OSMOTIC CONTROL OF BILAYER FUSION

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ABSTRACT We have used photography and capacitance measurement to monitor the steps in the interaction and eventual fusion of optically black lipid bilayers (BLMs), hydrostatically bulged to approximately hemispherical shape and pushed together mechanically. A necessary first step is drainage of aqueous solution from between the bilayers to allow close contact of the bilayers. The drainage can be controlled by varying the osmotic differences across the bilayers. If the differences are such as to remove water from between the bilayers, fusion occurs after a time that depends on the net osmotic difference and the area of contact. If there is an osmotic flow of water into the space between the bilayers, fusion never occurs. In the fusion process, a single central bilayer forms from the original apposed pair of bilayers. The central bilayer may later burst to allow mixing of the two volumes originally bounded by the separate bilayers; the topological equivalent of exocytosis.

INTRODUCTION

Fusion of two membranes is important in biological processes, some common examples being fertilization (1), synaptic nerve transmission (2), exocytosis (3), intercellular fusion (4) and indeed the normal somatic cell cycle itself, in which many membrane fusion events occur both at the cellular and subcellular levels. The mechanism or mechanisms by which membrane fusion occurs and is regulated are unknown, although agents such as polyethylene glycol (5) (or an impurity in it [6]), Sendai virus (4) and lysolecithin (3) are known to enhance fusion. Other agents, in particular the calcium ion (7), also seem to have important roles.

In the experiments reported here, we have investigated the possibility that osmotic differences may play a role in the fusion process. In these initial investigations we have used interacting black lipid bilayers (BLMs). Experiments with such simple model systems are a first step towards understanding the behavior of more complex natural membrane systems. Optically black lipid bilayers are so-called because of the very small percentage of light reflected from them, and are formed from hydrocarbon solutions of the appropriate lipids by drainage of the hydrocarbon (see Fig. 2 *a*). They have frequently been used as models for the behavior of real biological membranes. Their advantages and disadvantages in this respect have been cogently discussed by Haydon (8).

We report here the results of measurements on the interactions of pairs of bilayers formed from glycerol monooleate (GMO) (an uncharged lipid) in *n*-hexadecane. The behavior of bilayers made from egg yolk lecithin (a zwitterionic lipid) in *n*-decane or *n*-hexadecane was similar, but time-dependent effects made it difficult to ensure that equilibrium had been reached. These effects will be the subject of a separate communication.

MATERIALS AND METHODS

Glycerol monooleate was purchased from Nu-Chek-Prep., Inc. (Elysian, MN) and stored at −15°C until use, when it was used without further purification. The hydrocarbon solvents used (*n*-decane and *n*-hexadecane) were general purpose reagent grade (BDH Chemicals, Ltd., Parkstone, Poole, England), and were passed through an activated alumina column to remove polar impurities immediately before use. Sodium chloride was analytical reagent grade, and was baked at 550°C for 3 h to remove any organic impurities. The cells were machined from Kel-F® rods purchased from Afton Plastics Molding Company (Lakeland, MN). The stainless steel chamber and stainless steel cell supports were cleaned before use in hot detergent solution, then soaked in chromic acid for 1 h, followed by repeated rinsing with distilled water and a final rinse in double-distilled water. The Kel-F® cells on which the bilayers were actually formed were first cleaned in the same way as the stainless steel parts of the apparatus, followed by prolonged boiling in double distilled water. All experiments were performed at 30° ± 1°C).

The apparatus was assembled as shown in Fig. 1. A small amount of glycerol monooleate dissolved in hydrocarbon (~5 mg/ml) was introduced into the septum, either with Pasteur pipette, or from a reservoir of lipid solution (9). Depression of the syringe plungers *A* and *A'* (Fig. 1) bulged the lipid solutions to thick films of approximately hemispherical shape (Fig. 2 *a*). The thick films were then allowed to drain under gravity (Fig. 2 *a*) until the capacitance of each film had stabilized. The capacitance was measured using videobridge (model 2100; Electro Scientific Industries, Inc., Portland, OR) at a frequency of 20 Hz and an applied voltage of 20 mV. The films could be viewed either in grazing incidence white light, when interference colors from the thick (undrained) part of the film were clearly visible, or in grazing incidence laser light from a 2 mW He-Ne laser, which allowed a better view of the fully drained films. The area of the fully drained bilayer could be calculated from photographic measurements of the bright outline. The final capacitance per unit area for the fully drained bilayers agreed well with those of flat BLMs.

The method used to establish an osmotic difference across the bilayer was simply to flush ~10 ml of the required aqueous salt solution through

the Kel-F® cell, allowing excess solution to emerge from the septum into the aqueous solution in the main chamber. Lipid solution was then introduced across the septum, the solution in the main chamber stirred, and a sample of the solution in the main chamber taken for analysis by conductance measurement. The concentration of sodium chloride in the main chamber was kept close to the physiological value of ~0.15 M. The salt concentrations on each side of the bilayers were thus unambiguously established, and the osmotic difference was further confirmed by the rate of increase of capacitance (and hence of area) of the bulged bilayers as a result of water influx. Once a pair of bubbles, with a given osmotic difference, had been produced, the same conditions could be used repeatedly, since it was possible to pull the fused bilayers apart to re-establish the separated bubbles.

"Lenses" of solvent (9) in the films could be seen as bright points of light, especially under laser illumination. Bilayers containing such visible lenses were not used for fusion experiments.

Once two stable bilayers had been obtained, the micrometer-driven stage supporting the rear septum was moved until the bilayers were in "contact," i.e., separated only by a thin layer of aqueous solution. The micrometer reading corresponding to initial contact was noted and the micrometer stage then moved in by a further measured distance, Δz, to deform the contacting bilayers (Fig. 2 *b*). A record was kept of the time between initial contact and eventual fusion for cases where fusion occurred.

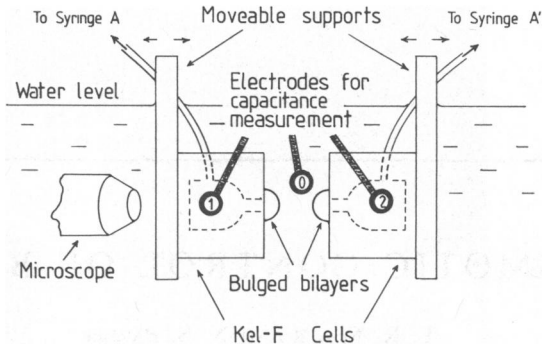


FIGURE 1 Schematic diagram of apparatus. The bilayers are formed across small (~0.5 mm diam) holes in a pair of Kel-F® cells. Capacitances are measured between Ag/AgCl electrodes at the points labelled 0, 1, and 2. The Kel-F® cells can be filled from a permanently connected syringe with aqueous solutions of composition differing from that of the solution in the chamber. A smaller syringe *A* or *A'* is used to bulge the lipid initially placed across the septum to form an approximately hemispherical film.

RESULTS

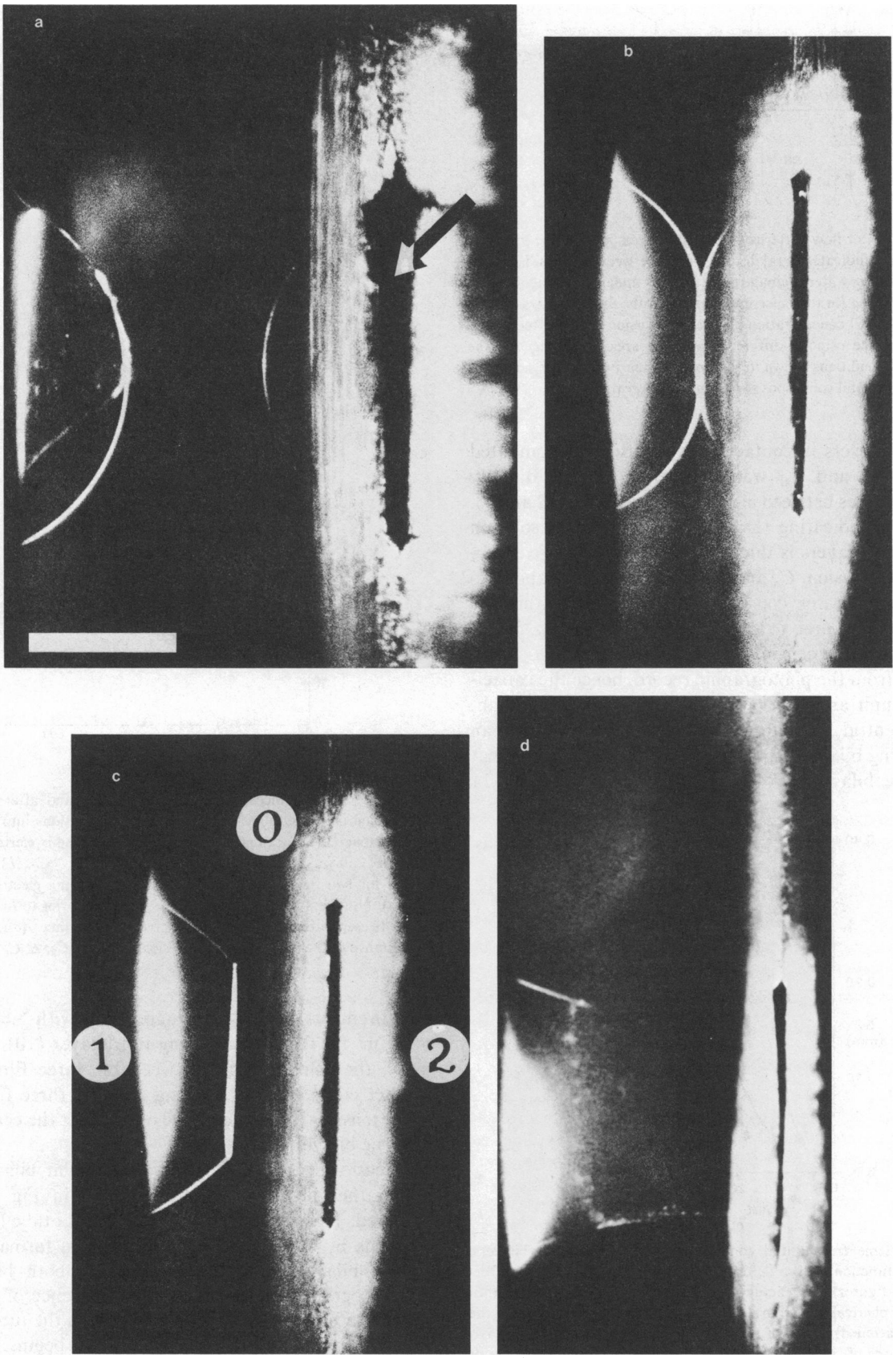
Typical changes in the conformation of the bulged bilayers on initial contact, fusion and, rarely, bursting of the central fused film are shown in Figs. 2 *b–d*.

With pure water on both sides of the bilayers fusion never occurred, the longest period of observation being 3 h. When salt solutions of different concentrations were set up on either side of the bilayers, the osmotic difference being such as to withdraw water from the space between the bilayers (Fig. 3, upper panel), the bilayers fused after a time that depended systematically on the net osmotic difference and the area of contact (Fig. 4). Fusion was never observed when an osmotic difference was set up in the reverse direction (Fig. 3, lower panel), the smallest difference tested being 0.001 osmol. Nominally equal salt concentrations gave irreproducible results, with fusion sometimes occurring after times as short as 50 s, but sometimes not at all, the longest time of observation being 90 min.

The result of fusion in the great majority of cases is shown in Fig. 2 *c*. Fusion of the two bilayers has produced a central film, and the compartments defined by the two original bilayers are still separated by this central film. The occurrence of fusion can be followed by capacitance measurement between electrodes 1 and 2. At fusion this capacitance steps to a new, higher value.

The capacitances C_A, C_B, and C_C of each of the three regions *A*, *B*, and *C* of the fused lipid films (Fig. 5 *a*) can be calculated from impedance measurements between any three different electrode combinations. In practice, the set 0 to [1 and 2], 1 to [2 and 0], and 2 to [0 and 1] was used to reduce the effects of stray capacitances. (The notation [1 and 2] means that electrodes 1 and 2 were electrically connected.) The simplified equivalent circuit for the fused films in the electrolyte is shown in Fig. 5 *b*. Although it was not possible to solve the full equations for C_A, C_B, and C_C in terms of the measured impedances explicitly, they could be solved iteratively using a computer program, which included corrections for the electrode impedances. With the bilayers separated, the solution gave values for C_A and C_B (the capacitance of the individual spherical caps) within 0.1% of their values measured directly, and C_C as zero.

FIGURE 2 (*a*) A pair of bulged glycerol monooleate/*n*-hexadecane spherical caps on Kel-F® supporting septa in 0.15 M NaCl, illuminated from the rear by white light at grazing incidence and viewed with a Nikon F2 camera (Nikon Inc., Instrument Div., Garden City, NY) fitted with an 85 mm lens on an extension bellows. Scale marker = 1 mm. Bulging was achieved with a micrometer-driven syringe attached to the supporting septum, so that the film enclosed a constant volume throughout the experiment. Excess hydrocarbon solvent drained to the Plateau borders (in contact with the septa), leaving a bilayer of glycerol monooleate containing ~20% of hydrocarbon (by volume) intercalated between the lipid chains. In this photograph the *right*-hand film is fully drained and the *left*-hand film about half-drained, so that its upper part is still relatively thick (several hundred nanometers). The Plateau border (arrow) of the *right*-hand film is particularly prominent in this example. (*b*) A pair of fully drained bilayers brought into juxtaposition. Note the contact angle of ~0° and general shape, similar to that found for a nonadhering bubble pressed against a solid surface. This and the following two photographs were taken with grazing incidence laser illumination. (*c*) Equilibrium structure of two films after fusion has occurred (the lighting arrangement only permits a good view of the *left*-hand film and *right*-hand Plateau border). The time from initiation of fusion to establishment of the final structure is a few milliseconds. Capacitance measurements with Ag/AgCl electrodes in the positions shown as 1, 2, and 0 allows calculation of the capacitance of central film. (*d*) Under some conditions (see text) the central bilayer bursts, leaving a tube connecting the two compartments. This arrangement, which allows intermixing of the contents of the two compartments, is topologically equivalent to exocytosis. The particles in the center of the picture are the remnants of a burst central bilayer.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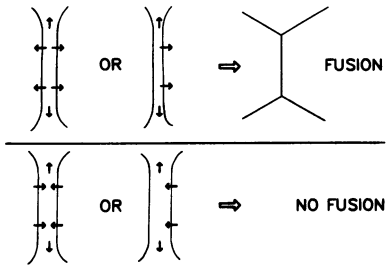

FIGURE 3 Water flow patterns when two bilayers are pressed together. Vertical arrows indicate lateral drainage from between bilayers. Horizontal arrows indicate water drainage through films under the influence of an osmotic difference (in the present experiments, the difference was established by an NaCl concentration difference). Fusion occurs after a time dependent on the osmotic difference and the area of contact for the experimental conditions shown in the *upper* panel. Fusion does not occur for the experimental conditions shown in the *lower* panel.

With the bilayers in contact but not fused the computed values of C_A and C_B were still equal to the directly measured values between electrodes (1 and 0) or (2 and 0), respectively, indicating that the film of aqueous solution between the bilayers is thick enough to provide a conduction path. On fusion, C_A and C_B fell, but C_C rose rapidly to a value that became constant for these films within the time (~10 s) required to take a set of readings (Fig. 5 *c*).

The area of the central region formed on fusion can be measured from the photographic record, hence the capacitance per unit area can be calculated. There is no doubt that the central structure is a single bilayer, rather than two adhering bilayers, since its specific capacitance is that of a single bilayer (5.84 ± 0.04 nF/mm^2, mean of 11

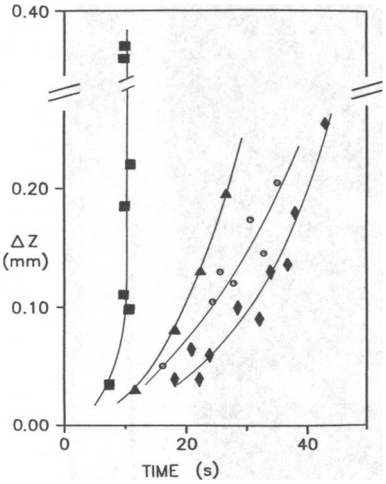

FIGURE 4 Time from initial contact to eventual fusion of bilayers, plotted as a function of film deformation Δz for various osmotic differences (boxed figures). Δz is actually the difference between the height of the original spherical cap from its Kel-F® support and the height of the deformed (flattened) region of the film after contact. To a first approximation, the area of contact is proportional to Δz. Each point is the result of a separate experiment. Net sodium chloride concentration difference: ■, 0.40 M; ▲, 0.20 M; ⊙, 0.028 M; ♦, 0.014 M.

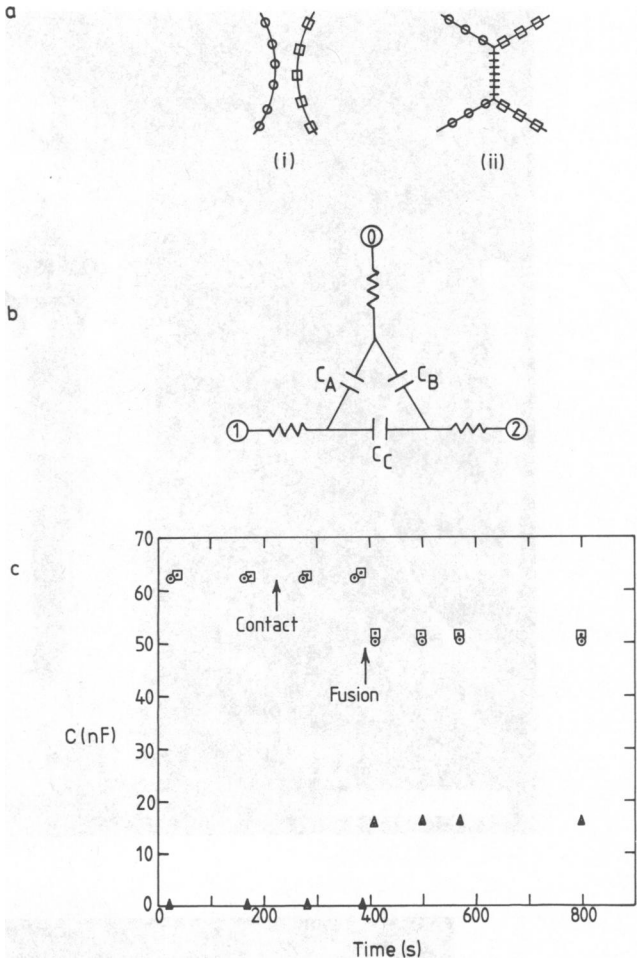

FIGURE 5 Capacitances of the bilayers before and after fusion. (*a*) Illustration of the subdivision of bilayer configurations into regions for which capacitances are determined. (*i*) separate hemispherical bilayers; (*ii*) fused bilayers. ⊖ = C_A; ⊟ = C_B; ⫼ = C_C. (*b*) Equivalent circuit for two fused bubbles (simplified by omitting electrode capacitances). Numbers represent electrodes. Note that prior to fusion $C_C = 0$ for both separated bilayers and bilayers in contact. (*c*) Change in capacitance of the three regions on fusion. ⊙, C_A; ⊡, C_B; △, C_C.

measurements) in excellent agreement with 5.80 ± 0.05 nF/mm^2 for the corresponding flat bilayer (10). Furthermore, the contact angle between the three films at the contact circle is 120°, showing that the three films have equal tensions and hence, once again, that the central film is a true bilayer.

Occasionally, the formation of a tubular bilayer, (Fig. 2 *d*) rather than the structure shown in Fig. 2 *c*, was observed. The question arises as to whether tube formation proceeds by a different mechanism from formation of a central bilayer. In our model system, both high-speed cinematography and the invariable presence of the remnants of a burst central film embedded in the tube surface suggest that a central bilayer always begins to form, bursting shortly thereafter to leave an open tube (Fig. 2 *d*) connecting the two originally separated compartments.

DISCUSSION

The "central bilayer" configuration has been observed by several previous groups of workers. For example, it has been observed by Neher (11) for dioleoyl lecithin bilayers, although complete equilibrium was not reached since some lenses of hydrocarbon solvent remained trapped in the central bilayer. Recently, Melikyan et al. (12) have observed similar structures for bilayers formed from dioleoyl lecithin, azolectin or phosphatidylethanolamine, although these workers also do not appear to have allowed sufficient time for the central bilayer to reach equilibrium, since the specific capacitances measured are lower than those for an "equilibrium" planar bilayer, as were the values measured by Neher.

Further support for the central bilayer model comes from the work of Breisblatt and Ohki (13) on the fusion of spherical bilayers of phosphatidylserine, performed under conditions where osmotic gradients across the bilayers exist which would promote fusion according to our model (Fig. 3). They find an intermediate stage where photographs of the juxtaposed spherical bilayers of phosphatidylserine show the 120° contact angle characteristic of fusion, although the authors themselves interpret these photographs as simply representing adhesion. Their experiments were performed in the apparent absence of calcium ions, which are normally required in high concentrations (>1 mM) for fusion of phosphatidylserine bilayers. It is tempting to conclude that osmotic effects alone were sufficient to induce fusion, but it is possible that the phosphatidylserine itself had retained some calcium during purification.

It is possible that osmotic flow through the bilayers is necessary to reduce the film of aqueous solution between the bilayers to some critical thickness at which the thin film collapses spontaneously (14, 15). Whether the aqueous film collapses to a stable thin film or collapses completely, permitting intimate contact of the bilayers and initiating fusion, may depend on the size of the osmotic pressure difference.

The importance of osmotic effects has previously been reported by Finkelstein and his co-workers in a series of seminal papers (16–18) on the fusion of multilamellar vesicles with planar bilayers. All of the experiments described in these papers start from an equilibrium condition where the osmotic pressures are equal on both sides of the planar bilayer, within the vesicle and in the layer trapped between the vesicle and the bilayer. Fusion occurs when the osmotic pressure in the vesicle is raised in some manner, giving a condition analagous to that in the center diagram of our Fig. 3 (top panel). The results of the two types of experiment thus appear compatible, although it is difficult to establish the subsequent condition in the space between the apposed bilayers, since this condition may be changing rapidly as part of the events leading up to fusion. Cohen et al. (reference 18; legend to Fig. 2, bottom line) have tentatively suggested that fusion occurs with the bilayers rupturing in the region of contact, presumably to re-form in the new configuration. This tentative model appears to us to be physically likely only if the bilayers are strongly adhering, as they are in the above experiments, but not in our own experiment.

It is, of course, possible that the presence of a small amount of residual hydrocarbon solvent (between 8% [reference 11] and 26% [reference 10] by volume in the hydrocarbon region, the exact figure depending on the assumptions used in the calculation) makes a significant difference to the behavior of our model system compared with real membranes. Certainly the central bilayer is stabilized by a reservoir of lipid in a Plateau border, and this could well account for the stability of the central bilayer compared with that in real systems. "Microlenses" of hydrocarbon solvent are probably not involved in initiating fusion, since we have never observed fusion between bilayers in pure water. Furthermore, initiation of fusion by microlenses is inconsistent with the increase in time from contact to fusion with increasing area of contact (Fig. 4) and consequently the occurrence of a greater number of microlenses in the region of contact.

The increase in time from contact to fusion with area of contact indicates that the rate of drainage of aqueous solution from between the bilayers by lateral flow is important when the osmotic difference is small. For high osmotic differences, the time to fusion is almost independent of the area of contact (Fig. 4), as is expected when the removal of water from between the bilayers is predominately under osmotic control.

We also note here that a stable central bilayer can be burst by application of a voltage pulse (reference 12 and our own observations), a procedure that may be relevant to electro-stimulated cell fusion.

We conclude that the presence of an appropriate osmotic gradient is a sufficient condition to promote fusion of juxtaposed uncharged lipid bilayers. Further details of both the physical mechanism of osmotic control and its possible occurrence in biological systems will appear elsewhere.

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