanatory. In the case of PS-containing bilayers, adhesion of the contacting bilayers would be due to their mutual binding to $Ca^{2+}$. The area of contact would be water-poor, if not actually dehydrated. Monolayer fusion occurs when a discontinuity occurs at the same place in the two contacting monolayers (*see below*), the result of which is that the monolayer-covered aqueous core of the vesicle becomes incorporated into the lens. As dissolved lipid from the lens inserts into the surface of the core, the increasing area/volume ratio leads to dispersal throughout the lens of the core aqueous phase. When iso- or hyperosmotic solutions of urea are perfused into the chamber after vesicles have adhered, the dispersal of the LUV aqueous phase marker (calcein) is reversed and the lenses become filled with large, aqueous droplets. Because the volume/area ratio of the droplets increases with volume, their formation from dispersed fragments of the vesicle core would be the expected response of the latter upon their swelling under the influx of urea and water.

## Interactions of Vesicles with Squalene-Based Membranes

In the presence of $Ca^{2+}$, LUVs adhere to but do not spontaneously fuse with squalene-based membranes. Binding was not reversible upon chelation of $Ca^{2+}$ by EDTA, but this is not surprising, given the low dissociation constant of $Ca^{2+}$ bound between phosphatidylserine lamellae [7]. There was no indication of lipid transfer or fusion in the bilayer region proper, although it appears that the outer monolayer of vesicles does fuse with the surface of

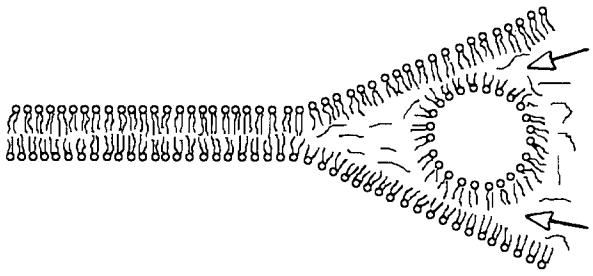

**Fig. 8.** Interpretation of images of vesicle-squalene torus interaction. Shown here is a cross-section through the membrane at the boundary of the torus. Hemifusion has stripped the vesicle of its outer monolayer and what remains is the aqueous core covered with the inner monolayer. The situation shown appears to be the stable state in the case of squalene-based membranes, but in the case of decane-based membranes, the solvent penetrates the bilayer (arrows) sufficiently that the monolayer-covered vesicle may migrate away from the edge of the planar membrane into the torus

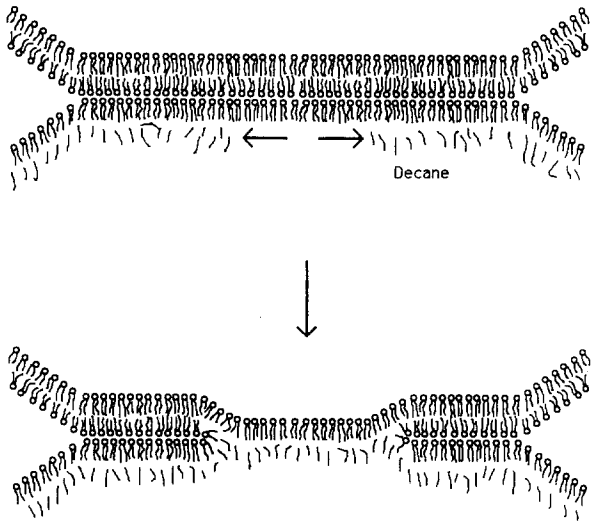


**Fig. 9.** Detail of proposed fusion mechanism. In the presence of $Ca^{2+}$, monolayers of acidic lipids tend to contract. The outer monolayer of the vesicles can contract only by separating from itself, which is possible at the region of vesicle-lens contact, where the rend is filled by decane and inner monolayer coming into contact

the torus; the only reasonable interpretation of the incorporation of a LUV into the torus at the Plateau border, such as seen in Fig. 5*D* or *E*, would seem to be hemifusion. Such a process is diagrammed in Fig. 8. Because squalene does not penetrate between phospholipid monolayers [21, 22], the aqueous contents of the vesicle, surrounded by its inner monolayer, remains at the edge of the bilayer. Hemifusion occurs at the torus of decane membranes also, but because decane would not be excluded from the intermonolayer region (arrow, Fig. 8), the aqueous core may move deeper into a decane torus. The behavior of squalene- and decane-based membranes is thus quite similar at the solvent interface. The predominant difference between the two types of membranes is the exclusion of the solvent from the membrane proper of squalene-based films, hence they are relatively inert.

The reason for the prevalence of fusion at the interface of a vesicle bilayer and the monolayer at the surface of bulk hydrocarbon is suggested by a previous analysis of vesicle-vesicle fusion [12; *see also* ref. 3 for a similar mechanism, but different driving force]. The key to the mechanism is the fact that cations such as $Ca^{2+}$, capable of causing fusion, induce a contraction of monolayers of fusion-susceptible lipids. Thus, in the vesicle-vesicle case, hemifusion occurs when upon binding $Ca^{2+}$, the external monolayer of the vesicles shrinks and pulls away from itself in the only region possible, the contact zone. (This is presumably the reason for the correlation, extensively documented by Ohki and Oshima [16], between the effectiveness of fusion induced by various divalent cations and the magnitude of the increase in surface tension those cations induce in monolayers.) The inner monolayers of the vesicles complete the hemifusion process by moving transversely into contact through the rends in the external monolayers. Vesicle-vesicle interactions differ from vesicle-planar membrane interactions in that the planar membrane is not under tension after divalent ion addition because the torus provides a reservoir of lipid to satisfy the tendency toward head group condensation. We propose that the head groups of planar membranes, rather than being pulled out of the contact region as occurs at the vesicle surface, are pushed apart by intrusion of solvent between the alkyl chains. Head group separation by this mechanism is also limited to the area of contact with the vesicle where exposure to water is avoided. That process is illustrated in Fig. 9. Since short chain alkanes exhibit a much greater penetration of monolayers than does squalene [8, 21–22], decane-based membranes are expected to be more susceptible to interactions with vesicles than would squalene-based membranes, as was observed.

## Osmotic Stress Is Not Always Sufficient to Induce Membrane Fusion

We were unable to demonstrate fusion of lipid vesicles with BLMs, although hemifusion was evident with decane-based planar membranes and with the

torus of squalene-based membranes. Applying osmotic stress, a manipulation that others have found to be effective in inducing fusion of vesicles with BLMs [2, 4–6, 23–25] or of BLMs with each other [9], caused the appearance of aqueous bubbles in the decane lenses, which formed after vesicle adhesion. Were these bubbles to expand to the point of herniating into the *trans* compartment, the result would be membrane fusion, at least in the phenomenological sense. In our experiments, however, they remained as stable structures. This is perhaps not surprising, for although one is accustomed to expecting bubbles to burst, there is actually no reason why the bubbles within the lenses could not continue to grow if aqueous phase influx continued. The lens contents are within a sandwich of two monolayers and these may separate over a wider and wider area, unlike an aqueous phase bounded by a single membrane, which would be forced to burst. Fusion was also not demonstrable when squalene-based membranes were used, for applying osmotic stress to vesicles bound to these membranes almost invariably led to membrane rupture.

We can rule out the possibility that horizontal membranes are incapable of fusion, for in another study [18] we observed very efficient fusion of synaptic vesicles with horizontal squalene-based membranes. Furthermore, if horizontal membranes thin differently than vertical membranes, they would be expected to retain more, not less solvent, which should, if it has any effect, promote hemifusion such as is seen at the torus. The most obvious difference between those cases where fusion was observed and the present results is that the vesicles used in the present study lacked proteins. Recently, Niles and Cohen [15] and Woodbury and Hall [23], using methods similar to ours, have shown that channels are essential for fusion because, under their conditions, channels allow the requisite osmotic swelling. We perfused with solutions of a permeant solute and could, therefore, induce osmotic stress without using vesicles containing channels. Since we did not observe fusion and since it was unlikely to have been due to the inadequacy of our methods, we are led to suggest that transbilayer proteins, whether or not specifically channel proteins, facilitate membrane fusion independent of effects they may have on osmotic water fluxes.

Although we did not reproduce the entire process of bilayer-bilayer fusion in this study, phenomena were observed that either corroborate earlier analyses of the mechanism of membrane fusion [12] or further our understanding of membrane interactions observed previously [15, 23]. The methods we have described should also be of use in future investigations of membrane fusion.

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