Parameters Affecting the Fusion of Unilamellar Phospholipid Vesicles with Planar Bilayer Membranes

FREDRIC S. COHEN,* MYLES H. AKABAS, JOSHUA ZIMMERBERG, and ALAN FINKELSTEIN
*Department of Physiology, Rush Medical College, Chicago, Illinois 61612; and Departments of Physiology/Biophysics and Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461. Dr. Zimmerberg's present address is National Institutes of Health Physical Science Laboratory, Bethesda, Maryland 20014.

ABSTRACT   It was previously shown (Cohen, F. S., J. Zimmerberg, and A. Finkelstein, 1980, J. Gen. Physiol., 75:251–270) that multilamellar phospholipid vesicles can fuse with decane-containing phospholipid bilayer membranes. An essential requirement for fusion was an osmotic gradient across the planar membrane, with the vesicle-containing (cis) side hyperosmotic with respect to the opposite (trans) side. We now report that unilamellar vesicles will fuse with "hydrocarbon-free" membranes subject to these same osmotic conditions. Thus the same conditions that apply to fusion of multilamellar vesicles with planar bilayer membranes also apply to fusion of unilamellar vesicles with these membranes, and hydrocarbon is not required for the fusion process. If the vesicles and/or planar membrane contain negatively charged lipids, divalent cation ($\sim$15 mM Ca^{++}) is required in the cis compartment (in addition to the osmotic gradient across the membrane) to obtain substantial fusion rates. On the other hand, vesicles made from uncharged lipids readily fuse with planar phosphatidylethanolamine planar membranes in the near absence of divalent cation with just an osmotic gradient. Vesicles fuse much more readily with phosphatidylethanolamine-containing than with phosphatidylcholine-containing planar membranes. Although hydrocarbon (decane) is not required in the planar membrane for fusion, it does affect the rate of fusion and causes the fusion process to be dependent on stirring in the cis compartment.

The mechanisms causing and controlling fusion of biological membranes are of interest to scientists from many diverse disciplines. Of the many possible components in the fusion process, the one certainty is that ultimately fusion occurs between two lipid bilayers—one from each membrane. Consequently, we have been investigating the fusion of phospholipid vesicles to planar phospholipid bilayer membranes. We have tried to answer several questions with this approach: first, can fusion occur in this system? Second, if it can, what conditions are necessary to obtain it and what parameters control its rate? Third, what are the forces and energetics in the process (that is, what mechanisms underly fusion in this system)? Finally, to what extent is the fusion process in this model system relevant to biological fusion events, particularly exocytosis?

We previously demonstrated that fusion can occur between phospholipid vesicles and planar phospholipid bilayer mem-

branes on the basis of two criteria: (a) the transfer of vesicular contents across the planar membrane ("exocytosis") (22), and (b) the incorporation into the planar membrane of a membrane-associated protein originally present in the vesicular membranes (4). The essential condition for obtaining fusion is an osmotic gradient across the planar membrane, with the cis side (the side containing the vesicles) hyperosmotic with respect to the opposite, trans, side (4). Although divalent cations at millimolar concentrations on the cis side greatly increase the rate of fusion, they are not essential to the process. We proposed that divalent cations increase the frequency and duration of close contact of vesicles with the planar membrane and that fusion results from osmotic swelling of these vesicles followed by rupture of vesicular and planar membranes in the region of contact (4). In support of this view, Zimmerberg et al. (23) reported that micromolar amounts specifically of Ca^{++} (rather than millimolar amounts of any divalent cation)

stimulated fusion if the planar membrane contained a calcium-binding protein, but an osmotic gradient across the planar membrane was still essential.

Two features detracted from the possible biological relevance of these previous studies: first, the vesicles were multilamellar, rather than unilamellar; second, the planar membranes contained large amounts of hydrocarbon solvent (decane). This paper addresses both issues. We find that the same conditions required for obtaining fusion of multilamellar vesicles to planar membranes also hold for unilamellar vesicles. We also show that fusion readily occurs with planar membranes largely devoid of hydrocarbon solvent; i.e., hydrocarbon is not required for fusion in this system. Hydrocarbon in the planar membrane, however, does modify the fusion process, which we describe and discuss. In the accompanying paper (2) we examine the nature of the interaction and attachment of vesicles with the planar membrane, and demonstrate a "pre-fusion" state that is separable from the osmotically driven fusion event.

MATERIALS AND METHODS

Preparation of Vesicles

CHOLATE DIALYSIS PROCEDURE: 4–6 mg of egg phosphatidylcholine (PC)[1] in chloroform were dried under nitrogen in a glass tube and then in a vacuum desiccator for 30 min. 0.1 ml of 50 mg/ml cholate (Aldrich Chemical Co., Milwaukee, WI) in "buffer" (500 mM KCl or NaCl, 10 mM HEPES, 1 mM MgCl$_2$, 0.1 mM EDTA, pH 8.0), 0.025 ml of porin stock suspension (1 mg/ml in "buffer") and 0.375 ml of "buffer" were then added to the lipid-containing tube. (The exact lipid to cholate ratio required to reconstitute porin into the vesicular membrane varied somewhat among batches of porin and was empirically determined for each batch.) The mixture was vortexed for 1 min, sonicated briefly in a cylindrical bath sonicator (Lab Supplies Company, Inc., Hicksville, NY) to clarity, transferred to a 12,000-mol-wt cutoff dialysis tube (Spectra/Por 2 membranes, Fischer Scientific Co., Pittsburgh, PA), and dialyzed overnight at 4°C against 200 ml of "buffer." Vesicles were removed from the dialysis tube and stored on ice. Egg PC was from Avanti Biochemical Co. (Birmingham, AL) (see reference 13).

SONICATION-FREEZE-THAW PROCEDURE: 5 mg of the desired lipid in either chloroform or chloroform:methanol (2:1) was dried under nitrogen in a glass tube and then in a vacuum desiccator for 30 min. 0.475 ml of "buffer" and 0.025 ml of porin stock suspension (see above) were added to the lipid-containing tube; the mixture was vortexed for 30 s, transferred to a polycarbonate screw-top tube, and sealed under nitrogen. The cloudy suspension was clarified by sonication for ~10 min at 4°C in a Branson Sonifier Model W185 with Cup Horn attachment (Branson Sonic Power Co., Danbury, Conn.). It was then rapidly frozen in a dry ice/ethanol bath for 70 s and allowed to thaw at room temperature. This freeze-thaw cycle was repeated three to five times, during which time the suspension became cloudy. The cloudy vesicle suspension was then sonicated in a cylindrical bath sonicator for 15 s to disperse the vesicles. These vesicles, now ready for use, were prepared fresh daily and stored on ice. All lipids were from Avanti Biochemical Co. (see reference 7).

Reconstitution of porin by both vesicle-formation techniques was assayed by the vesicles' inability to retain K$^+$ when dialyzed against an isosmotic Li$^+$ solution. (The K$^+$ initially inside a vesicle with at least one porin channel in its membrane will exchange for Li$^+$ during the dialysis.) After dialysis, the amount of K$^+$ that remained trapped inside porin-free vesicles was determined, using a K$^+$-selective electrode, from the amount of K$^+$ released from the vesicle by gramicidin A. This technique was also used to determine the average diameter of vesicles prepared in the absence of porin. The volume-average diameter of both cholate dialysis and freeze-thaw vesicles was ~700 Å.

Porin: Porin is a large (110,000 mol wt) intrinsic membrane protein that forms channels in the outer membrane of gram-negative bacteria and is responsible for the permeability of that membrane to small solutes (12). It is easily purified in large quantities, and remains stable even under such harsh conditions as sonication and exposure to concentrated detergent solutions. It is a particularly good marker for fusion because of its characteristic voltage-dependent properties (18, 19), which make it easy to distinguish porin-induced

[1] Abbreviations used in this paper: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

conductance from nonspecific leaks in the planar membrane. Fig. 1 illustrates the voltage-dependent properties of porin inserted from a detergent solution into a planar membrane. Channels are open at voltages below ±100 mV and begin to close at larger voltages of either polarity. The rate of decline of the macroscopic conductance induced by many porin channels increases with voltage. Porin channels are similar in many respects to the voltage-dependent anion channel obtained from the outer membrane of mitochondria (5, 17) and used as a membrane marker in earlier fusion experiments (4). We used porin, rather than voltage-dependent anion channel, in the present experiments because of the ease in preparing large, purified quantities.

Porin was initially obtained as a generous gift from Dr. Carl Schnaitman (University of Virginia). This, and two subsequent preparations of porin, were prepared by a modification of the procedure of Rosenbusch (15) (C. Schnaitman, personal communication); the details of the preparation are given in reference 1. Porin was stored dry as a lyophilized powder in the refrigerator or freezer for at least two years without significant loss of activity. Porin stock suspensions were stored in the refrigerator and showed no loss of activity over several months.

Planar Membranes: Two types of planar phospholipid bilayer membranes were used in the present study: (a) "hydrocarbon-free" membranes formed by folding together two monolayers (10), and (b) decane-containing membranes formed by the brush technique (11). Experiments were performed at room temperature in a chamber that consisted of a 125 μm thick Teflon partition separating two lucite compartments, each containing 2 to 3 ml of the desired salt solution. Each compartment contained a small magnetic stirring bar which permitted vigorous mixing of the compartment's contents. When required, the contents of either compartment could be intermittently or continuously perfused with a fresh solution via Teflon tubes connected to a peristaltic pump; the total compartmental volume could be exchanged several times in 1 min.

"Hydrocarbon-free" membranes were formed across a 0.36-mm diameter hole that was pre-coated with squalene (14) purified on an alumina column; decane-containing membranes were formed across either a 0.36- or a 1.0-mm diameter hole from 2 to 5% lipid solutions in n-decane. The salt solutions in the two compartments generally consisted of 100 mM KCl or NaCl, 10 mM MES, 1 mM EDTA, pH 6.0; for the hydrocarbon-free membranes, 2 mM MgCl$_2$ was often included in the salt solution to aid in membrane formation. Asolectin (lecithin type II from Sigma Chemical Co. [St. Louis, MO]) was purified by the method of Kagawa and Racker (6) to remove neutral lipids; its composition after this procedure is 40% PC, 33% phosphatidylethanolamine (PE), 14% phosphatidylinositol, and smaller percentages of lysophosphatidylcholine and cardiolipin (9). All other lipids used to form planar membranes were purchased from Avanti. Salts were reagent grade and used as obtained; water was deionized and then distilled in a glass still.

Electrical measurements were made under voltage clamp conditions through a single pair of saturated calomel electrodes contacting the aqueous solutions through saturated KCl bridges. Generally, a 20 mV potential (cis side positive) was applied across the planar membrane and the resulting transmembrane current monitored on a chart recorder and an oscilloscope screen; the transmembrane current is directly proportional to membrane conductance (which equals current/voltage), since the voltage is held constant. The compartment to which vesicles were added is called the cis compartment; the opposite compartment is referred to as the trans compartment.

RESULTS AND DISCUSSION

Fusion of Unilamellar Vesicles to "Hydrocarbon-Free" Planar Membranes

CONDITIONS FOR AND CHARACTERISTICS OF FUSION: When cholate-dialysis, porin-containing unilamellar vesicles are added to the aqueous phase on one side (the cis side) of a "hydrocarbon-free" planar bilayer membrane, no incorporation of porin channels occurs in the planar membrane, regardless of the incubation time. Thus, porin does not spontaneously transfer from vesicular to planar membranes. If an osmotic gradient of ~200 mosM or higher is now established across the planar membrane by addition of urea or KCl to the cis compartment, a few porin channels may insert over the next several minutes (Fig. 2). Under voltage-clamp conditions, these insertions appear as discrete current (conductance) jumps. If Ca^{++} is now added to the cis compartment to a concentration of 5 to 15 mM, the rate of
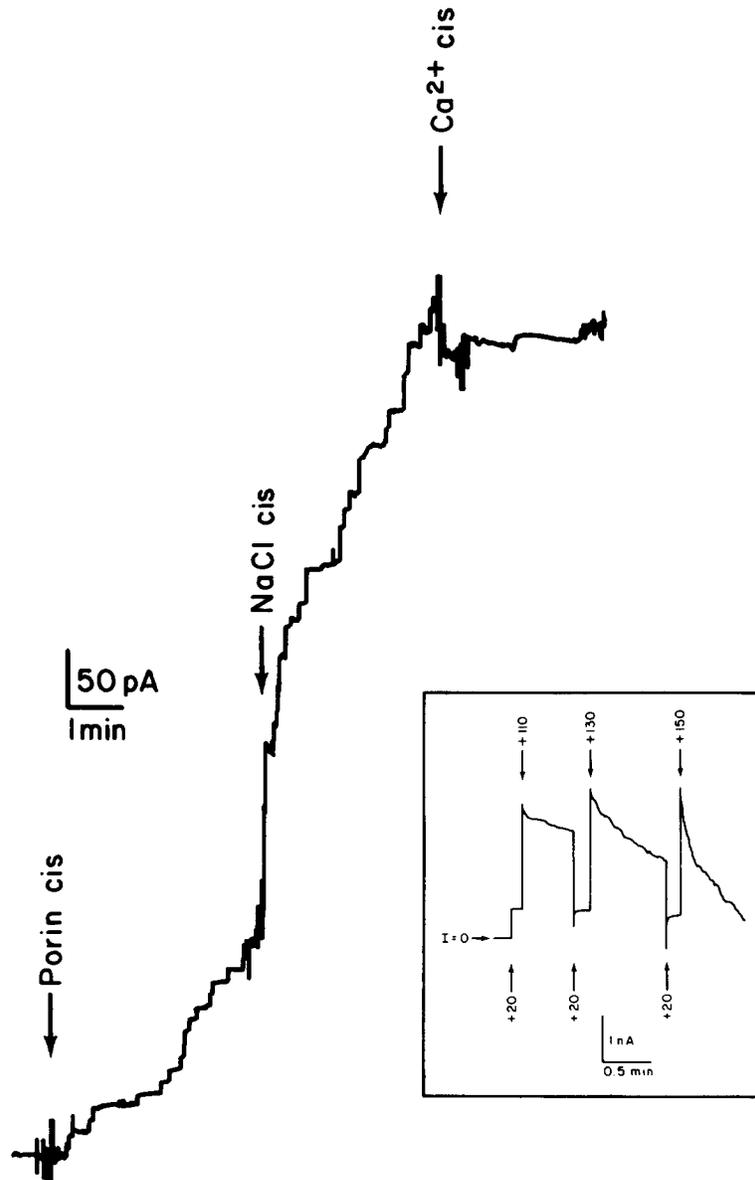
FIGURE 1   The insertion of porin channels and the voltage-dependent behavior of porin-induced conductance in planar lipid bilayers. An asolectin membrane clamped at V = 20 mV separates symmetric solutions (100 mM NaCl, 10 mM MES, 1 mM EDTA, pH 6.0). At the start of the record, porin in cholate buffer (0.1 mg/ml porin, 1 mg/ml cholate, 500 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0) is added to the cis compartment to a concentration of 1.7×10^{-3} mg/ml. Shortly after this addition, the transmembrane current begins to increase in a step-like manner. 3½ min later an osmotic gradient is formed by addition of NaCl to the cis side to a concentration of 50 mM. Aside from the instantaneous increase in the membrane conductance due to the increase in the salt concentration, this does not affect the rate of channel insertion into the planar bilayer. The addition to the cis side of $CaCl_2$ to a concentration of 10 mM stops the step increases in conductance. Ca^{++} seems to inhibit the insertion into the planar membrane of porin in detergent solution, perhaps by chelating the cholate in the compartment. In the inset, the voltage dependence of the porin-induced conductance is shown. The transmembrane voltage is clamped to the voltages indicated. At voltages >100 mV the conductance declines; the larger the voltage the faster the decline. The decline in the conductance is indicative of the closing of the porin channels.

channel insertion is greatly stimulated (Fig. 2). This slows steadily with time, and insertions eventually cease to occur after several minutes to more than an hour. If, while channel insertion is occurring, the osmotic gradient is abolished by addition of osmoticant to the trans compartment, channel insertion ceases; it resumes when the osmotic gradient is re-established (Fig. 3).

The osmotic gradient can be created by making either the cis side hyperosmotic (Fig. 2) or the trans side hypoosmotic (Fig. 4). Thus, insertion of porin channels into the planar

membrane is caused by the osmotic gradient (cis side hyperosmotic with respect to the trans side) and not by a direct effect of osmoticants on the vesicles. (We have never observed porin insertion when the trans side was hyperosmotic with respect to the cis side).

Insertion rates can be so high (as in Figs. 2–4) that individual events cannot be resolved. If, however, fewer vesicles are added to the cis compartment, one can clearly resolve the individual current jumps and show that each represents the simultaneous insertion of many porin channels into the planar
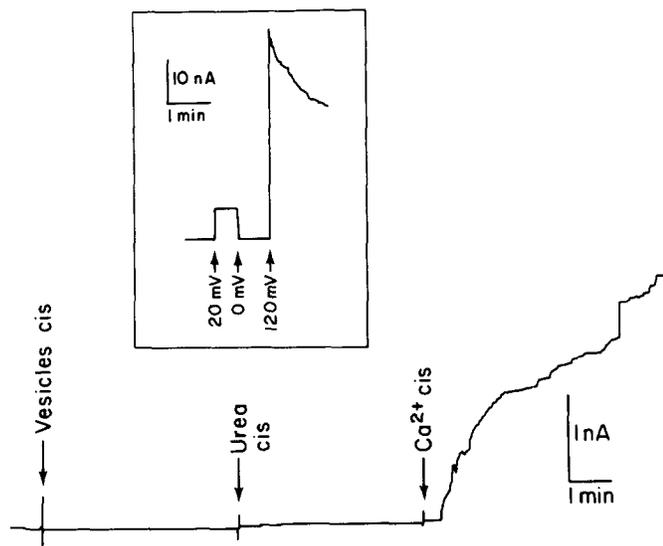
FIGURE 2 Fusion of unilamellar vesicles to a "hydrocarbon-free" planar membrane. An asolectin membrane clamped at V = 20 mV separates symmetric solutions (100 mM NaCl, 10 mM MES, 3 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). At the start of the record, cholate dialysis PC vesicles ($\sim 10^{11}$/ml) are added to the cis compartment. No fusion events (current jumps) occur during the next 3 ½ min. Urea is then added to the cis compartment to a concentration of 400 mM. Two or three fusion events (current jumps) occur over the next 4 min. After the addition of $CaCl_2$ to the cis compartment to a concentration of 15 mM, the rate of fusion (current jumps) dramatically increases. (Inset) Demonstration of the voltage dependence of the fusion-induced conductance. After fusion has proceeded for 12 min (during which time $CaCl_2$ was also added to the trans compartment to a concentration of 15 mM), the voltage across the planar membrane is stepped from 0 to 20 mV and back to 0, and then stepped from 0 to 120 mV. Note that in the latter case, the current (conductance) declines in a manner characteristic of porin channels closing, thus demonstrating that the current jumps seen in the main record resulted from the insertion of porin channels into the planar membrane.

membrane. ("Simultaneous" here means "within 200 μs", which is our time resolution). In Fig. 5, for example, only one current jump has occurred. When the voltage across the planar membrane was increased from 20 to 100 mV, the current decreased stepwise, each step corresponding to the turn off of one to three porin channels. In this record, about 15 porin channels were associated with the current jump. If the voltage is increased from 20 mV to ≥ 100 mV across a membrane in which there have been numerous insertions, a macroscopic decline of current occurs, characteristic of many porin channels turning off (see Fig. 1 and inset to Fig. 2). We routinely checked in this way that the large conductances obtained in many of our experiments resulted from insertions of porin channels, and not from nonspecific "leaks" induced in the planar membranes.

An osmotic gradient across the planar membrane is absolutely essential to the above phenomena; Ca^{++} by itself does not induce channel insertion, even at symmetrical concentrations of 100 mM. In Fig. 3, for example, we see that 15 mM Ca^{++} alone is ineffective, but the subsequent application of an osmotic gradient induces massive incorporation of channels into the membrane. 5–15 mM Ca^{++} in the cis compartment does, however, greatly increase the rate of channel insertion when an osmotic gradient already exists (see Fig. 2),

particularly if the planar and/or vesicular membrane contain negatively charged lipids. Ca^{++} is not unique in this respect. Mg^{++} and Ba^{++} are also effective, although about twofold higher concentrations of Mg^{++} are required to achieve similar rates.

The osmotic requirement and Ca^{++} stimulation of channel insertion into planar membranes exist only if porin has been incorporated into vesicles. Porin-free vesicles added to the cis compartment, whether followed or preceeded by the addition of an osmoticant and Ca^{++}, induce no change in the conductance of planar membranes. Porin added directly from its stock suspension, with or without an osmotic gradient and Ca^{++}, produces no change in planar membrane conductance, nor does the addition of porin-free vesicles alter this result. When porin solubilized in cholate is added to the cis compartment, channels insert singly, or infrequently as small multiples, rather than as large multiples (see Fig. 1), and the insertion rate is not increased by an osmotic gradient across the membrane. Ca^{++} added to the cis compartment actually inhibits, rather than stimulates, channel insertion, possibly because Ca^{++} chelates with cholate. Thus, only if porin is first reconstituted in vesicles does an osmotic gradient and Ca^{++} promote incorporation of porin channels into planar membranes.

SIMILARITY OF RESULTS WITH UNILAMELLAR AND MULTILAMELLAR VESICLES: All of the features of porin channel insertion into planar membranes from unilamellar
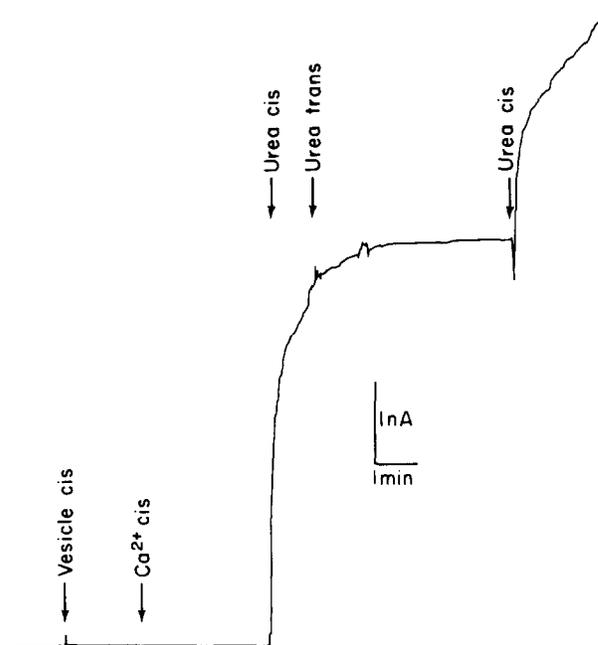

FIGURE 3 Demonstration that an osmotic gradient is required across the planar membrane for fusion to occur. A "hydrocarbon-free" asolectin membrane clamped at V = 20 mV separates the same symmetric solutions as in Fig. 2. At the start of the record, unilamellar cholate dialysis PC vesicles ($\sim 10^{11}$/ml) are added to the cis compartment, and no fusion events (current jumps) occur even after the addition of $CaCl_2$ (15 mM) to the cis side. However, when urea is added to the cis side to a concentration of 400 mM, fusion events (current jumps) occur at a rapid rate. These cease to occur shortly after the abolishment of the osmotic gradient across the planar membrane by the addition of urea to the trans side to a concentration of 400 mM; they resume, when the osmotic gradient is re-established by a second addition of urea (increment of 400 mM) to the cis side.
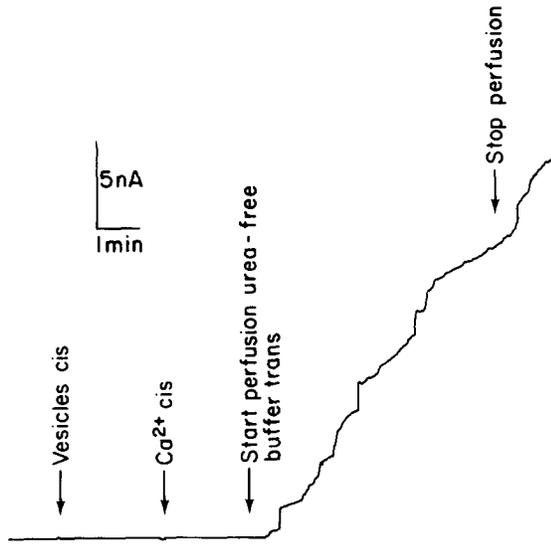
FIGURE 4 Demonstration that fusion is induced when the osmotic gradient across the planar membrane is created by making the trans side hypoosmotic. A "hydrocarbon-free" asolectin membrane separates symmetric solutions (100 mM NaCl, 300 mM urea, 10 mM MES, 3 mM CaCl$_2$, 0.1 mM EDTA, pH 6.0). At the start of the record, cholate dialysis PC vesicles ($\sim 10^{11}$/ml) are added to the cis side followed 2 min later by the addition of CaCl$_2$ (15 mM) to the same side. No fusion events (current jumps) occur until the trans side is perfused with urea-free buffer (100 mM NaCl, 10 mM MES, 3 mM CaCl$_2$, 0.1 mM EDTA, pH 6.0), thus establishing an osmotic gradient across the planar membrane.

vesicles—the simultaneous insertion of many channels, the requirement for an osmotic gradient across the planar membrane (cis side hyperosmotic), the stimulation of insertion rate by addition of divalent cations to the cis compartment—pertain to insertion of porin channels from multilamellar vesicles. More importantly, these are the same features previously described for the fusion of multilamellar vesicles with decane-containing planar bilayer membranes (4, 22). We therefore conclude that the insertion of porin channels into planar bilayers described in Figs. 2–5 results from fusion of unilamellar vesicles with planar membranes. Furthermore, the fusion process is the same for unilamellar and multilamellar vesicles.

This last point is of considerable practical importance. The unilamellar vesicles described in this section were made by the cholate dialysis procedure. Although we readily reconstituted porin into vesicles made from PC, we were not as successful with other lipids; the titration of lipid, porin, and cholate proved rather critical and difficult to reproduce. On the other hand, there was no difficulty reconstituting porin into any desired lipid mixture by the sonication-freeze-thaw technique. Although vesicles formed this way are said to be unilamellar (7), a significant fraction are undoubtedly not (C. Miller, personal communication). Since, however, the fusion process is apparently similar for both unilamellar and multilamellar vesicles, we could study it using sonicated-freeze-thaw vesicles without qualms. Most of our experiments were with vesicles prepared in this way.

The number of channels incorporated into a planar membrane by a single fusion event ranged from one to several hundred, with a mean of about twenty. (The number of channels in a fusion event was determined from the size of the current jump. The single channel conductance of porin

in 100 mM KCL is $\sim 1.5 \times 10^{-10}$ mho [18; personal observations of the authors].) This mean may be artifactually high, as it was often difficult to resolve small current steps from the background noise. The large range may reflect the heterogeneous size distribution of sonicated-freeze-thaw vesicles. This was very apparent in electron micrographs of negatively stained preparations, with vesicle diameters ranging from 200 to several thousand angstroms. When assayed by the K$^+$ release method (see Materials and Methods), porin-free vesicles had an average diameter of ~ 700 Å.

Cholate dialysis and sonication-freeze-thaw vesicles offer a significant advantage over large multilamellar vesicles for studying fusion. Because much greater numbers of the smaller vesicles can be added to the cis compartment, much higher rates of fusion are obtainable. In typical experiments, between 10^3 and 10^4 fusion events occur in 10 min, whereas fewer than 100 events occur with large multilamellar vesicles (4). (Normalizing for the number of vesicles added to the cis side, approximately the same number of fusion events are seen over a wide variety of vesicle sizes.) This permits a more detailed study of the parameters affecting the fusion process.

"Hydrocarbon-Free" vs. Decane-containing Planar Membranes

The earlier study by Cohen et al. (4) left unresolved the role of hydrocarbon in their planar membranes: was it essential to
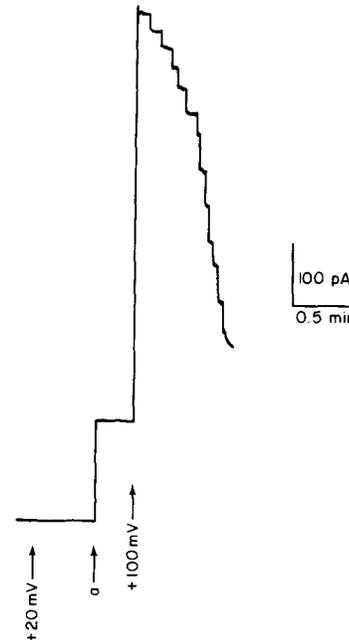

FIGURE 5 Demonstration of simultaneous insertion of many channels into a planar membrane. An asolectin membrane clamped at V = +20 mV separates assymmetric solutions. The cis side contains cholate dialysis PC vesicles ($\sim 10^9$/ml) and 200 mM urea, 100 mM NaCl, 10 mM MOPS, 10 mM CaCl$_2$, 0.1 mM EDTA, pH 7.0. The trans side contains 100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, pH 7.0. No change in the membrane conductance has occurred before the start of the record. About 1 min after the start of the record, at point a, there is a single step jump of 160 pA in the transmembrane current. Half a minute later, the voltage is switched from +20 to +100 mV. The current begins to decline immediately in step-like fashion, each step indicative of the closing of one to three porin channels. This shows that the single current jump at point a resulted from the simultaneous insertion of many porin channels into the planar membrane (in this case about 15).

or did it significantly affect the fusion process? Low fusion rates with large multilamellar vesicles precluded the use of "hydrocarbon-free" membranes, since such membranes can only be formed across holes significantly smaller than 1 mm in diameter—the size of their decane-containing membranes. The decrease in membrane surface area alone would have
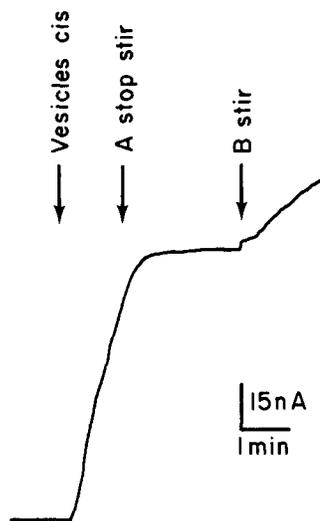
reduced the number of fusion events in an experiment to a mean of one or less. In the present system, with many more fusion events, we could investigate the effect of hydrocarbon on fusion. By forming decane-containing and "hydrocarbon-free" bilayers of essentially the same area, we could directly compare the two types of membranes.

Fusion of vesicles to "hydrocarbon-free" and decane-containing planar membranes occurs under the same set of conditions. Thus, an osmotic gradient across the planar membrane (cis side hyperosmotic) is essential to the process, and the rate of fusion is dramatically increased by millimolar concentrations of Ca^{++} in the cis compartment, when the planar and/or vesicular membrane contain negatively charged lipids. (The concentration of Ca^{++} that stimulates fusion in "hydrocarbon-free" membranes (~15 mM) is slightly higher than that which stimulates fusion in decane-containing membranes (5–10 mM). Also, stimulation by Ca^{++} is usually delayed ~30–60 s in "hydrocarbon-free" membranes but not in decane-containing membranes.) Osmotic gradients about twice as large as those applied to decane-containing membranes are required for comparable rates of fusion to "hydrocarbon-free" membranes; this may simply reflect a smaller water permeability of the latter. Thus, the presence of large amounts of hydrocarbon in the planar membrane is not a requirement for fusion; fusion occurs between unilamellar vesicles and "hydrocarbon-free" membranes. (Because the hole in the partition across which "hydrocarbon-free" membranes were formed was pre-coated with squalene, we cannot be certain that these membranes are absolutely hydrocarbon-free, although the amount of squalene in the bilayer is minimal (20, 21). We have also obtained fusion with membranes formed across a hole that was pre-coated with vaseline. Even
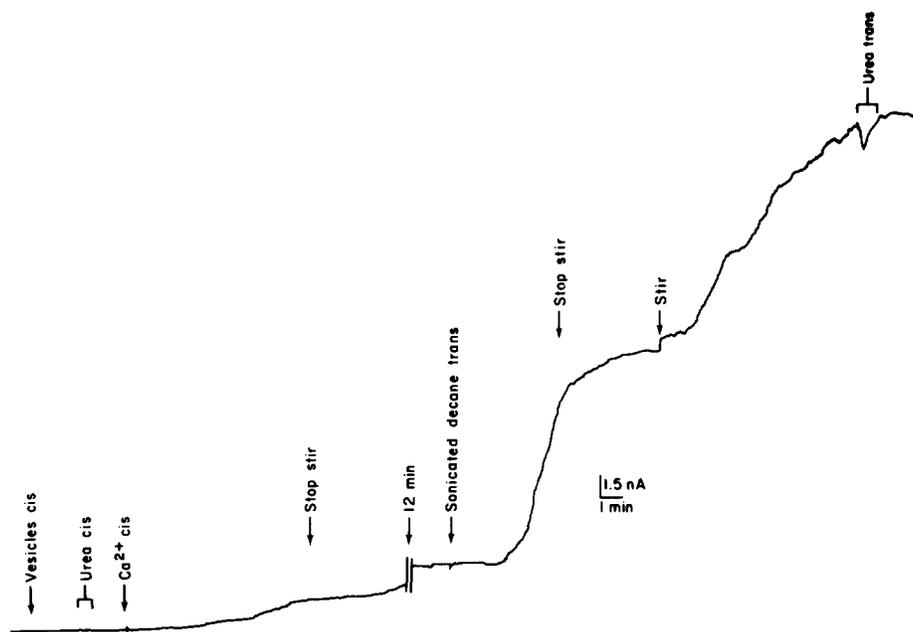

FIGURE 6 Stirring dependence of fusion to a decane-containing membrane. An asolectin membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 1 mM EDTA, pH 6.0). Before the start of this record, 200 mM urea and 10 mM CaCl₂ were added to the cis side. With both compartments stirred, PC vesicles (~10¹¹/ml) are added to the cis compartment. About 20 s later fusion begins. Stirring is stopped at point A, and within 1 min fusion ceases. When stirring is resumed at point B, fusion resumes.

FIGURE 7 The effect of sonicated decane on fusion to a "hydrocarbon-free" membrane. An asolectin "hydrocarbon-free" membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10mM MES, 2 mM MgCl₂, 0.1 mM EDTA, pH 6.0). Vesicles (egg PC; ~10¹¹/ml) are added to the cis compartment, followed by 450 mM urea and 15 mM CaCl₂. After the addition of calcium, fusion begins and is not affected when stirring ceases 8 min later. Stirring is resumed in the intervening 12 min, at the end of which fusion has nearly plateaued. 2 min after the addition to the trans compartment of 50 μl of sonicated decane in buffer (1 ml decane in 15 ml buffer), the fusion rate accelerates. When stirring is now stopped, the fusion rate slows and eventually ceases after ~4 min; fusion resumes when stirring is begun again. This decane-accelerated fusion is still dependent on the existence of the osmotic gradient. The reduction of the osmotic gradient by the addition of 300 mM urea to the trans compartment stops fusion.

in these membranes there may be small amounts of hydro-carbon in the membrane.)

Fusion with decane-containing membranes differs from that with "hydrocarbon-free" membranes in one significant respect: the former is sustained only while the aqueous phases are stirred. Fusion, which is proceeding normally, ceases within 1 min after stirring is stopped and commences again shortly after it is resumed (Fig. 6). This phenomenon is repeatable several times on the same membrane. In contrast, fusion of vesicles to "hydrocarbon-free" membranes is totally unaffected by stirring. It is only stirring in the vesicle-containing (cis) compartment that influences fusion to decane-containing membranes; fusion rate is unaffected by stirring in the trans compartment.

What is the possible meaning of these differences between "hydrocarbon-free" and decane-containing membranes? We believe that there are two steps in the fusion process: first, attachment of vesicles to the planar membrane leading to a "pre-fusion" state, and second, osmotic swelling of these attached vesicles that can ultimately result in fusion. In the accompanying paper (2) we show that these two steps are experimentally separable and that it is the formation of the pre-fusion state that is stirring dependent in decane-containing membranes. A possible interpretation of the stirring effect, therefore, is that the pre-fusion state cannot form in the bilayer proper of decane-containing membranes, but can occur in the toroidal region. Stirring in the cis compartment may then sweep these vesicles into the bilayer region where they can osmotically swell and fuse. The differences between PE and PC, which we attribute to the pre-fusion process (2), are masked in decane-containing membranes, possibly because attachment occurs in the toroidal region.

If the pre-fusion state is formed in the toroidal region of decane-containing membranes, where is it formed in "hydro-carbon-free" membranes? At present we cannot say with certainty that this occurs in the bilayer proper. The energy barrier to vesicle-planar membrane attachment, which we discuss in the accompanying paper, may be fortuitously low in the boundary region between monolayer and bilayer. If so, other factors must exist in biological systems that reduce the energy barrier for vesicle attachment to the plasma membrane.

A "hydrocarbon-free" membrane can be converted to a decane-containing membrane by adding a suspension of decane droplets to the trans compartment (Fig. 7). The capacitance of the planar membrane declines to ~60% of its previous magnitude, presumably because of decane entry into the membrane, and fusion is affected in two ways: first, the rate increases dramatically, and second, it is now stirring-dependent.

Effect of Lipid Composition on Fusion ("Hydrocarbon-Free" Bilayers)

NEGATIVELY CHARGED LIPIDS: An osmotic gradient and 15 mM Ca^{++} are both necessary to obtain fusion when vesicles and/or planar membranes contain negatively charged lipids (e.g., Figs. 2 and 8). (Occasionally low rates of fusion occur with just an osmotic gradient, as in Fig. 2 and 8). Examples include fusion of PC and PC:PE (1:1) vesicles with either asolectin, PC:PS (4:1), or PE:PS (4:1) membranes; fusion of PC:PS (4:1) and PE:PS (4:1) vesicles with PC, PE, and PC:PE (1:1) membranes, and fusion of PC:PS (4:1) and PE:PS (4:1) vesicles with asolectin, PC:PS (4:1) and PE:PS
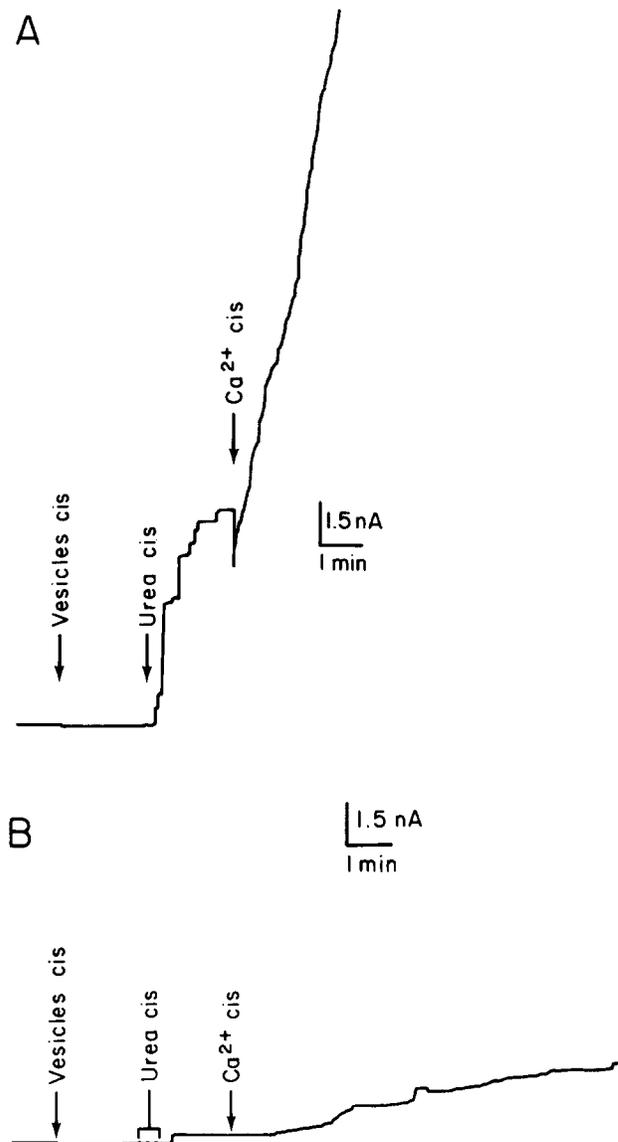

Figure 8   Demonstration that vesicles fuse much more readily to a PE-containing, negatively charged "hydrocarbon-free" membrane than to a PC-containing, negatively charged "hydrocarbon-free" membrane. (A) Fusion to a plant PE:PS membrane. A plant PE:PS (4:1) membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0). Vesicles (egg PC:PS, 4:1; ~10^{11}/ml) are added to the cis compartment, and in the subsequent 2 min no fusion occurs. When an osmotic gradient is formed by addition of urea to the cis side to a concentration of 450 mM, fusion occurs. (The reason for the exceptionally large size of two of the fusion events is unclear.) $CaCl_2$ is then added to the cis side to a concentration of 15 mM. This induces a high rate of fusion. (The reason for the absence of the large events after the addition of Ca^{++} is also unclear.) Fusion continues at a high rate for over 12 min. (B) Fusion to a plant PC:PS membrane. A plant PC:PS (4:1) membrane clamped at V = 20 mV separates the same symmetric solutions as in A. Vesicles (egg PC:PS, 4:1 [the same vesicle preparation as used in A]; ~10^{11}/ml) are added to the cis compartment, and in the subsequent 2 min no fusion occurs. When urea is added to the cis side to a final concentration of 450 mM, there is only one fusion event during the next 2 min (in contrast to the many seen in A under the same circumstances). $CaCl_2$ is then added to the cis side to a concentration of 15 mM. Fusion begins ~1 min later and continues at a slow rate. This slow rate contrasts with high fusion rates to plant PE:PS (seen in A) and asolectin membranes.
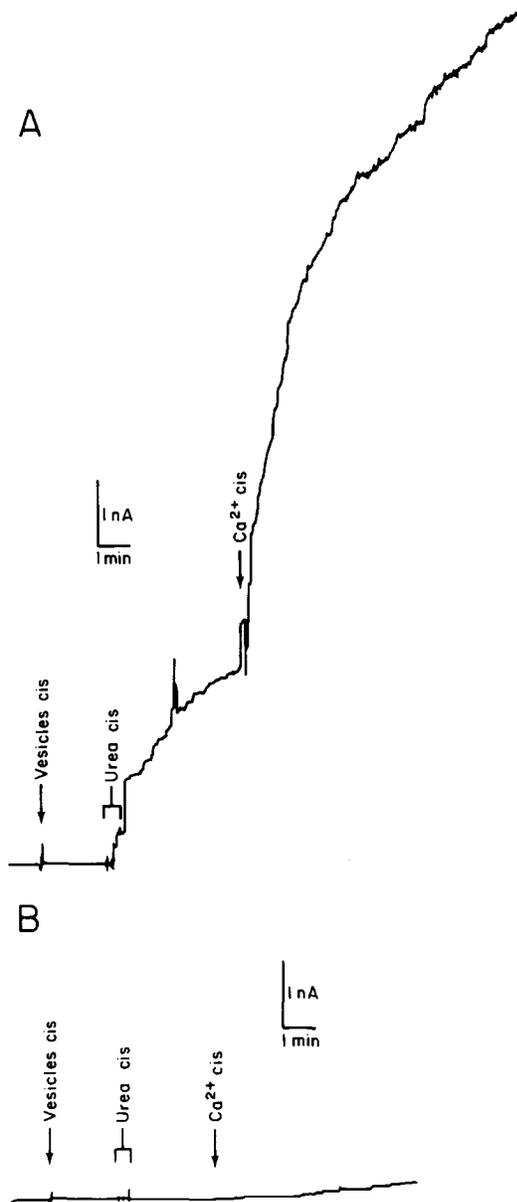
A

B

FIGURE 9 Fusion of uncharged vesicles to uncharged "hydrocarbon-free" membranes. (A) Fusion to a PE membrane. A membrane (E. coli PE) clamped at V = 20 mV separates symmetric solutions (100 mM KCL, 10 mM MES, 2 mM MgCl₂, 0.1 mM EDTA, pH 6.0). When egg PC:egg PE (1:1) vesicles (~10¹¹/ml) are added to the cis compartment, no fusion occurs within the next 2 min. After urea (450 mM) is added to the cis side, fusion begins. 4 min later, CaCl₂ (15 mM) is added to the cis side, and the rate of fusion increases. The rate slows steadily over the next 10 min. (B) Fusion to a PC membrane. A diphytanoyl PC membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 2mM MgCl₂, 0.1 mM EDTA, pH 6.0). Vesicles (egg PC:egg PE,1:1 [same as in A]; ~10¹¹/ml) are added to the cis side. There is no fusion during the next 2 min. When urea is added to the cis side to a concentration of 450 mM, there is still no fusion. When, in addition, CaCl₂ (15 mM) is added to the cis side, a very low rate of fusion results. Thus, although PC:PE vesicles readily fuse with a "hydrocarbon-free" PE membrane, they fuse poorly with a "hydrocarbon-free" PC membrane.

(4:1) membranes. Although the propensity of lipids to transfer from one membrane to another (3, 8, 16) complicates the interpretation of those experiments with charged lipids in only one membrane, our results do suggest that the fusion

process does not require negatively charged lipids in both membranes. In fact, it appears that it does not require them in either membrane. Vesicles made from uncharged lipids (PC:PE [1:1] or PC) readily fuse with planar PE membranes with just an osmotic gradient (Fig. 9A). (For reasons of membrane stability, we always included small concentrations of divalent cation (generally 2 mM Mg⁺⁺) when working with "hydrocarbon-free" membranes. We therefore cannot definitively declare that fusion can occur with "hydrocarbon-free" membranes in the complete absence of divalent cations. We can say, however, that fusion does occur with decane-containing membranes in the complete absence of divalent cations.) (15 mM Ca⁺⁺ increases the fusion rate, possibly because of negatively charged contaminants in the lipids. The significant rates of fusion that occur with no Ca⁺⁺ present illustrate that these presumed negative contaminants are not necessary for fusion. When known amounts of negatively charged lipids are present in the vesicular or planar membrane, such high fusion rates do not occur in the absence of Ca⁺⁺.) Thus, neither Ca⁺⁺ nor negatively charged lipids appear to be necessary for fusion to occur.

PE VS. PC: Vesicles fuse much more readily with PE-containing than with PC-containing planar membranes. This is apparent in three ways. First, good fusion rates occur with PE membranes (see Fig. 9A), but not with PC membranes (Fig. 9B). Second, an osmotic gradient alone will induce reasonable numbers of fusion events (which quickly cease) with PE:PS (4:1) planar membranes but not with PC:PS (4:1) membranes (Fig. 8). Third, after Ca⁺⁺ addition, fusion rates are some 30-fold higher with PE:PS (4:1) membranes than with PC:PS (4:1) membranes (Fig. 8). (An osmotic gradient alone will not induce significant fusion with a negatively charged planar membrane containing both PE and PC [e.g., asolectin], but after Ca⁺⁺ addition, the fusion rate is the same

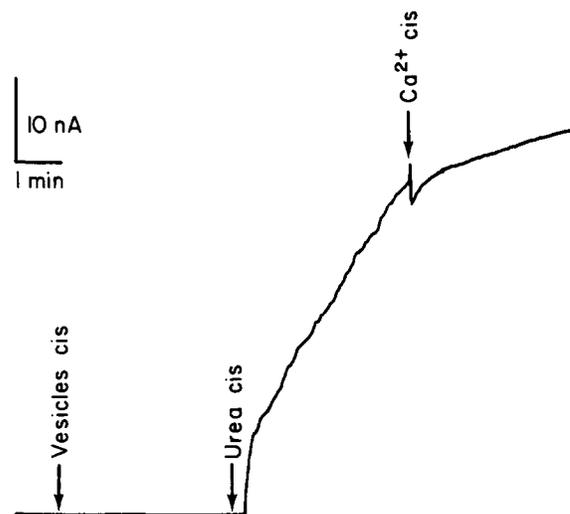
FIGURE 10 Fusion of PC vesicles to a decane-containing PC membrane. A diphytanoyl PC membrane clamped at V = 20 mV separates symmetric solutions (100 mM KCl, 10 mM MES, 1 mM EDTA, pH 6.0). Egg PC vesicles (~10¹¹/ml) are added to the cis side with continuous stirring. There is no fusion during the subsequent 4 minutes. Urea (200 mM) is then added to the cis side, and fusion begins immediately. The rate of fusion is fairly steady for the next 4 min. The addition of CaCl₂ (10 mM) to the cis side appears to slow the rate of fusion. Thus, although uncharged vesicles fuse poorly to a hydrocarbon-free PC membrane (Fig. 9B), they fuse readily to a decane-containing PC membrane (this figure).

as for a PE:PS [4:1] membrane. PC in the planar membrane appears to inhibit fusion if just an osmotic gradient is present, but has little effect on fusion after Ca^{++} is added.)

Distinctions between PE and PC are obscured in decane-containing membranes. Here, presumably because of an effect of decane, an osmotic gradient alone produces high rates of fusion between PC vesicles and PC planar membranes (Fig. 10). This rate of fusion is not increased by the addition of Ca^{++}. (In the experiment shown in Fig. 10, the fusion rate actually appears to be slowed by Ca^{++}.) For the decane-containing membranes it is thus unambiguous that negatively charged lipids are not required for fusion; only an osmotic gradient is required. Similarly, whereas fusion rates are higher in negatively charged PE-containing "hydrocarbon-free" membranes than in negatively charged PC-containing "hydro-carbon-free" membranes, no systematic differential effects exist between negatively charged PE-containing and PC-containing decane-containing membranes.

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