

# ROLE OF CHANNELS IN THE FUSION OF VESICLES WITH A PLANAR BILAYER

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**ABSTRACT** Fluorescence microscopy combined with electrical conductance measurements were used to assess fusion of phospholipid vesicles with a planar bilayer. Large unilamellar vesicles (0.5–3  $\mu\text{m}$  diam.) filled with the fluorescent dye, calcein, were made both with or without porin channels. Vesicle-bilayer fusion was induced by increasing the osmolarity of the solution on the side of the bilayer to which the vesicles were added. Fusion was detected optically by the fluorescent flash due to release of vesicular contents. Although both porin-containing and porin-free vesicles give the same kind of flash upon content release, the conditions necessary to induce release are very different. Only 4% of the porin-free vesicles fuse (release their contents) when subjected to 3 M urea. However, the same conditions induce 53% of the porin-containing vesicles to fuse and most of these fusions occur at a lower osmolarity ([urea] < 400 mM). Thus channels greatly enhance fusion in this model system.

A physical model based on the postulate that fusion is induced by an increase in surface tension, predicts that three conditions are necessary for fusion in this system: (a) an open channel in the vesicle membrane, (b) an osmotic gradient across the bilayer, and (c) the vesicle in contact with the planar membrane. These are the conditions that experimentally produce fusion in the model system.

## INTRODUCTION

Membrane fusion has long been studied in the vesicle-planar-bilayer system. Vesicles may be formed from cell-membrane fragments or purified lipids, and planar bilayers can be formed from a variety of lipids. Previous studies have considered the effects on fusion resulting from changes in bilayer, vesicle, and solution composition. This model system allows a wide range of experimental conditions.

The most common way to induce fusion is with osmotic forces (Miller et al., 1976; Cohen et al., 1984). Usually this is done by changing the osmotic composition of the solution on one side of the bilayer such that the vesicle side (defined as the *cis* side) is hyperosmotic. Although the role of an osmotic gradient is not clear in cellular exocytosis (Rand and Parsegian, 1986), it does seem clear that an osmotic gradient can induce fusion in some biological systems (Zimmerberg and Whitaker, 1985). A variety of osmotic agents, including  $\text{Ca}^{++}$ , urea, and small sugars have been shown to induce exocytosis, although not necessarily by the same mechanism.

The vesicle-bilayer system allows simple detection of fusion. Usually fusion is detected as the bilayer conductance increases after transfer of ion channels from the vesicle membrane to the bilayer. There is convincing

evidence that this transfer is a result of vesicle-bilayer fusion (Cohen et al., 1980; Woodbury and Hall, 1988). However, such an assay makes it impossible to study the effect of channels as such on fusion.

To determine what role, if any, channels play in the fusion process, we used a new fusion assay first suggested by MacDonald (Kendall and MacDonald, 1982). The assay, as developed in our laboratory, uses fluorescence microscopy to visualize large unilamellar vesicles filled with the fluorescent dye, calcein. Fusion is detected by the flash of light observed as vesicles fuse with the bilayer and release their contents. Vesicles were made with or without the large ion channel, porin. This made it possible to assess the role of the channels themselves in the fusion process.

Unexpectedly, the fluorescence assay for fusion gives very different results for vesicle containing the ion channel porin than for vesicles without porin. As previously reported, fusion (content release) was observed with porin-containing vesicles when the *cis* solution was made hyperosmotic. In contrast, only highly hyposmotic solutions were effective at inducing fusion in porin-free vesicles.

These experimental results are predicted by a simple model which postulates that fusion is induced osmotically by an increase in surface tension. This surface-tension-induced fusion (STIF) model predicts that the following conditions should promote fusion in the vesicle-bilayer system: (a) an open channel is in the vesicle membrane, (b) an osmotic gradient is across the bilayer, (c) the vesicle is in contact with the bilayer.

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## METHODS

### Chemicals and Solutions

All solutions contained 400 mM KCl, 0.1 mM CaCl<sub>2</sub>, and 15 mM Hepes (*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.0, and were adjusted to have an osmolarity of 740–755 mOsm, except as noted (e.g., urea-containing solutions). The osmolarity of all solutions was measured with a 5120-A vapor-pressure osmometer (Wescor Inc., Logan UT).

Porin was generously given to us by Dr. H. Nikaïdo (University of California, Berkeley) and by Dr. F. Cohen (Rush Medical College, Ill.).

The fluorescent dye, calcein, was chosen as a suitable marker of vesicle contents. The proposed structure of calcein is shown in Fig. 1. Calcein is a derivative of fluorescein and thus has the advantage of less overlap between excitation and emission spectra than some other dyes (e.g., rhodamine). Calcein is larger and more highly charged than fluorescein and thus is less membrane permeable. This makes it suitable as a marker of the aqueous interior of vesicles.

Calcein was first obtained as the sodium salt (Fluorescein Complexon) from Kodak Laboratory Chemicals (Rochester, NY). Before use, the dye was purified by evaporation, reprecipitation, and elution from a Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) column. Contaminating volatile solvents were first removed by storing calcein desiccated and under vacuum for 24 h. Second, the dry calcein was dissolved in a minimum of water at pH 6 and then reprecipitated at pH 2.5 by addition of acid (3 N HCl). The precipitate was collected with a scintered glass filter and rinsed with a small volume of 3 mM HCl in distilled water. Finally, the precipitated calcein (now an acid) was prepared and checked for purity on TLC plates as described (Allen, 1984) using a 4 cm × 10 cm column of Sephadex G-25-150 and eluting with distilled water. Calcein was also obtained as the acid from Sigma Chemical Company, St. Louis, MO. Dye from this source was not reprecipitated but was eluted from Sephadex as described above. In both cases, the pure calcein fraction was adjusted to pH 7.0 and 745 mOsm (~170 mM calcein).

### Optical Equipment

A fluorescence microscope model UEM (Carl Zeiss Inc., Thornwood, NY) was used in preliminary experiments. Similar results were obtained in later experiments by using a (model M; Nikon Inc., Instrument Div., Garden City, NY) microscope modified for fluorescence. The modification added the same Zeiss filters (FITC) to the Nikon, but used a quartz halogen lamp (24 V 250 W) instead of a mercury arc lamp (100 W). The quartz halogen lamp was normally powered at 23 V to prolong bulb life. When recording on video tape the voltage was raised to 26 V for better detection of faint vesicles. Both microscopes had a 26 times objective with a 6.8 mm working distance (numerical aperture 0.41, Zeiss no. UD 46 20 46). The objective was mounted so that the optical axis was horizontal by means of an adapter containing an elliptical front surface mirror. The microscope was equipped with an image-intensified camera (RCA Broad-

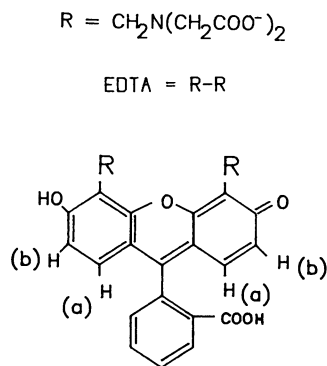


FIGURE 1 Proposed structure of the fluorescent dye, calcein. In this figure the two R groups are located differently from other published structures of calcein (Wallach et al., 1959; Kodak Laboratory Chemicals, 1985). Only this structure however, is consistent with the H-NMR spectra which shows splitting between two sets of two protons (a and b) at 6.7 ppm and at 7.3 ppm (Woodbury and Jacobs, unpublished results).

cast Systems, RCA Corp, Camden, NJ) connected to a television monitor and a AG-6200 video recorder (Panasonic Co., Div. of Matsushita Electric Corp. of America, Secaucus, NJ).

A fluorimeter (Spex Fluorolog, Spex Industries Inc., Metuchen, NJ) was used to measure intensity of calcein fluorescence as a function of concentration. Round, 1 cm cuvettes were filled with different calcein concentrations (in standard KCl solution) and were excited at 460–461 nm. Light emission from 500–530 nm was recorded at 90° to the exciting light path.

### Planar Bilayers

The bilayer chamber was made of Teflon and divided into two compartments by a septum made of 0.13-mm thick Teflon film. Membranes were formed on a small (250–300 μm diameter) hole punched in the center of the septum. The hole and the membrane were observed through a 0.15-mm thick glass window (no. 1 thickness cover slip) and 5 mm of *trans* solution. The volumes of *trans* and *cis* compartments were ~350 and 650 μl respectively.

Before each use the chamber was cleaned according to the following procedure. The Teflon chamber was rinsed in H<sub>2</sub>O, drained, and set upright in a Teflon container. The chamber was washed with acid by filling the container with 80% H<sub>2</sub>SO<sub>4</sub>, 20% HNO<sub>3</sub>, and bath sonicating 10–20 min. The acid was returned to the acid bottle and the chamber rinsed in H<sub>2</sub>O and then EtOH (95%). The wash and rinse was repeated except base (0.1 N KOH in 100% EtOH) was used. Finally, the container was filled with EtOH (100%) and sonicated for 15–60 min. Before use, the chamber was dried at 70°C.

The planar bilayer was formed from bacterial phosphatidylethanolamine (PE) and soy phosphatidylcholine (PC) in a 2 to 1 ratio. The lipids, reported to be >99% pure, were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Before forming the membrane, the hole was treated with ~0.5 μl of a pentane solution containing 5 mg lipid/ml and allowed to dry. The chambers were then filled with solution.

A membrane was formed by blowing an air bubble over the hole with a 10 μl pipet coated with membrane-forming solution (15 mg lipid/ml decane). Pipets were coated by dipping them in this solution and then blowing the solution back out. This left a lipid/decane solution coating the inner wall of the pipet. This coated pipet was used to blow a bubble in the *cis* chamber and the bubble was brushed over the hole in the septum; the membrane formed spontaneously shortly thereafter. The membranes thus formed had a capacitance of 100–250 pF. This capacitance changed <20% during the course of an experiment.

### Vesicles

The preparation of large (0.5–3.0 μm) unilamellar vesicles was based on the procedure described by Kim and Martin (1981) but differed from their procedure in certain significant details. The following three solutions were prepared: (a) an organic solution composed of 6.7 mg PC (Phosphatidylcholine type II-S from Sigma Chemical Co.), 2.0 mg cholesterol, and 0.3 mg triolein dissolved in 1 ml chloroform and 1 ml diethyl ether; (b) a KCl solution containing 400 mM KCl, 0.5 mM EDTA ([ethylenediamine]tetraacetic acid), and 15 mM Hepes, pH 7.0; and (c) a MgCl<sub>2</sub> solution made by adding 1.5 mM MgCl<sub>2</sub> to the KCl solution.

Two different emulsions were formed by shaking together the above solutions on a calibrated vortex (as described by Kim and Martin, 1981). First, an organic-in-aqueous emulsion was formed by adding 1 ml organic solution to 5 ml of the MgCl<sub>2</sub> solution and shaking 20 s in a 12 ml vial. Second, an aqueous-in-organic emulsion was formed by injecting (with a no. 22, 1.5 inch needle) 1 ml of the MgCl<sub>2</sub> solution containing 100–150 μg porin into 1 ml organic solution and shaking 150 s in a 2.5 ml vial. Finally, a double emulsion was formed by adding the aqueous-in-organic emulsion to the organic-in-aqueous emulsion and shaking 90 s in the 12 ml vial. (The organic-in-aqueous emulsion was shaken for an additional 15 s just before the final addition.) The double emulsion was transferred to a 50-ml

round bottom flask. The flask was then mounted on a Rotavapor (Buechi, Flawil, Switzerland) and rotated at  $\sim 150$  rpm.

The organic solvent was removed by evaporation. This was done under a vacuum of 18–20 inches Hg at a temperature of  $30^{\circ}\text{C}$  for 10–30 min. After  $\sim 5$  min the viscosity of the solution markedly decreases. Then the vacuum is raised to 25–26 inches Hg for 60 min to remove trace organic solvent. The remaining solution ( $\sim 5.5$  ml) was diluted 1:1 with the KCl solution and filtered through a polycarbonate filter with a pore size of  $3\ \mu\text{m}$ . The solution was divided into 10 aliquots and stored at  $4^{\circ}\text{C}$  in 1.5 ml centrifuge tubes. Vesicles that did not contain porin were made the same way only without porin.

Shortly before each bilayer experiment, vesicles were loaded with calcein. Stored aliquots of porin-containing and porin-free vesicles were concentrated by centrifuging for 5 min at 16,000 *g*. All but  $50\ \mu\text{l}$  of supernatant was removed and the pellet was resuspended in  $350\ \mu\text{l}$  of isosmotic calcein solution ( $\sim 170$  mM). Porin-containing vesicles were soaked in calcein at  $22^{\circ}\text{C}$  for 10 min. Porin-free vesicles were soaked at  $30^{\circ}\text{C}$  for 30 min as they load with calcein more slowly. The vesicle-containing solutions were then diluted with  $950\ \mu\text{l}$  of cold ( $4^{\circ}\text{C}$ ) KCl solution. Vesicles that did not load with calcein floated in this solution and were removed with the supernatant after centrifugation for 5 min at 16,000 *g*. The pellet, which consisted of calcein-loaded vesicles, was resuspended in 1 ml of cold isotonic KCl buffer, and again centrifuged. This final supernatant was removed and the pellet resuspended in the same cold buffer to give  $100\ \mu\text{l}$  of vesicle solution. Vesicles thus prepared contained  $\sim 10$ – $100$  mM calcein and enough KCl to balance the osmolarity. These vesicles slowly leak calcein but are clearly visible for the 30 min duration of the experiment.

To verify that most vesicles made with porin really contained porin, porin-free vesicles were also prepared with a short calcein soak (10 min at  $22^{\circ}\text{C}$ ). The pellet from these porin-free vesicles was  $<25\%$  the size of porin-containing vesicles, demonstrating that porin substantially aids the entry of calcein into vesicles. Thus, over 75% of the vesicles made with porin contain active porin channels.

### Ejection Pipet Fabrication

Pipets for ejecting vesicles near the bilayer were formed by pulling 1.8 mm OD glass pipets to  $\sim 7\ \mu\text{m}$  and using a microforge to smooth and shrink the tip to  $4$ – $6\ \mu\text{m}$ . A right angle bend was made  $\sim 4$  mm from the tip with a small bunsen burner. The pipet was filled with  $\sim 15\ \mu\text{l}$  of vesicle solution and loaded into a holder mounted on a 3-axis micro manipulator. For each experiment the tip of the pipet was moved within  $\sim 40\ \mu\text{m}$  of the membrane. This delicate task was simplified by using the pipet as a light pipe and illuminating the tip by shining bright light directly at the other end. Vesicles were ejected for 5–20 s by applying pressure to a 10 ml syringe connected through tubing to the pipet.

### Vesicle-Bilayer Adhesion

Adhering vesicles were seen as fluorescent disks that remain fixed in the same plane of focus as the bilayer. Adhesion was confirmed by stirring the *cis* chamber and observing the movement of the vesicles. Vesicles not adhering to the bilayer quickly moved out of focus. If fewer than five adhering vesicles were observed 4–8 min after ejection near the bilayer, the pipet was again moved near the bilayer and more vesicles were ejected. After 5–50 vesicles adhered to the membrane, the experiment was begun and urea solutions were perfused into the *cis* chamber.

### Optical Detection of Content Release

Fig. 2, a plot of fluorescence versus dye concentration, shows the property of self-quenching fluorescence from calcein. Note that the total fluorescence increases to a maximum and then decreases with increasing dye concentration. The apparent calcein concentration at which self-quenching begins depends on path length and thus on vesicle size. The curve through the solid symbols in Fig. 2 shows an intensity maximum at  $\sim 30\ \mu\text{M}$  (point D). These data were obtained with a 1 cm path length. The

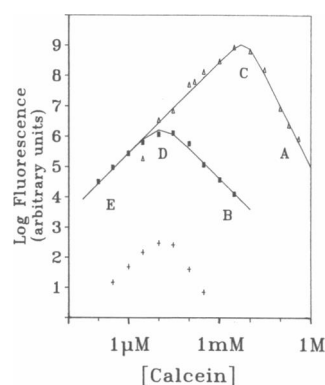


FIGURE 2 Concentration dependence of calcein fluorescence measured with a fluorimeter with a 1 cm path length (solid symbols) and measured with a fluorescence microscope using a  $30\ \mu\text{m}$  path length (triangles). The two sets of data were scaled to overlap at low calcein concentrations. The microscope measurements were obtained by placing  $19\ \mu\text{l}$  of dye solution under a  $25 \times 25$  mm cover slip and measuring the total fluorescence of the video image (Woodbury, 1986). Neutral density fil-

ters were used to expand the intensity range. Decreased fluorescence due to quenching by divalent cations is also shown (crosses). The quenched data shown is for 1 mM free  $\text{Co}^{++}$  (1.5 mM added  $\text{Co}^{++}$ ). The solid lines are empirical fits to the data points. All measurements were made with calcein dissolved in a solution of 400 mM KCl, 15 mM HEPES (pH 7.0), and 0.5 mM EDTA.

curve through the triangles shows a maximum at  $\sim 6$  mM (point C). These data were obtained with a  $30\ \mu\text{m}$  path length. Below this concentration (point E), fluorescence decreases due to a decrease in the number of molecules that can fluoresce. Above this concentration (point A or B), self-quenching occurs.

The property of self-quenching is the basis of a useful method for detecting content release. The method depends on filling vesicles with self-quenched calcein, and is qualitatively modeled with the aid of the equation for hemispherical diffusion (Woodbury, 1986). If vesicles are filled with 100 mM calcein they fluoresce weakly (point A, Fig. 2). When a vesicle fuses with a planar bilayer then the dye within the vesicle is released and diffuses away. As the dye is diluted by diffusion, its fluorescence increases, until its concentration is  $\sim 6$  mM (point C). Further dilution of the dye leads to a decrease in fluorescence down to background (point E). This process is seen in the microscope as a flash. For vesicles not filled with self-quenched concentrations of calcein, the release of contents appears as a disappearance. Although the number of observed flashes or disappearances seen on a given video tape varies with observer, probably because different observers have different thresholds of vision, the ratio of hypertonicity-induced flashes to total flashes is nearly independent of observer. This is discussed below.

### Experimental Protocol

In a typical experiment a bilayer was formed in a chamber filled with standard KCl solution (400 mM KCl, 15 mM HEPES pH 7.0, and 0.1 mM  $\text{CaCl}_2$ ). A glass pipet filled with vesicles (equilibrated in isosmotic solution) was moved close to the *cis* side of the bilayer and vesicles were ejected toward the bilayer. The pipet was removed and the *cis* side perfused with a few milliliters of standard KCl solution.

A video recorder was used to record all optical and electrical events occurring at the bilayer. Optical events (e.g., the release of dye from vesicles) were recorded as the video signal from a camera attached to the microscope. Electrical events were recorded on the audio channels of the video tape as the frequency-modulated current through the bilayer. Optical and electrical events were induced as described below by changing the osmolarity of the *cis* solution.

Solutions containing standard KCl solution and different amounts of urea were perfused through the *cis* chamber as follows (protocol A): control (0 mM urea), 100 mM urea, 400 mM urea, 1.5 M urea, 0 mM urea. About 4 ml (over five chamber volumes) of each solution were perfused at a rate of 2–3 ml/min. Brief stirring preceded perfusion of each new solution. Additional protocols are listed in Table I. Experiments

TABLE I  
PERFUSION PROTOCOLS USED TO INDUCE FUSION

Protocol name	Urea concentration			Amount (each)
	1st	2nd	3rd	
A	100 mM	400 mM	1.5 M	4 ml
B	400 mM	3.0 M		3 ml
C	3.0 M			2 ml

Each protocol began by perfusing the *cis* chamber with standard KCl solution (400 mM KCl, 15 mM Hepes pH 7.0, and 0.1 mM CaCl<sub>2</sub>). Next the osmolarity was increased by perfusion with different urea solutions according to protocol A, B, or C. Each new solution contained the listed amount of urea in standard KCl solution. Finally the osmolarity was lowered to the original value by perfusion with 5 mL of the original KCl solution. The volume of the perfused chamber was ~650 μL.

were analyzed by reviewing the video-taped record of vesicles that released contents with each urea perfusion.

### Analysis of Recorded Experiment

Most optical events occurred during the first minute or two of perfusion and were separated by several seconds. Events were analyzed at a time resolution of ~0.4 s. This was done by recording the FM-demodulated current trace on a chart recorder equipped with an event marker. The chart recorder was run at a rate of 20 cm/min, and the event marker was triggered by hand every time a flash or disappearance was visually observed on the monitor.

In most experiments with porin-containing vesicles there were step-wise increases in membrane conductance after urea addition. These increases were never seen with porin-free vesicles and were attributed to the insertion of porin channels into the planar bilayer (Cohen et al., 1980).

Because detection of content release depended on subjective observation, the variation in the visual observation from a number of observers was measured. Table II shows the variation of five different observers who

viewed the same video tape segment. Although the absolute count of flashes by each observer vary, the percent of flashes scored after hyperosmotic solution addition is highly consistent between all observers. This shows that the optical results presented here do not depend on scoring marginally detectable flashes (assuming the difference in values between different observers is due to threshold detection).

## RESULTS

### The Effect of Channels and Urea

Although both porin-containing and porin-free vesicles produce the same kind of flash upon dye release, the conditions necessary to induce release are distinctly different. Increasing the osmolarity is ineffective in inducing flashes from porin-free vesicles but is effective with porin-containing vesicles.

The difference in the number of induced flashes was measured using the three different protocols listed in Table I and described in the Experimental Protocol section. Protocol A provided the most gradual increase in osmolarity and also elicited the largest total number of flashes with porin-containing vesicles (Table III). Protocols B and C provided the largest transient decreases in osmolarity (3–0 M) and were most effective in inducing flashes from porin-free vesicles. Table II lists the results from a typical B experiment that was analyzed by five different people.

The difference between porin-free and porin-containing vesicles is manifest as a large difference in the percent of flashes that occurs during urea increase. These percentages are presented in Table III and Fig. 3. The key result is that porin-containing vesicles flash after a gradual increase in osmolarity, whereas porin-free vesicles do not. A sudden decrease in osmolarity, however, causes porin-free vesicles to flash.

TABLE II  
DETECTION OF FLASHES FROM PORIN CONTAINING VESICLES

Perfusion solution	Flashes observed							Average
	DW1	DW2	DC1	DC2	GE	JH	ME	
<u>Experiment 1</u>								
Hyperosmotic flashes	29	27	21	21	17	22	22	23
Hyposmotic flashes	14	12	7	9	6	7	5	9
Total flashes	43	39	28	30	23	29	27	31
Percent hyperosmotic flashes	67%	69%	75%	70%	74%	76%	81%	73%
<u>Experiment 2</u>								
Hyperosmotic flashes	16	14	11	6	9	9	13	11
Hyposmotic flashes	26	23	16	17	26	20	19	21
Total flashes	42	37	27	23	35	29	32	32
Percent hyperosmotic flashes	38%	38%	41%	26%	26%	31%	41%	35%
Average hyperosmotic flashes	53%	54%	58%	51%	45%	53%	59%	53%

The same segment of video tape was viewed once or twice by five different observers. The tape contained the record of two different experiments (1 and 2), each of which involved changing the osmolarity of the *cis* chamber according to protocol B (Table I). Each observer was required to count the number of flashes that occurred after each solution change. Flashes that occur following addition of solutions containing 0.4 and 3 M urea are termed hyperosmotic flashes and flashes that occur after perfusion to decrease osmolarity to the original value are termed hyposmotic flashes. Except for DW the observers knew neither the osmolarity of each solution nor if the vesicles contained porin. (A summary of the results from all experiments, both with and without porin, is presented in Fig. 3.)

TABLE III  
PERCENT OF FLASHES INDUCED WITH AN INCREASE IN UREA, COMPARED WITH THE TOTAL NUMBER OF FLASHES, *N* (THOSE INDUCED WITH AN INCREASE OR A DECREASE)

Protocol name	Porin-containing		Porin-free	
		<i>N</i>		<i>N</i>
A	89%	(525)	24%	(66)
B	51%	(174)	10%	(29)
C	54%	(198)	2%	(92)

Note that for porin-free vesicles there is a low percentage (2–10%) of flashes due to urea increases of up to 3 M. The higher percentage with protocol A, 24%, is because fewer flashes are induced after washout of a lower urea concentration (1.5 M not 3.0 M).

### Conditions Necessary for Content Release

All of the results presented thus far are for vesicles adhering to a planar bilayer; the effects of porin and osmolarity changes were also observed for free-floating vesicles and vesicles adhering to the torus. (The torus is a relatively thick decane ring that supports the planar bilayer. It is covered by a monolayer of lipid). Bursting of free-floating vesicles does not occur under the standard conditions used for vesicle-bilayer fusion. In fact, free-floating vesicles tend to lose water (shrink) when exposed to a hyperosmotic gradient. This was detected as a slow (seconds) decrease in vesicle fluorescence due to increased self-quenching of the more concentrated fluorescent dye, calcein.

Table IV shows the correlation between various experimental conditions and content release as manifested by a flash. Also shown in the table is the expected direction of transient water flow, that is the flow expected after a

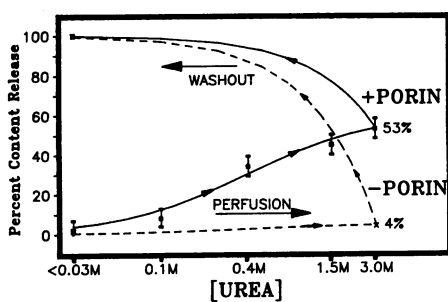


FIGURE 3 The extent of urea-induced content release from vesicles bound to a bilayer is shown for vesicles made with and without the ion channel porin. Perfusion with up to 3 M urea (in standard 400 mM KCl solution) causes ~53% of all the porin-containing vesicles to release their contents. This percent release was found to be independent of protocol (see Table III, protocols B and C). A final perfusion with urea-free buffer produces maximal release. The arrows on the traces show the time sequences of the points. The same perfusion sequence performed with porin-free vesicles shows that perfusion up to 3.0 M urea alone causes only 4% of the vesicles to release their contents. Each point is the average from 5–15 different experiments. Error bars show 95% confidence limits.

TABLE IV  
THE EFFECTS OF OSMOLARITY, PRESENCE OF PORIN CHANNELS, AND VESICLE-BILAYER ADHESION ON CONTENT RELEASE OF VESICLES

Experimental conditions			Results	
Osmolarity of <i>cis</i> solution	Porin?	Adhesion?	Content release?	Direction of transient water flow
Decreasing	no	no	yes	inward
"	no	yes	yes	"
"	yes	no	yes	"
"	yes	yes	yes	"
Increasing	no	no	rarely	outward
"	no	yes	rarely	"
"	yes	no	rarely	"
"	yes	yes	yes	"

An increase in osmolarity means that up to 3 M urea was added to the *cis* solution; a decrease in osmolarity means that the 3 M urea was washed out. The heading "Porin?" refers to whether or not porin channels were reconstituted into the vesicle membrane. A "no" under "Adhesion?" means that the experiment was done with vesicles adhering to the torus or other nonbilayer structure near a planar bilayer (similar results were also obtained from free-floating vesicles in the absence of a planar bilayer). The entries under "Content release?" indicate whether or not flashes were observed for the conditions stated. For each condition, content release is compared with the extent of release for the opposite change in osmolarity. The last column lists the expected direction of transient water flow immediately after the change in osmolarity.

change in osmolarity. An inward transient flow could increase the hydrostatic pressure inside the vesicle and thus the vesicle surface tension. With one exception content release is correlated with conditions expected to produce transient inward water flow. The exception is the case of an osmolarity increase for porin-containing adherent vesicles. It is this case which is found experimentally to give rise to the largest number of correlated fusion events (Woodbury, 1986, Table IV). In this case the experimental conditions are expected to produce not only a transient outward flow of water, but new steady-state conditions that eventually produce a net increase of water inside the vesicle. This case is discussed in detail below.

### THEORY OF SURFACE-TENSION-INDUCED FUSION (STIF)

Vesicles are most likely to fuse when they contain porin, have adhered to the bilayer, and have been exposed to a hypertonic urea solution. This and previous results (Finkelstein et al., 1986) clearly implicate osmotic forces in vesicle fusion. A possible link between osmotic forces and fusion is through the surface tension of the vesicle membrane in relation to that of the bilayer.

When the vesicle fuses with the bilayer, its surface tension must become equal to that of the bilayer. Energetic considerations favor movement of lipid from a high-surface tension system to a low-surface-tension system. Thus, fusion will be energetically favorable only when the vesicle

has a higher surface tension than the bilayer. (The energetic pathway taken by a high-surface-tension vesicle fusing with a low surface-tension planar bilayer is unclear and difficult to determine, nevertheless the endpoints are easy to determine). The surface tension of the vesicle is fixed by the law of Laplace while the surface tension of the planar bilayer is fixed by its composition (Needham and Haydon, 1983). Thus an increase in vesicle surface tension (via the law of Laplace) will tend to promote fusion.

This surface-tension-induced-fusion (STIF) model postulates that an increase in the surface tension of the vesicle membrane induces fusion with a bilayer. Because we do not have a good idea of the activation energy separating the fused and unfused states, we cannot produce a quantitative rate theory of fusion. Nonetheless, the model makes qualitative and testable predictions about the conditions necessary for fusion. These conditions suggest some possibilities for how exocytosis might be induced in cellular systems. The cause of the postulated increase in vesicle surface tension might be very different in a biological system, but in model systems it seems to be an osmotically-induced increase in intravesicular hydrostatic pressure.

For the particular experimental conditions of this study, it is possible to estimate the expected changes in surface tension and thus test the STIF model. We will make a number of simplifying assumptions in order to make the problem tractable. These assumptions will be introduced where appropriate in the course of the calculation. They include the simplification that the process of fusion does not alter the water or urea permeabilities of the contact region between vesicle and bilayer, that the permeabilities of the porin channel to urea and water are nearly equal, and that until fusion occurs, the lipid composition of the vesicles remains unaltered. Using these assumptions, we can relate surface tension to the conditions of our experiments.

Surface tension of the vesicle membrane is proportional to the hydrostatic pressure inside the vesicle (Law of Laplace). Hydrostatic pressure can in turn be controlled by appropriate changes in the osmotic pressure of the bathing solution. The causal chain is thus:

Osmotic Gradient → Increase in Hydro-Static Pressure → Increase in Surface Tension → Vesicle Fusion

Adding hyposmotic solution is the simplest way to increase the hydrostatic pressure inside a vesicle. In this case, there is a transient flow of water into the vesicle. The water flow continues until the vesicle bursts or its contents become isotonic. If hyperosmotic solution is added the vesicle will shrink due to a flux of water out of the vesicle. If the osmotically active solute can readily cross the membrane, then the vesicle may eventually return to its original size as the solute enters the vesicle. Under these conditions there will be no increase in hydrostatic pressure.

Thus, a consideration of transient water flow due to

osmotic gradients correctly predicts the results given in the first seven entries in Table IV but does not predict an increase in hydrostatic pressure for the standard conditions used here (the last entry of Table IV). Transient fluxes would therefore not be expected to increase the surface tension of a porin-containing vesicle adhering to a bilayer, although it is these conditions which were found most likely to induce fusion. Steady-state fluxes however are expected to increase surface tension under just these conditions, as shown below.

### Calculation of the Steady-State Pressure in a Vesicle

Consider a channel-containing vesicle in contact with a planar membrane as shown in Fig. 4. Let  $A_t$  (subscript  $t$  for *trans*) be the vesicular area of contact ( $\text{cm}^2$ ) with the *trans* solution (assumed to be a single bilayer), and  $A_c$  (subscript  $c$  for *cis*) be the area of contact with the *cis* solution, then:

$$A_t + A_c = A, \quad (1)$$

where  $A$  is the total surface area of the vesicle. The water flux ( $\text{cm}^3/\text{s}$ ) through area  $A_t$ ,  $F_{mt}$ , and that through area  $A_c$ ,  $F_{mc}$ , are (subscript  $m$  for membrane):

$$F_{mt} = A_t(p_w V_w/RT)[-P + \Pi_t] \quad (2)$$

and

$$F_{mc} = A_c(p_w V_w/RT)[-P + \Pi_c - \Pi_t]. \quad (3)$$

$P$  is the hydrostatic pressure (atmospheres) in the vesicle (the hydrostatic pressure in the *cis* and *trans* compartments are equal and taken as 0).  $\Pi_t$  and  $\Pi_c$  are the osmotic pressures in the vesicle and *cis* compartment with respect to the *trans* compartment. The square brackets thus contain the total driving force for water flow. The quantity  $(p_w V_w/RT)$  is the hydraulic conductivity (centimeters/seconds/atmospheres) of the membrane to water flow where  $p_w$  is the osmotic permeability (centimeters/seconds) of a membrane to water,  $V_w$  is the partial molar volume of water ( $\text{cm}^3/\text{mol}$ ),  $R$  is the gas constant, and  $T$  the absolute temperature ( $RT \sim 24 \text{ l}^* \text{atm}/\text{mol}$ ).

There is also a water flux through the porin channel.

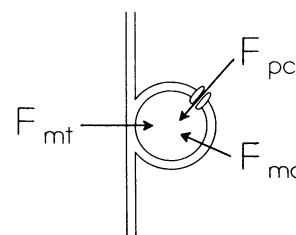


FIGURE 4 The flux of water or urea into a vesicle adhering to a membrane.  $F_{pc}$  is the flux through pores in the *cis* membrane and  $F_{mt}$  and  $F_{mc}$  are the fluxes through the membrane. The arrows show the direction of a positive flux. The area of contact between the vesicle

and the bilayer is denoted as  $A_t$  (for area in contact with the *trans* solution) and the vesicular surface area of no contact is  $A_c$  (for area in contact with the *cis* solution).

This flux,  $F_{pc}$  (subscript p for porin) is given by:

$$F_{pc} = na_p(p_p V_w / RT)[-P + \Pi_v - \Pi_c], \quad (4)$$

where  $a_p$  is the minimum cross-sectional area of the channel,  $p_p$  is the permeability of the channel to water and  $n$  is the number of channels in the *cis* membrane. In steady state (no swelling):

$$F_{mt} + F_{mc} + F_{pc} = 0 \quad (5)$$

that is, the net flow must equal zero. Substituting in the values for each flux term and solving for  $P$ , the pressure inside the vesicle, gives:

$$P = \Pi_v - [\Pi_c(A_c + A_w)/(A + A_w)], \quad (6)$$

where

$$A_w = na_p p_p / p_w \quad (7)$$

is the equivalent area of the channel for water flow. For the simple case of a nonadhering vesicle, the area of contact with the bilayer is zero,  $A_c = A$ , and the hydrostatic and the osmotic pressures are equal and opposite.

Next, consider the flux of an osmotically active molecule, such as urea, through  $A_c$  and  $A_t$  into the vesicle. In this case, hydrostatic pressure does not affect the flow and the concentration gradient is the only driving force. Thus

$$F_{mt}(\text{urea}) = A_t p_u [C_t - C_v] \quad (8)$$

$$F_{mc}(\text{urea}) = A_c p_u [C_c - C_v] \quad (9)$$

and

$$F_{pc}(\text{urea}) = na_p p_p [C_c - C_v] \quad (10)$$

where  $p_u$  is the permeability (centimeters/seconds) of the membrane to urea, and  $C_c$ ,  $C_t$ , and  $C_v$  are the concentrations (mol/liter) of urea in the *cis*, *trans*, and vesicular compartments respectively.

In Eq. 10, the permeability of the channel to water,  $p_p$  (sub p for pore), is assumed to be the same as the permeability of the channel to urea. This is a reasonable assumption because the porin channel is large and molecules inside it can be treated, to a first approximation, as though they were in water. Thus, because the aqueous diffusion constants of water and urea are nearly equal, their diffusion constants inside the porin channel should also be nearly equal. Because permeability (in a bulk medium) and diffusion constant are related only by geometric factors, the permeability of the porin channel to water and the permeability to urea should be nearly equal.

If urea is added only to the *cis* compartment than  $C_t$  is zero and the steady-state equation for urea flow is:

$$F_{mt}(\text{urea}) + F_{mc}(\text{urea}) + F_{pc}(\text{urea}) = 0. \quad (11)$$

Substituting the values for each term and solving for the

steady-state urea concentration inside the vesicle,  $C_v$ , gives:

$$C_v = C_c(A_c + A_w)/(A + A_w), \quad (12)$$

where

$$A_w = na_p p_p / p_u \quad (13)$$

is the equivalent area of the channel for urea flow. Again, for the simple case of a nonadhering vesicle ( $A_c = A$ ) at a steady state, the concentration of urea in the vesicle,  $C_v$ , equals the concentration of urea outside,  $C_c$ , and there is no osmotic or hydrostatic pressure.

We now have an equation for the steady-state urea concentration in the vesicle (Eq. 12) and an equation for the pressure in the vesicle as a function of osmotic pressure (Eq. 6). Osmotic pressure is itself a function of urea concentration:

$$\Pi = RT(C_1 - C_2). \quad (14)$$

Thus, the hydrostatic pressure inside the vesicle can be written as a function of the relative area of vesicle-bilayer contact, the relative permeabilities of the pore to water and

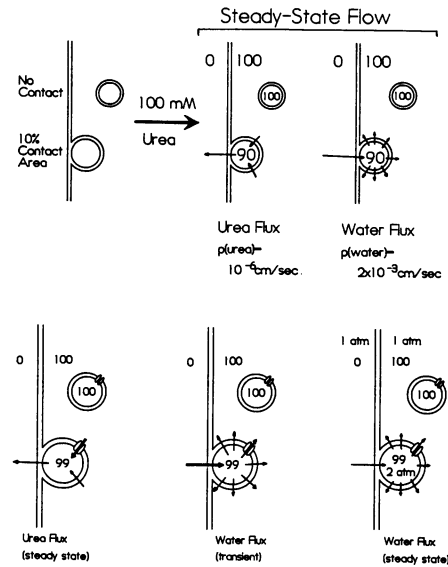


FIGURE 5 Demonstration of how a hydrostatic pressure can develop in a pore-containing vesicle adhering to a bilayer with an osmotic gradient across it. Each compartment is labeled with a number showing its urea concentration in millimolar. The top three panels show the expected urea and water flux for vesicles without pores. The water flux is due only to osmotic gradients, and no hydrostatic pressure is developed. The bottom left panel shows the steady-state concentration of urea and the direction of urea flux for vesicles with a pore. The bottom right panel shows the water flux and the steady-state hydrostatic pressure that must develop so that water efflux matches water influx. The central panel indicates the increase in water influx due to the increased osmotic driving force before a hydrostatic pressure develops.

urea, and the *cis* urea concentration:

$$P = RTC_c(A_c + A_u)/(A + A_u) - RTC_c(A_c + A_w)/(A + A_w) \quad (15)$$

or

$$\frac{P}{RTC_c} = \frac{A_c + A_u}{A + A_u} - \frac{A_c + A_w}{A + A_w} \equiv K, \quad (16)$$

where  $K$  is the normalized pressure.

### Without a Channel, there is no Steady-State Hydrostatic Pressure

We can now consider the steady-state consequence of adding hypertonic urea to a vesicle-bilayer system. First consider a vesicle without a channel. The top half of Fig. 5 shows how urea addition affects both free-floating and adhering vesicles. If 100 mM urea is added to the *cis* side of the bilayer then, in the case of a free-floating vesicle, the urea will slowly enter the vesicle until it contains 100 mM urea and there will be no further net urea or water flux. This is because urea is a membrane-permeable solute and equilibrates rapidly across the membrane, even though it is about a thousand times less permeable than water.

The urea and water fluxes are more complicated for an adhering vesicle. If we assume that the vesicle has a 10% area of contact then the concentration of urea inside the vesicle at steady state would be 90 mM (Eq. 12, where  $n = 0$ ). Thus there is a steady flow of urea through the vesicle to the *trans* side and an opposite flow of water through the vesicle to the *cis* side. This steady-state flux is due solely to the osmotic gradient across each membrane and generates no hydrostatic pressure inside the vesicle.

### With a Channel, a Hydrostatic Pressure is Generated

Now consider the case that these vesicles contain a pore in their membranes, as shown in the lower half of Fig. 5. For a free-floating vesicle this will not change the internal urea concentration and there will still be no net urea or water flow. However, for an adhering vesicle the situation is more complicated. If we assume, for this example, that the flow of urea through the pore is about ten times larger than the flow of urea through the membrane,  $A_u = 10 \times (A)$  (this is the case if a 0.2- $\mu$ m diam vesicle contains one porin channel), then the concentration of urea inside the vesicle at steady state is 99 mM. This initially increases the osmotic driving force for water influx through the area of contact,  $A_i$ , and decreases the osmotic driving force for water efflux through the *cis* membrane,  $A_c$ . Thus there is a transient influx of water. The net entry of water increases the hydrostatic pressure inside the vesicle; this increase will continue until the hydrostatic pressure increases the efflux of water to match the osmotically-driven influx. The

TABLE V  
VALUE OF CONSTANTS USED IN EQ. 15

Parameter	Symbol	Value	Reference
Water permeability	$p_w$	21 $\mu\text{m}^2/\text{s}$	Hanai et al. (1966)
Urea permeability	$p_u$	0.04 $\mu\text{m}^2/\text{s}$	Lippe (1968)
Surface area lipid*	$A_l$	46 $\text{\AA}^2$	Engelman et al. (1972)
Molecular weight lipid*	$MW_l$	0.65 kD	
Molecular weight porin	$MW_p$	36.5 kD	Benz et al. (1978)
Radius of porin channel mouth	$r_p$	4.5 $\text{\AA}$	Benz et al. (1978)

\*Note: lipid values are the average per molecule for a membrane made of 2:1, PC and Cholesterol.

steady-state water flux is shown in the last panel of Fig. 5. Thus, a hydrostatic pressure is generated inside a vesicle only if it contains pores and is adhering to a bilayer with an osmotic gradient across it. According to the STIF model such a vesicle is driven towards fusion if the increased hydrostatic pressure raises the surface tension of the vesicle membrane so that a reduction in system free energy will take place upon fusion.

### An Example

Here we present an illustrative example using reasonable values for the parameters in Eq. 15. We assume the ion channel is porin, that the vesicles are large (0.5–3.0  $\mu\text{m}$  dia), and that there is 100 mM urea on the *cis* side. Table V lists the available values for each of the measurable parameters in Eq. 15. Additional values needed for the calculation are listed in Table VI. Figs. 6 and 7 show plots of the steady-state hydrostatic pressure inside the vesicle as a function of the area of contact,  $A_i$ , and the mass ratio of porin to lipid.

Two important points emerge from the calculations illustrated in these figures. First, we see in Fig. 7 that

TABLE VI  
ADDITIONAL CONSTANTS USED IN EQ. 15

Parameter	Symbol	Value	Formula
Area porin channel	$a_p$	$6.4 \times 10^{-15} \text{ cm}^2$	$\pi(r_p)^2$
Length of porin channel	$l_p$	80 $\text{\AA}$	
Diffusion of water or urea in water	$D$	$5.0 \times 10^{-6} \text{ cm}^2/\text{s}$	
Permeability of porin channel	$p_p$	6.2 cm/s	$D/l_p$
Porin flow	$a_p p_p$	$4.0 \times 10^{-14} \text{ cm}^3/\text{s}$	$\pi D(r_p)^2/l_p$
Relative channel area for urea	$A_u$	$n^* 4.0 \times 10^{-8} \text{ cm}^2$	$na_p p_p/p_u$
Relative channel area for water	$A_w$	$n^* 1.8 \times 10^{-11} \text{ cm}^2$	$na_p p_p/p_w$
Area of 1 $\mu\text{m}$ diam vesicle	$A$	$3.1 \times 10^{-8} \text{ cm}^2$	$\pi d^2$
[Urea] <i>cis</i>	$C_c$	100 mM	



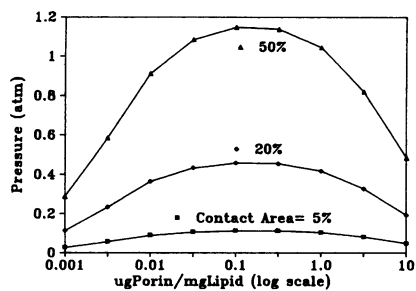


FIGURE 6 Pressure versus [porin] for a 2  $\mu\text{m}$  diam vesicle. The steady-state hydrostatic pressure inside a vesicle adhering to a membrane is shown as a function of the number of porin channels in the vesicle membrane (expressed as micrograms of porin per milligram lipid). This figure is calculated from Eq. 15 and the values given in Tables V and VI. Note that there is an optimum channel density (0.1  $\mu\text{g}/\text{mg}$ ) for maximum hydrostatic pressure (1.15 atm at 50% contact area). This density corresponds to 20 porin channels for a 1  $\mu\text{m}$  diameter vesicle. The optimum density is calculated assuming porin is a monomer. If porin is a trimer the optimum density will be 0.3  $\mu\text{g}/\text{mg}$ .

pressure increases with area of contact. This implies that conditions that increase adhesion also increase fusion. Fig. 6 shows that there is an intermediate value for the porin-to-lipid ratio which maximizes pressure. This value is  $\sim 0.1$   $\mu\text{g}$  porin/mg lipid for the values given in Tables V and VI. This corresponds to 20 porin channels for a vesicle  $\sim 1$   $\mu\text{m}$  diam. This predicted value is close to the average number of channels, 15, seen in real vesicles of approximately the same size, 0.5–3  $\mu\text{m}$  diameter (Woodbury and Hall, 1988).

The surface tension,  $\gamma$ , of the vesicle membrane can now be calculated from the steady-state hydrostatic pressure,  $P$ , using the Law of Laplace:  $\gamma = rP/2$ , where  $r$  is the radius of the vesicle.

Fig. 8 shows a plot of surface tension as a function of the urea concentration ( $C_c$ ), with the vesicle diameter ( $d$ ) as a parameter. Also shown on the figure is  $\gamma_B$ , an estimate of the tension necessary to break a membrane (Kwok and Evans, 1981). In calculating the curves in Fig. 8, an optimum number of porin channels and a contact area of

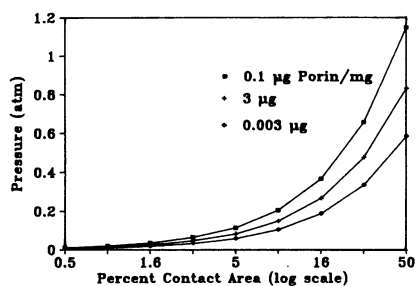


FIGURE 7 Pressure versus contact area for a 2  $\mu\text{m}$  diam vesicle. The steady-state hydrostatic pressure inside a vesicle adhering to a membrane is shown as a function of the percent area of contact between the vesicle and the bilayer. The abscissa is drawn with a log scale such that the left side of the graph represents very little contact (0.5%) and the right side of the graph represents a maximum of 50% contact area.

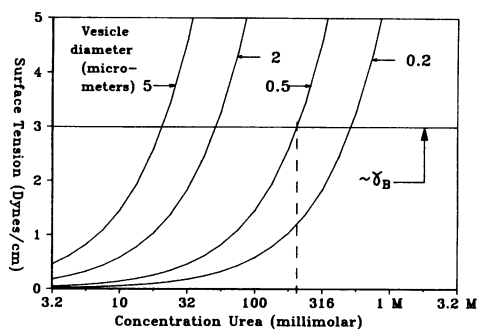


FIGURE 8 The membrane surface tension of an adhering vesicle is plotted as a function of urea concentration on the *cis* side of the bilayer. Each trace in the figure represents a different size of vesicle, as labeled. The tension necessary to break a membrane,  $\gamma_B$  (see text) is indicated. An intermediate value is assumed for porin density (0.1  $\mu\text{g}/\text{mg}$  lipid, see Fig. 6) and for the area of contact (5%, see Fig. 7); other conditions are as given in Tables V and VI.

5% ( $A_i/A = 0.05$ ) were assumed. Under these conditions,  $\sim 200$  mM urea is required to induce sufficient pressure to burst a 0.5  $\mu\text{m}$  diam vesicle ( $\gamma = \gamma_B$ ). The relationship (if any) between the pressure required to burst a vesicle and the critical tension for vesicle-bilayer fusion postulated by the STIF model remains to be established. But it seems reasonable to assume that the critical tension for fusion needs to be less than the bursting tension. Although the surface tension of a bilayer with the composition used in our system is not known, it is probably 1–4 dyn/cm (Needham and Haydon, 1983). This is close to  $\gamma_B$ , the bursting tension for vesicles reported as 3–4 dyn/cm for lecithin vesicles (Kwok and Evans, 1981). If the vesicle bursting tension really is close to the bilayer surface tension, then it might explain why many vesicles burst instead of fuse with higher surface tension (decane) membranes (Woodbury and Hall, 1988) and fewer vesicles burst with lower surface tension (squalene) membranes (Niles and Cohen, 1987).

## CONCLUSIONS

The detection of ion channels transferred from vesicles to a bilayer has long been used as an assay for vesicle fusion (Moore, 1976; Cohen et al., 1980). However, this simple assay cannot be used to study the effect of channels as such on fusion. To determine the role of channels in the fusion process, we used a newly developed fusion assay. Fusion is detected optically as the release of dye from vesicles fusing with the bilayer. As expected (Cohen et al., 1984), fusion was observed with channel-containing vesicles when the *cis* solution was made hyperosmotic. In surprising contrast, the same treatment was ineffective at inducing fusion in channel-free vesicles. These results are qualitatively predicted with a simple model based on osmotic and hydrostatic flow through the vesicle membrane.

The model relates vesicular hydrostatic pressure to urea concentration and the number of porin channels in the vesicle. A positive pressure is generated when the following conditions are met: (a)  $n > 0$ , there must be at least one channel or pore in the *cis* membrane (assuming no channels in the *trans* membrane); (b)  $C_c > 0$ , there is a *trans*-bilayer osmotic gradient with the *cis* chamber hyperosmotic; and (c)  $A_t > 0$ , the vesicle must be bound to the membrane. The conditions which produce a hydrostatic pressure inside the vesicle used here are: pores, an osmotic gradient, and adhesion (mnemonic: POGA). This method of generating a hydrostatic pressure applies only to the experimental system studied here. It is possible, indeed likely, that if surface-tension-induced fusion is involved in exocytosis and fusion in biological systems, other quite different mechanisms might generate the increased pressure or even increase vesicle surface tension without a concomitant change in pressure. Activation of ion pumps, contraction of actin filaments, or the opening of appropriate channels could all lead to an increase in the surface tension of the vesicle membrane.

Inducing fusion by opening a channel in the vesicle membrane is an interesting possibility worth further consideration. The steady-state STIF model predicts fusion (a positive vesicular pressure) not only when channels are in the *cis* membrane but also when they are in the *trans* membrane if the *cis* solution is hyposmotic. This may be the case in biological systems where a gap-junction-like protein could both form a channel in the *trans* membrane and bind the vesicle to the cellular membrane (Breckenridge and Almers, 1987, Zimmerberg et al., 1987). Fusion could also be induced in the absence of an osmotic gradient. If the channel were permeable to some external ions, and impermeable to a major internal ion, then the Gibbs-Donnan effect would cause an increase in intravesicular pressure that could lead to fusion.

Future experiments could test additional predictions of the surface-tension model and its biological relevance. Pertinent questions are: (a) Are channels present in vesicles of biological fusion systems? This question comes from a prediction of the steady-state STIF model which says that POGA (pores, osmotic gradients, and adhesion) are necessary for fusion. Since adhesion and osmotic gradients are important in at least some cases of biological fusion, it follows to determine if pores are also required. (b) Do open channels in the vesicle membrane aid the incorporation of other proteins from the vesicle membrane into planar bilayers? The answer to this question may be important for the reconstitution of some biological proteins into bilayers by vesicle fusion. For example, this may explain why initial studies to reconstitute the  $\text{Na}^+$  channel were only successful when the channel was blocked in the open state with the toxin BTX (Krueger et al., 1983). (c) What role does  $\text{Ca}^{++}$  play in biological fusion? The role of  $\text{Ca}^{++}$  has long been a question in the study of biological fusion. One possibility is that it controls the opening of a channel in the vesicle

membrane which initiates fusion (Stanley and Ehrenstein, 1985).

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