# Video Fluorescence Microscopy Studies of Phospholipid Vesicle Fusion with a Planar Phospholipid Membrane

## *Nature of Membrane-Membrane Interactions and Detection of Release of Contents*

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ABSTRACT Video fluorescence microscopy was used to study adsorption and fusion of unilamellar phospholipid vesicles to solvent-free planar bilayer membranes. Large unilamellar vesicles (2–10 μm diam) were loaded with 200 mM of the membrane-impermeant fluorescent dye calcein. Vesicles were ejected from a pipette brought to within 10 μm of the planar membrane, thereby minimizing background fluorescence and diffusion times through the unstirred layer. Vesicle binding to the planar membrane reached a maximum at 20 mM calcium. The vesicles fused when they were osmotically swollen by dissipating a KCl gradient across the vesicular membrane with the channel-forming antibiotic nystatin or, alternatively, by making the *cis* compartment hyperosmotic. Osmotically induced ruptures appeared as bright flashes of light that lasted several video fields (each 1/60 s). Flashes of light, and therefore swelling, occurred only when channels were present in the vesicular membrane. The flashes were observed when nystatin was added to the *cis* compartment but not when added to the *trans*. This demonstrates that the vesicular and planar membranes remain individual bilayers in the region of contact, rather than melding into a single bilayer. Measurements of flash duration in the presence of cobalt (a quencher of calcein fluorescence) were used to determine the side of the planar membrane to which dye was released. In the presence of 20 mM calcium, 50% of the vesicle ruptures were found to result in fusion with the planar membrane. In 100 mM calcium, nearly 70% of the vesicle ruptures resulted in fusion. The methods of this study can be used to increase significantly the efficiency of reconstitution of channels into planar membranes by fusion techniques.

### INTRODUCTION

Fusion between membranes is a fundamental process occurring in a wide range of biological functions, including release of neurotransmitters and hormones

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(Katz, 1969; Castel et al., 1984), fertilization of egg by sperm (Wasserman, 1987), intracellular membrane trafficking (Lodish et al., 1981), viral infections of cells (White et al., 1983), endocytosis (Goldstein et al., 1979), and cell division (Mabuchi, 1986). In exocytosis, for example, fusion is the incorporation of a secretory granule's membrane into the plasmalemma with the concomitant release of the granule's contents to the extracellular medium. In spite of fusion's ubiquity, little is known about its physical basis or the molecular events that lead to the melding of two membranes into one. As the phospholipid bilayer is the backbone of biological membranes, it is necessary to describe the phenomenology of fusion on the level of phospholipids and it is relevant to determine which characteristics of biological fusion are achieved with lipids alone. With these goals in mind, we are studying a model system: the fusion of phospholipid vesicles to planar bilayer membranes.

The fusion process in this model system has been experimentally separated into two steps. In the first, divalent cations promote a tight adsorption of vesicles to the planar membrane. It is only in the second step that fusion occurs, via osmotic swelling of the vesicles in intimate contact with the planar membrane (Akabas et al., 1984). This separation into two steps used an ion channel reconstituted into vesicles as a marker for fusion. There were two major limitations to this assay. First, quantitative studies of vesicle adsorption were not possible because adsorption does not affect the membrane conductance. Second, only fusion events could be scored; lysing of vesicles resulting from swelling with the consequent release of vesicular contents into the vesicle-containing compartment (*cis*) escaped detection. Assaying release of contents is important because, in biological fusion, contents are presumed to be efficiently transferred. The one previous study that demonstrated release of contents could not assay for vesicle lysis (Zimmerberg et al., 1980).

We have now developed optical techniques that overcome the above-mentioned limitations and have used them to study the nature of adsorption and the sidedness of release of contents. We have measured the dependence of vesicle adsorption on divalent cations and probed the region of contact between the vesicle and planar membranes. We have studied the manner in which divalent cations influence the sidedness of release of contents. The techniques used have direct utility for improving the efficiency of incorporation of channels into planar membranes.

## MATERIALS AND METHODS

### *Overview of Method*

To study binding of vesicles and release of their contents, we loaded vesicles with the fluorescent dye calcein (4′,5′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein) at high concentrations and used a planar bilayer setup built to accommodate a fluorescence microscope with video equipment. The vesicles were sufficiently large (2–10 μm) and the video camera was sufficiently sensitive for the fluorescent vesicles to be observed. This enabled us to view the binding of vesicles to the planar membrane directly. When fluorescent molecules were released, they diffused and became diluted, and the region occupied by dye increased. Release of dye resulting from a vesicle rupture could thus be

observed as a flash of light. The side of the planar membrane to which dye was released as determined by allowing cobalt, added to one side or the other, to quench the calcein fluorescence. Release of dye into cobalt-containing solution resulted in light flashes of short duration, whereas release into cobalt-free solution led to flashes of long duration. In this study, fusion is operationally defined as the transfer of dye to the *trans* side of the planar membrane.

### *Preparation of Vesicles*

Large unilamellar vesicles filled with calcein were prepared by a modification of the double-emulsion method of Kim and Martin (1981). An outline of the procedure is shown in Fig. 1. In this method, the aqueous material to be entrapped is added to lipid dissolved in chloroform. Vortexing results in an emulsion of aqueous spherules suspended in chloroform with a monolayer of lipid at each water-chloroform interface (Fig. 1, step *a*). These spherules will become the internal contents of the vesicles. Lipid dissolved in diethyl ether is emulsified in the buffer that is to become the external medium of the vesicles; this emulsion is termed the "mother liquor" (step *b*). The spherules of entrapped material in chloroform are then injected into the mother liquor. This results in each spherule being surrounded by a shell of chloroform and ether solvent, which in turn is suspended in the outside buffer (step *c*). A lipid monolayer is present at each solvent-water interface. When the solvent is evaporated, the monolayers appose to form a lipid bilayer and vesicles form (step *d*).

We used 10 mg of lipid mixture, consisting of 8 mg crude asolectin (type 2S, Sigma Chemical Co., St. Louis, MO) and 2 mg ergosterol recrystallized once from ethanol, dissolved in chloroform. This mixture was split equally into two 1-dram vials. To one vial, marked "inside," enough chloroform was added to bring the volume to 0.5 ml. A water-in-chloroform emulsion was made in this vial by carefully adding, dropwise, 0.5 ml of dye solution, consisting of 200 mM calcein, 10 mM MES, 5 mM *n*-propyl gallate, and 50 μg/ml nystatin, pH 6.5 (666 mosmol). Calcein (Hach Chemicals, Ames, IA) was purified by the method of Ralston et al. (1981). The *n*-propyl gallate served as an antioxidant to retard fading of the dye under illumination (Giloh and Sedat, 1982). This vial was vortexed at a moderate rate for 40 s to produce spherules of dye. The final diameters of the vesicles are determined by this step: the longer the vortexing, the smaller the vesicles. The lipid mixture in the other vial, marked "outside," was dried under a stream of nitrogen gas and resuspended in 0.5 ml of diethyl ether by vortexing. 2.5 ml of outside buffer, consisting of 700 mM sucrose (856 mosmol), was added to this vial, and the emulsion of mother liquor was made by vortexing for 30 s. The emulsion of the inside vial was pulled up into a Pasteur pipette with a tip drawn to a narrow diameter (<0.5 mm) and injected into the mother liquor. This double emulsion was vortexed for 30 s at a moderate setting. Vortexing determined the distribution of vesicle diameters (Kim and Martin, 1981). This produced aqueous dye spherules, each surrounded by a shell of chloroform-ether solvent, mixed in sucrose buffer with a lipid monolayer at each solvent-water interface. The emulsion was transferred to a 50-ml round-bottomed flask, which was attached to a rotary evaporator and lowered into a water bath at 37°C. The solvent was removed by rotary evaporation with a vacuum pump. Nitrogen was streamed into the flask at a pressure adjusted to avoid boiling of the solvent, which would prevent formation of vesicles. After the solvent was evaporated (which took ~20 min), the vesicles were transferred to a 15-ml conical centrifuge tube, diluted to 8 ml with 700 mM glucose, and pelleted by centrifugation at 1,200 *g* for 15 min in a clinical centrifuge. The vesicles were separated from external dye either by washing them twice in 700 mM glucose or by filtration through a column of Sephadex G-25 (1 × 15 cm) and elution with 700 mM

sucrose. The vesicle pellets were resuspended in 0.25 ml of 700 mM sucrose. The density of vesicles in the preparation was $\sim 10^5$/ml.

Vesicle pellets were fixed for electron microscopy by incubation in 1% osmium tetroxide for 1 h. The pellets were dehydrated in graded series of ethanol and propylene oxide, embedded in Epon 812, and sectioned. Thin sections were stained with uranyl acetate and lead citrate.

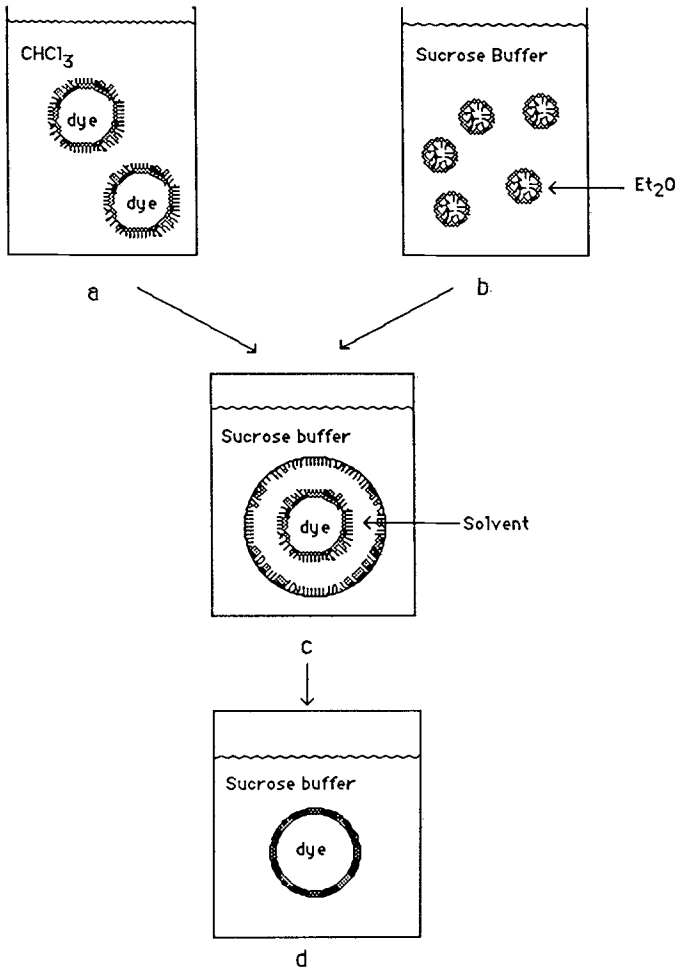


FIGURE 1. Schematic description of formation of large unilamellar vesicles. (*a*) An emulsion of dye-containing aqueous spherules in chloroform is formed. A monolayer of lipid forms at each interface. Each spherule will become the interior compartment of one vesicle. (*b*) An emulsion of ether with dissolved lipid in outside buffer (e.g., sucrose) is prepared. This is the "mother liquor." (*c*) A double emulsion results from mixing the solutions of steps *a* and *b*. The dye spherules are separated from the outside buffer by a shell of chloroform-ether solvent. Lipid monolayers form at each organic solvent-water interface. (*d*) Evaporation of solvent leads to unilamellar vesicles.

### *Video Fluorimeter*

The dye-filled vesicles were observed in a video fluorimeter, as illustrated in Fig. 2. A fluorescence microscope, without a stage, was laid on its back and a membrane chamber was inserted where the stage would normally be. A pipette coupled to a micromanipulator was used to deliver vesicles to the planar membrane. Images were projected in real time onto a video camera via a series of lenses and data were recorded on video tape for later analysis.

The membrane chamber was constructed from milled Teflon, with a black Teflon septum, 125 $\mu$m thick, separating the two compartments. The planar membrane was formed in a circular orifice (160 $\mu$m diam) bored through the center of the septum. The front and rear walls of the chamber were made of glass coverslips (0.17 mm thick),

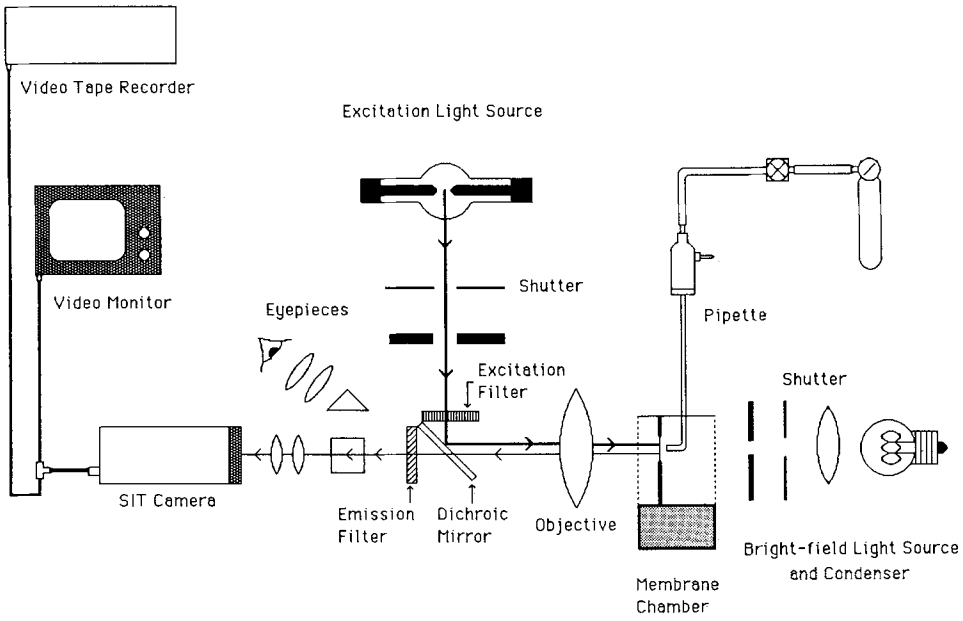


FIGURE 2. Diagram of the membrane fluorescence microscope and video equipment. The light sources, filters, dichroic mirror, objective, condenser, and eyepieces are components of an upright microscope laid on its backbrace. See text for details.

providing unobstructed light paths to both sides of the planar membrane. To minimize image degradation, the distance between the front coverslip and the septum was kept short but experimentally convenient (3 mm). The distance between the septum and the rear coverslip was greater (10 mm), since the rear compartment did not affect image quality under epi-illumination. Ag/AgCl electrodes were placed in each compartment through holes bored in the Teflon and connected to the headstage of a current-measuring amplifier. A small magnetic stirring bar was placed in the rear compartment. The chamber was mounted in a Lucite holder attached to a micromanipulator (model 1951, Narishige Scientific Instruments, Tokyo, Japan), which in turn was mounted on a magnetic base on a vibration-free table (Micro-g, Technical Manufacturing Corp., Woburn, MA). The membrane was brought into focus with the micromanipulator.

We used a Laborlux 11 (E. Leitz, Inc., Rockleigh, NJ) with epi-fluorescence attachments turned on its back brace and bolted to the vibration-free table. The rear (*cis*) compartment of the membrane chamber faced the base of the microscope, which contained the bright-field light source and adjustable condenser lens. An electric shutter (Uniblitz SD-122B, Vincent Associates, Rochester, NY) was placed between the rear of the chamber and the condenser, which allowed us to block the bright-field light path to the planar membrane. Bright-field illumination was used for membrane formation. The objective turret of the microscope, facing the *trans* compartment, was located at the front of the chamber. The objective used for all studies was a Nikon MPlan 20×/NA 0.4 ELWD with a working distance of 11 mm.

The calcein in vesicles adjacent to the planar membrane was excited by epi-illumination; the plane of the membrane was normal to the epi-illumination path. The planar membrane and attached vesicles were viewed through the front compartment of the chamber. The observation path consisted of the objective lens, a fluorescence attachment (Ploemopak 2.4, E. Leitz, Inc.), a binocular eyepiece assembly (Periplan 10×/18, E. Leitz, Inc.), a projection lens, and video camera (SIT 66, Dage-MTI, Michigan City, IN). As illustrated in Fig. 2, the light source for fluorescence excitation was a mercury bulb (HBO 100 W/2) in a housing at the end of the fluorescence attachment; the light path from the bulb was at a 90° angle to the observation path. The light source could be blocked by an electric shutter. The excitation light was passed through a 450–490-nm band-pass filter (BP450-490, E. Leitz, Inc.) in a filter cube (N2, E. Leitz, Inc.), reflected by a dichroic mirror (RKP 510, E. Leitz, Inc.) at a 45° angle to the incident light path, and passed through the objective lens into the front compartment of the chamber. Emission of calcein fluorescence was collected by the objective, transmitted through the dichroic mirror with a lower transmission limit of 510 nm, and passed through a high-pass filter (LP515, E. Leitz, Inc.), isolating the emission wavelengths of calcein >515 nm. The emitted light was magnified by a 15× eyepiece placed in tandem with a 0.3× projection lens, and focused onto the photocathode of the SIT camera. The binocular eyepiece assembly contained partially silvered prisms that allowed emitted light to be directed either to the eyepieces or to the video camera.

The video camera was operated at settings that enabled detection of 200 mM calcein in the vesicles. The gain was manually set at 75% of maximum, thereby disabling a reflex circuit that automatically scaled the gain. Contrast was fixed at maximum. When required, the voltage-clamped membrane current was digitized, and a digital display generated by a modified video date-time generator (Video Timer VTG-33, For-A Corp. Ltd., West Newton, MA) was superimposed on the video image. The composite video signal was displayed on a video monitor (V20, Electrohome Ltd., Kitchener, Ontario, Canada). The total magnification of the optical and video system was 1,000. The video signal was recorded on video tape (3/4-in. U-matic) by a video tape recorder (VO-5800H, Sony Corp. of America, Park Ridge, NJ). All data analysis was performed from the video tape.

### *Delivery of Vesicles*

The pipette used to deliver vesicles to the planar membrane was fashioned after those used to patch-clamp black lipid membranes (Andersen, 1983). A 6-in.-long shank of glass capillary tubing, 0.8 mm o.d. and 0.5 mm i.d. (7740, Corning Glass Works, Corning, NY), was pulled over a gas flame to yield a long, tapered tip. The pipette was cut to yield a right circular break with an outer diameter of 0.2 mm and then forged to make a right-angle bend 5 mm back from the tip. The tip was fire-polished until its inner diameter was 50 μm. Vesicles in sucrose buffer were introduced into the pipette by back-filling from a 3-in.-long 30-gauge needle. The pipette was placed in an electrode holder (EH-2R, E. W.

Wright, Guilford, CT) joined by a Lucite block to a hydraulic micromanipulator (MO-103, Narishige Scientific Instruments). The back end of the pipette, which protruded through the top of the holder, was connected by polyethylene tubing to an electric valve (Picospritzer, General Valve Corp., E. Hanover, NJ), which was connected to a supply of pressurized nitrogen. The pipette was lowered into the rear compartment of the chamber and the tip was brought close to the membrane (5–10 $\mu$m) by means of the micromanipulator. Vesicles were pressure-ejected toward the membrane by opening the valve for 3–4 ms.

### *Chamber Buffers*

The following buffers were used in the chamber: KCl buffer: 400 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.5, 760 mosmol; KCl plus cobalt citrate buffer: 350 mM KCl, 40 mM cobalt citrate, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.5, 770 mosmol; sucrose buffer: 650 mM sucrose, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.5, 830 mosmol; sucrose plus cobalt citrate buffer: 600 mM sucrose, 40 mM cobalt citrate, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.5, 820 mosmol. Cobalt citrate was made by dissolving citric acid in water and then slowly adding cobalt hydroxide powder (Alfa Products, Danvers, MA) in very small increments with vigorous stirring to prevent precipitation.

Vesicle swelling was induced by the addition of aliquots from a stock of either 50 mg/ml nystatin (Mycostatin, Sigma Chemical Co., St. Louis, MO) or 6 M urea to the rear compartment as described in the Results.

### *Planar Membrane Formation*

A planar membrane was formed in the orifice of the black Teflon septum by the union of two phospholipid monolayers (Montal and Mueller, 1972). The lipid used in most experiments was asolectin (type 4S, Sigma Chemical Co.) washed in acetone and ether (Kagawa and Racker, 1971). The composition of this lipid mixture has been determined by Miller and Racker (1976). In experiments in which nystatin channels were incorporated into the planar membrane, the lipid mixture consisted of 4:1 wt/wt asolectin/ergosterol. In all experiments, the hole was precoated with 2% squalene in pentane and the lipid monolayers spread from a 1% solution in hexane on the surfaces of buffer in the two compartments. Membrane thinning was detected electrically by the growth of capacitance, and optically, with bright-field illumination, by the development of a well-defined torus between the edge of the orifice and the planar bilayer. When the monolayers had been raised over the hole, the front compartment contained 0.7 ml of buffer and the rear compartment volume was 1.5 ml.

## RESULTS

### *Microscopy of the Vesicle Preparation*

We determined that our vesicle preparation consisted of vesicles and aggregates of nonvesicular material by viewing thin sections with electron microscopy (Fig. 3*A*). Vesicle profiles were predominantly unilamellar, with membrane thicknesses of 5–10 nm. The profiles appeared circular to elliptical in shape, with diameters ranging from 300 nm to 20 $\mu$m. The profiles of the nonvesicular material were electron dense, much larger than the vesicles, and had little trapped aqueous space. The size distribution of the vesicle preparation is illustrated by

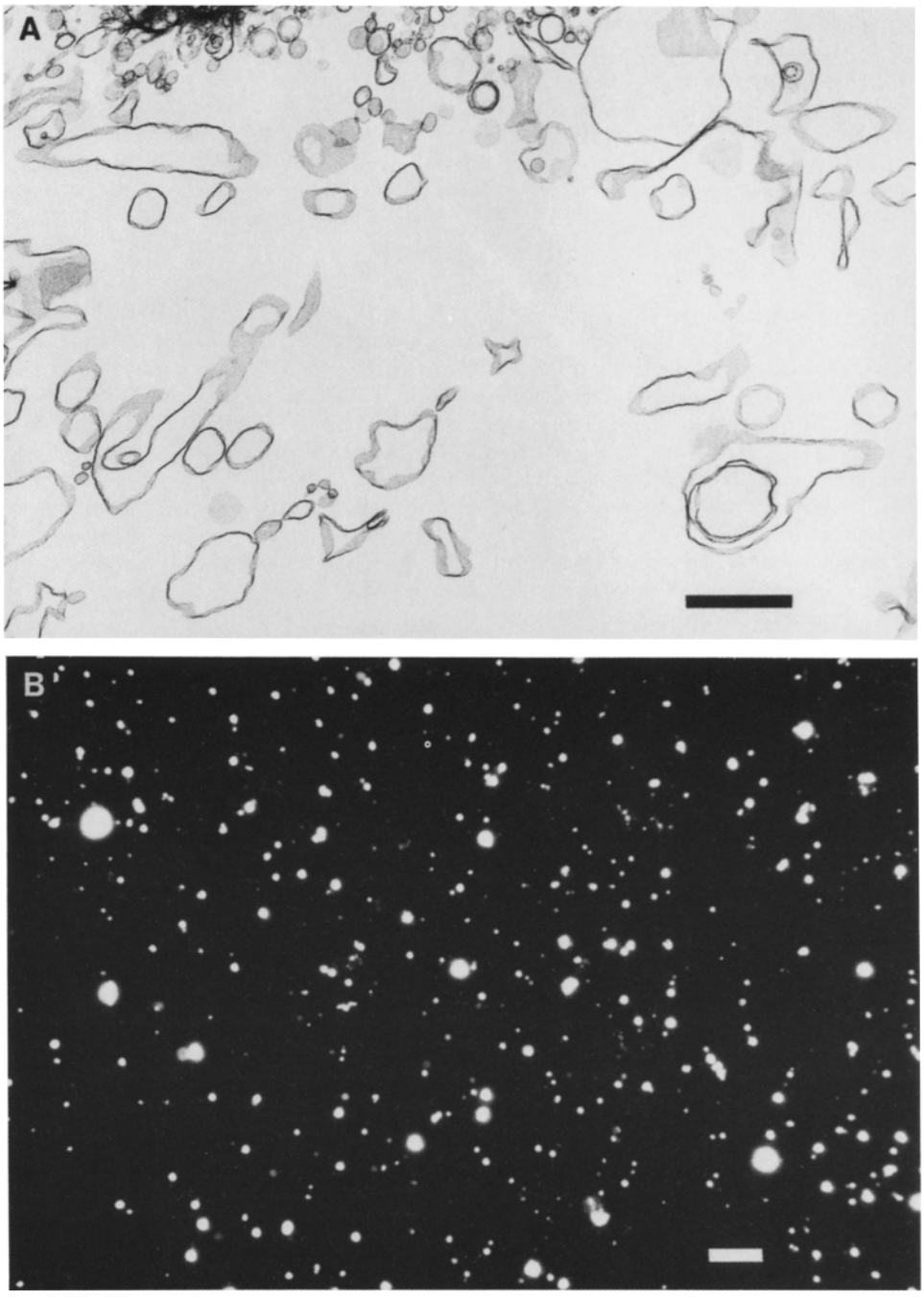


FIGURE 3. The vesicle preparation. (*A*) Electron micrograph of thin-sectioned vesicles. The scale bar denotes 5 μm. (*B*) Fluorescence micrograph of vesicles loaded with 200 mM calcein as described in the text. The scale bar denotes 10 μm.

the fluorescence micrograph of Fig. 3*B*. The vesicles were readily visible in the fluorescence microscope and had a heterogeneous size distribution.

*Visualization of Vesicles in the Video Fluorimeter*

*Vesicle access to planar membrane is diffusion limited.* In initial experiments, vesicles were added over a magnetic stir bar in the rear (*cis*) compartment. To observe attached vesicles, we waited for the vesicles to diffuse through the unstirred layer (~100 μm) and then perfused the chamber to remove the unbound vesicles. Unbound vesicles contributed a huge fluorescence background and made it impossible to observe bound vesicles. Very few bound vesicles were observed when we used this protocol; the procedure proved impractical. As elaborated in the Discussion, the problem is that large vesicles can take hours to diffuse through the unstirred layers, and the large volume per vesicle limits the number of vesicles that can be added.

We overcame these problems by maneuvering a vesicle-containing glass pipette until the tip was in focus, thereby reducing the distance the vesicles must travel to reach the planar membrane to within 5–10 μm. Submicroliter quantities of vesicle solution were pressure-ejected from the pipette onto the membrane, in spurts of 20 psi each, 3–4 ms in duration. The pipette was then backed away from the membrane, reducing the background fluorescence from vesicles in the pipette. The pipette was repositioned for additional ejection of vesicles as needed. In this way, vesicles could be delivered to the planar membrane repeatedly during an experiment, the fluorescence background remained low, and the need to perfuse out unbound vesicles was obviated.

*Appearance of vesicles bound to the planar membrane.* The calcein-loaded vesicles appeared as bright circular profiles, 2–10 μm in diameter. Because the vesicles contained 5 mM *n*-propyl gallate, they were resistant to fading for ~5 min. Without the antioxidant, the vesicles faded within seconds. The settings of the SIT camera were such that the vesicles were observed readily and they often locally saturated the video image. After pressure ejection, most vesicles flowed out of view within several seconds, without adhering to the membrane. This flowing occurred both from stirring and gravitational settling of vesicles (calcein-containing vesicles are more dense than some of the chamber buffers used). The remaining vesicles adhered to the planar membrane. These vesicles stayed in focus despite stirring, often moving slowly in the plane of the membrane. When the objective was focused in the front compartment and then slowly refocused onto the membrane by moving the chamber toward the objective, these bound vesicles were the first to come into view, and all were in a single plane of focus.

We observed that vesicles adsorbed directly to the planar membrane rather than first attaching to an auxiliary structure, such as the torus, and then diffusing into the membrane. In fact, vesicles that adsorbed to the torus never entered the bilayer region. If vesicles entered the ring where the torus merged with the bilayer (annulus), they remained in this ring, regardless of whether they entered from the bilayer or the torus. With time, the annulus became decorated with fluorescent vesicles.

*Vesicle binding requires calcium.* The number of vesicles bound to the planar membrane depended on the calcium concentration in the *cis* compartment. We quantitatively determined this by shooting vesicles at the membrane while varying the calcium concentration in the *cis* compartment. After allowing the convection transient to subside, we counted the total number of vesicles either passing through or remaining within a 10- × 10-cm square drawn on the video screen for a period of 5 s. This provided the total number of vesicles available for binding with the planar membrane. At the end of the 5-s period, the number of vesicles remaining within the square was counted. Binding for a given concentration of calcium was expressed as the ratio of remaining vesicles to the total number of vesicles. The averages of these ratios are shown in Fig. 4. The threshold concentration of calcium for binding is 10 mM. An apparent saturation

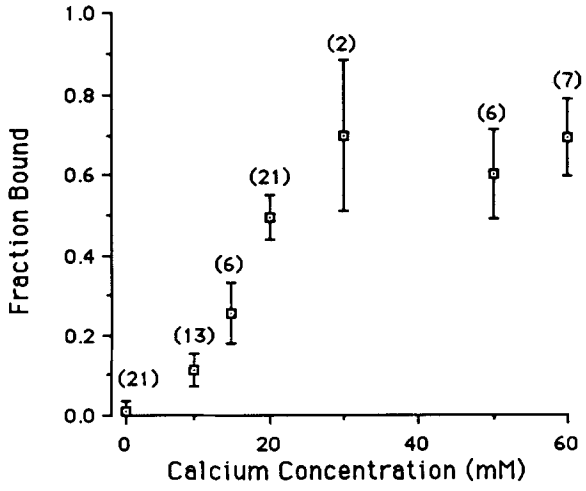


FIGURE 4. Vesicle binding to a planar membrane as a function of calcium concentration. Vesicles were ejected at a planar membrane bathed in the indicated concentration of free calcium ion. After the convection transient subsided, the total number of vesicles in focus within an arbitrary area of the planar membrane during a 5-s observation period was counted. At the end of this observation period, the number of vesicles remaining within the area was counted. The plotted fraction bound is the average ratio of remaining to total vesicles for the indicated number of vesicle ejections. Error bars denote standard deviations of the observations.

of binding is reached between 20 and 30 mM calcium. Under conditions of maximum binding, 70% of the available vesicles bind. 20 mM calcium was included in the buffer of the *cis* compartment in all subsequent experiments.

### *Visualization of Vesicle Rupture*

*Vesicle rupture and calcein release appear as a flash.* We loaded the vesicles with 200 mM calcein. When a vesicle osmotically ruptured, the dye was rapidly released and diluted into the surrounding medium. This spreading of dye led to an increased cross-sectional area where dye molecules captured photons and, therefore, to an increased area for emission of fluorescence, which appeared as a flash of light. Each flash was a bright circular spot that gradually increased and

then decreased in diameter over several video fields (see Fig. 5). We observed that the fluorescence of intact vesicles increased as we increased the concentration of calcein in the vesicles from 1 to 60 to 200 mM. At 200 mM, with the camera set at high gain, the output of the camera in the region of the vesicles was saturated locally, resulting in an image of maximum brightness. Furthermore, with 200 mM calcein in the vesicles, the images of osmotically released dye always locally saturated the camera, whereas lower calcein concentrations did not do so reproducibly. Since the saturation level of the video camera provided a fixed reference of fluorescence intensity for the characterization of each flash, we always loaded the vesicles with 200 mM calcein. With these conditions, we were able to study release of vesicular contents on the level of single vesicles in real time.

*Osmotic swelling of the vesicles.* Two different methods were used to swell the vesicles. The first method relied on a differential permeability change in the vesicular membrane to allow influx of an external solute but retention of internal contents. We pressure-ejected vesicles at a membrane bathed in KCl buffer with 20 mM $CaCl_2$ added to the *cis* compartment, to enable vesicle adhesion to the planar membrane, and then added 70 μg/ml of the channel-forming antibiotic nystatin to the *cis* side. Two-sided nystatin channels formed in the vesicle membrane (Holz and Finkelstein, 1970) because nystatin was added to the internal solution of the vesicle, and ergosterol, which is required for the formation of nystatin channels, was incorporated in the vesicle membrane (see Materials and Methods). Formation of double-sided nystatin channels was necessary to effect vesicle rupture. (Substantially larger concentrations of nystatin are required to form channels when added to one side of a membrane than when added to both sides [Marty and Finkelstein, 1975]. Under our conditions, flashes occurred only when nystatin was present on both sides of the vesicular membrane.) The cutoff diameter of the two-sided nystatin channel is 8 Å (Holz and Finkelstein, 1970), and therefore the channel is permeable to KCl but not to calcein. KCl entered the vesicles, water followed, and the vesicles swelled. By this procedure, which we refer to as dissipation of a KCl gradient, all osmotically active vesicles in the *cis* compartment swelled.

In the second method, osmotic water flow from the *trans* to *cis* compartments was used to swell vesicles bound to the planar membrane. We ejected vesicles at a planar membrane bathed on both sides by sucrose buffer with 20 mM $CaCl_2$ in the *cis* compartment. 400 mM urea was then added to the *cis* compartment to make it hyperosmotic. The vesicles initially shrank because of the hyperosmolarity. 60 μg/ml nystatin was added to the *cis* side to aid entry of urea into the vesicles. Because sucrose is impermeant through nystatin channels (Holz and Finkelstein, 1970), vesicles did not swell by dissipating the sucrose gradient across the vesicle membrane in a manner analogous to the first method. Since urea is permeant through nystatin channels, however, the vesicles reswelled owing to the entry of urea and water. Net swelling of the vesicles bound to the planar membrane occurred because of the osmotic gradient across the planar membrane, which caused water to flow from the *trans* to *cis* compartments and into the bound vesicles.

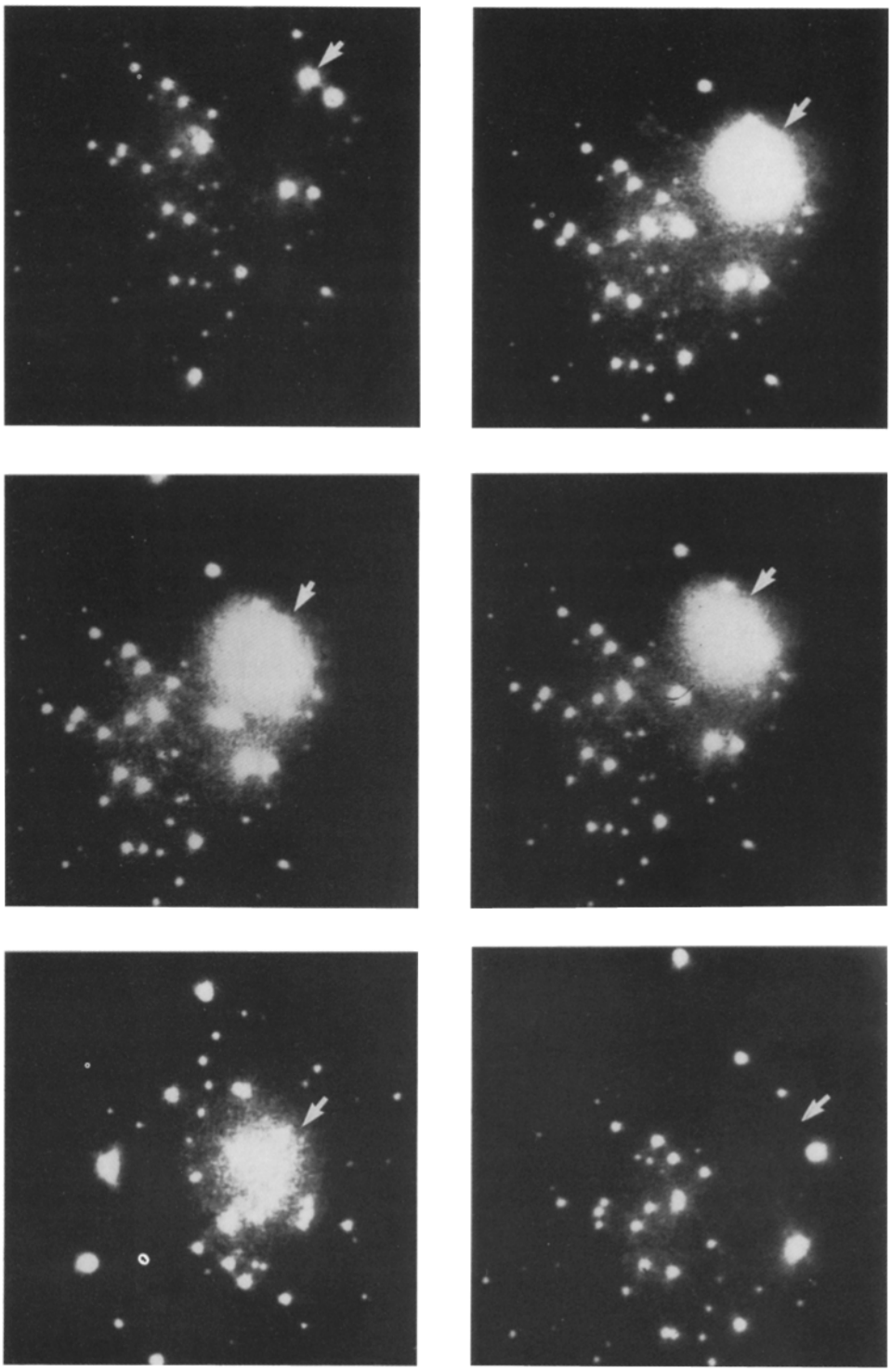

FIGURE 5.

*Channels required for flashes.* For both of the above methods, the presence of channels in the vesicle membrane is necessary to allow net swelling. Indeed, in the absence of nystatin, the vesicles did not osmotically rupture either with an osmotic gradient across the planar membrane or with a KCl gradient across the vesicles. When nystatin was added to the *cis* compartment, channels formed in the vesicular membrane and flashes of light were observed.

*Nature of Region of Contact*

When a vesicle adsorbs to the planar membrane, two possibilities arise. Either the two bilayers in contact maintain their integrity or fundamental structural rearrangements occur (as illustrated in Fig. 6). In the latter case, "semi-fusion" could occur, in which the monolayers of the planar and vesicular membranes facing each other would meld to form a single bilayer in the region of contact that separates the interior of the vesicle from the *trans* compartment. Because a single bilayer would exist in the region of contact for semi-fused vesicles, double-sided nystatin channels would form in this region with addition of nystatin to the *trans* compartment (Fig. 6*B*). This would cause the vesicles to swell osmotically and rupture.

To test the possibility that semi-fusion occurred, we determined whether adding nystatin to the *trans* compartment could dissipate a KCl gradient. We formed planar membranes from an asolectin/ergosterol mixture (4:1 wt/wt), bathed by the KCl buffer with 20 mM $CaCl_2$ on the *cis* side. Vesicles were pressure-ejected at the membrane. The number of bound vesicles that flashed per ejection of vesicles, $f$, was obtained. We then added nystatin to the *trans* compartment until an appreciable conductance was measured and we obtained $f$. Typically, the membrane conductance reached $10^{-4}$ S for a 160-$\mu$m-diam membrane, which shows that a large number of channels formed in the planar membrane. We then added nystatin to the *cis* compartment of the same membrane and again evaluated $f$. For six membranes, in the absence of nystatin, $f =$

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FIGURE 5. (*opposite*) Light flash owing to calcein release by an osmotically ruptured vesicle. The videotaped record of this flash, obtained at 60 video fields/s, was converted to 16-mm movie film, running at 24 movie frames/s. Individual movie frames in the record were selected and made into the photographs shown here. The temporal sequence is from left to right, running from top to bottom. The panel at the top left was taken from the last movie frame just before the vesicle rupture. The intact vesicle is indicated by the arrowhead. This panel is displaced upward with respect to subsequent panels. The next panel, at the top right, was taken four movie frames (167 ms) after the first panel. The arrowhead in this panel and the three panels that follow points to the vesicle undergoing the flash. The next three panels were obtained 9 (375 ms), 14 (584 ms), and 21 (876 ms) movie frames, respectively, after the first panel. The last panel, at the bottom right, is the 24th movie frame from the beginning of the sequence. Note that the vesicle has disappeared from its original position (arrowhead). See Materials and Methods for the vesicle composition. The *cis* compartment contained sucrose plus 40 mM cobalt citrate buffer, 400 mM urea, and 60 $\mu$g/ml nystatin. The *trans* compartment contained sucrose buffer (see Materials and Methods).

0.08 ± 0.13 (mean ± SD); with nystatin *trans*, $f = 0.12 \pm 0.19$; and after nystatin addition *cis*, $f = 4.23 \pm 1.47$. Therefore, semi-fusion did not occur.

*Experimental Determination of the Vesicle Rupture Site*

*Quenching of calcein fluorescence by cobalt.* The flashes of light that result from rupture of swelling vesicles in contact with the membrane could be due either to fusion, in which case rupture occurs in the region of contact and dye is released to the *trans* compartment, or to lysis, which results in dye release to the *cis* side. We determined the side of the planar membrane to which dye was released by including cobalt in either the *cis* or *trans* compartment. Cobalt quenches calcein fluorescence to ~1–4% of its unquenched value by forming a 1:1 complex with an intrinsic association constant of $6 \times 10^7$ $M^{-1}$ with the dye

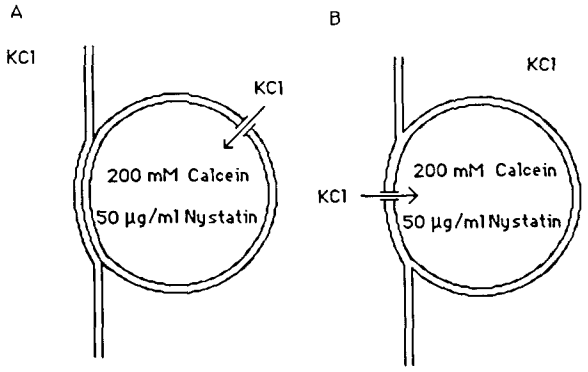


FIGURE 6. Possible membrane configurations in the region of contact. In both *A* and *B*, a vesicle adsorbed to a planar membrane is shown. The planar membranes are bathed on both sides by KCl, which is isosmotic with the vesicles' contents, consisting of 200 mM calcein and 50 µg/ml nystatin. (*A*) Two bilayers in the region of contact. The vesicular and planar membranes do not coalesce in the adhesion zone but remain distinct bilayers. In this case, nystatin will form channels and cause vesicular swelling only when added to the *cis* (vesicle-containing) compartment. (*B*) One bilayer in the region of contact. The outer monolayer of the vesicular membrane has coalesced with the *cis*-facing monolayer of the planar membrane to form a single bilayer in the adhesion zone. For this situation, nystatin will cause swelling when added to either side of the planar membrane. Experimentally, nystatin causes swelling only when added to the *cis* compartment. Thus, two distinct bilayers exist in tight association in the region of contact.

(Wallach and Steck, 1963; Kendall and MacDonald, 1983). The basis for distinguishing fusion from lysis is that with cobalt present on the *trans* side, flashes resulting from fusion would be quenched. With cobalt present on the *cis* side, flashes owing to fusion would be unchanged and lysis events would be quenched. We established the feasibility of this method by measuring the differences between quenched and unquenched flashes and establishing that the two possibilities are experimentally distinguishable.

*Effects of cobalt on the visualization of calcein release.* To distinguish quenched and unquenched flashes, we quantitatively studied the effects of cobalt on the spatial and temporal nature of flashes. The nature of the flashes was independent of the method of osmotic rupture. (The primary difference between the two methods of swelling was in the number of vesicles that ruptured. With hyperos-

motic urea in the *cis* compartment, 12% of the vesicles bound to the planar membrane ruptured. When vesicles were swollen in isosmotic KCl with nystatin, 37% of the vesicles ruptured. Thus, at least half of the fluorescent vesicles were, in actuality, osmotically inactive fluorescent objects. This was verified by using a hemocytometer to measure the fraction of objects surviving osmotic shock in distilled water. Nearly 50% of the objects were visually intact after incubation in distilled water for 20 min. This is consistent with the electron micrographs showing a large number of nonvesicular objects. Since we were concerned only with vesicles that rupture, our analysis of flashes and conclusions were unaffected by osmotically inactive fluorescent objects in the preparation.) With cobalt absent on both sides of the planar membrane, 659 flashes in five separate experiments were recorded on video tape. Each flash was examined on the video monitor by playing the video tape back in search mode to the first video field before the eruption of the flash. The initial diameter of the fluorescent vesicle yielding the flash was measured with a ruler. The tape was then played forward, field by field. When the flash image saturated the camera, a video image of maximum brightness was produced. We refer to this image as the "saturated image." For each video field, the diameter of the saturated image was measured. This measurement was continued until a video field was reached in which the flash no longer produced a saturated image. Each vesicle rupture was thus characterized by three measurements: (*a*) the initial diameter of the vesicle, (*b*) the diameter of the saturated image at fixed time points after the beginning of the flash, and (*c*) the number of video fields over which the flash image saturated the camera. Since each video field represents 16.7 ms, the third measurement furnished the flash duration.

The same protocol was used with 40 mM cobalt citrate on both sides of the membrane, in which 671 vesicle ruptures in six experiments were analyzed. For quenched and unquenched flashes that were of the same duration, the diameters of the saturated images were not significantly different. The theoretical reasons for this empirical observation are elaborated upon in the Appendix.

By examining frequency histograms of flash durations, we determined that the primary effect of quenching flashes with cobalt was a shortening of the flash durations (Fig. 7). Histograms obtained from swelling the vesicles by dissipating a KCl gradient were identical to those obtained from inducing swelling by adding urea to the *cis* compartment; hence, these data were pooled. To compare histograms from vesicles that contained the same amount of dye, we grouped the flash data into classes according to the initial diameter of the vesicle. The diameters were grouped integrally from 2 to 10 $\mu$m. For each diameter group, standard histograms were generated for the number of flashes lasting a given number of video fields for both the quenched and unquenched situations. The histograms of diameters ranging from 6 to 10 $\mu$m were not significantly different ($P < 0.4$ for all two-sample Kolmogorov-Smirnov tests; Beyer, 1968), so these data were pooled.

As we increased the concentration of cobalt, the difference between the standard histograms of flash durations became statistically different for the smallest vesicles first. For example, at 20 mM cobalt citrate, the histograms were significantly different for 2-$\mu$m-diam vesicles but not for 4-$\mu$m-diam vesicles. It

was not until 40 mM cobalt citrate was used that the histograms were significantly different for all diameter classes, and therefore we used 40 mM cobalt citrate for all quenching experiments. Since the intrinsic association constant for the 1:1 complex of cobalt and citrate is $10^5$ $M^{-1}$ (Martell and Smith, 1977), the concentration of free cobalt in the chamber at pH 7.5 was ~5 mM. Histograms of quenched and unquenched flash durations for vesicle diameters of 3 and 4

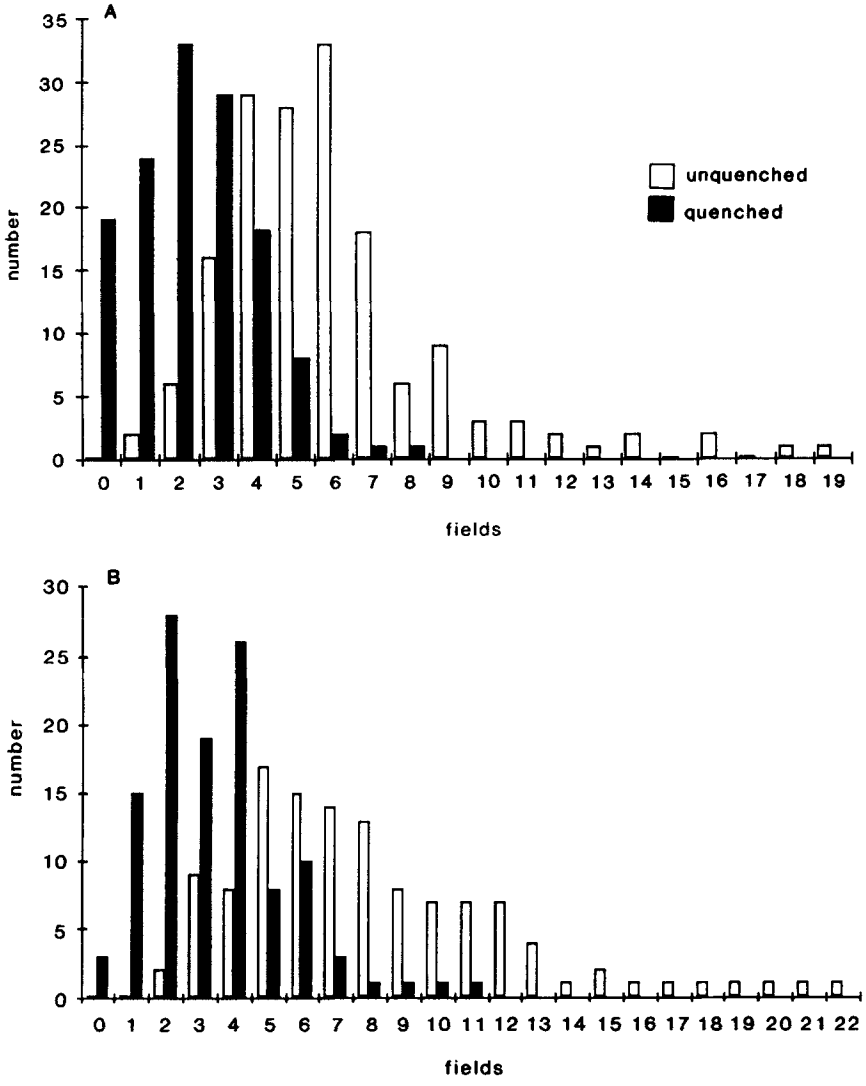


FIGURE 7. Frequency histograms for the durations of unquenched flashes (open columns) and flashes quenched in 40 mM cobalt citrate (filled columns). (*A*) 3-μm-diam vesicles. (*B*) 4-μm-diam vesicles. Note that the unquenched flashes last for more video fields than the quenched flashes.

$\mu$m are shown in Fig. 7. For vesicles of 3 $\mu$m initial diameter, the differences between the histograms are readily apparent. The variability in duration for quenched flashes is less than that for unquenched flashes. Moreover, the modes of the durations for the two cases are separated by four video fields. Unquenched flashes tend to last for six video fields, while quenched flashes tend to last only two video fields. For 4-$\mu$m-diam vesicles, however, the histograms overlap considerably, despite the separation in mode durations by three video fields.

*Assignment of flashes in asymmetric cobalt.* When cobalt was present on only one side of the membrane, we calculated the probability that a flash was quenched or unquenched from the standard histograms of flash durations. For the standard histograms, each flash used was an independent observation obtained under a fixed condition. Moreover, the set of conditions was exhaustive (i.e., covered all possibilities: the flash had to be either quenched or unquenched). This allowed calculation of the probability that any arbitrary flash, obtained in an experiment with cobalt present only on one side of the planar membrane, was quenched or unquenched. We assumed for this calculation that, for any given vesicle, all of the calcein was released to one side of the membrane. These probabilities were calculated from the pair of standard histograms as follows. Let $P(U \mid t)$ be the conditional probability that a flash was unquenched, given that its duration was $t$ video fields, and let $P(Q \mid t)$ be the corresponding conditional probability that the flash was quenched, given that it lasted $t$ video fields. According to Bayes' theorem (cf. Brownlee, 1965),

$$P(U \mid t) = \frac{P(t \cap U)}{P(t \cap U) + P(t \cap Q)}, \tag{1a}$$

and

$$P(Q \mid t) = \frac{P(t \cap Q)}{P(t \cap U) + P(t \cap Q)}, \tag{1b}$$

where $P(t \cap U)$ is the compound probability that an unquenched flash was $t$ video fields in duration, and $P(t \cap Q)$ is the compound probability that a quenched flash was $t$ video fields in duration. The compound probabilities were computed from the standard histograms of flash durations: $P(t \cap U)$ was computed for each flash duration $t$ by dividing the number of unquenched flashes lasting for $t$ video fields by the sum of the quenched and unquenched flashes $t$ video fields in duration. $P(t \cap Q)$ was calculated similarly by dividing the number of quenched flashes of duration $t$ by the sum of the quenched and unquenched flashes $t$ video fields in duration. The conditional probabilities for each flash duration were then computed by Eqs. 1*a* and 1*b* and organized into look-up tables, with one look-up table for each initial diameter. These conditional probabilities were used to estimate the likelihood that any vesicle rupture resulted in fusion with the planar membrane.

*Likelihood of vesicle rupture leading to fusion.* The likelihood that a flash of light was due to a vesicle fusing with the planar membrane was estimated from the duration of saturated images when cobalt was present only on one side of the membrane. This likelihood is referred to as the "likelihood of fusion." Planar

membranes were formed with 40 mM cobalt citrate in one compartment of the chamber and a buffer of the same composition but without cobalt citrate in the other compartment. The cobalt-free buffer was adjusted to have the same osmolarity as the cobalt-containing solution (see chamber buffers in Materials and Methods). We added 20 mM calcium to the *cis* compartment to promote binding of vesicles to the planar membrane. 50 μg/ml nystatin was added to the *cis* compartment before the injection of vesicles, which were swollen by the two methods described. Each experiment was performed by repeatedly ejecting

TABLE I

*Percentage of Vesicles Fusing with the Planar Membrane*

| Diameter | Percent of vesicles fusing | Number of vesicles |
|---|---|---|
| μm | ±SEM | |
| Cobalt *cis*/swelling with KCl | | |
| 2 | 38±5 | 46 |
| 3 | 25±5 | 23 |
| 4 | 37±7 | 12 |
| 5 | 36±11 | 10 |
| ≥6 | 78±10 | 12 |
| Cobalt *trans*/swelling with KCl | | |
| 2 | 56±3 | 91 |
| 3 | 59±4 | 44 |
| 4 | 40±5 | 35 |
| 5 | 48±8 | 16 |
| ≥6 | 21±10 | 13 |
| Cobalt *cis*/swelling with urea | | |
| 2 | 62±3 | 96 |
| 3 | 54±4 | 62 |
| 4 | 54±4 | 57 |
| 5 | 40±4 | 41 |
| ≥6 | 54±9 | 18 |
| Cobalt *trans*/swelling with urea | | |
| 2 | 54±3 | 98 |
| 3 | 61±4 | 78 |
| 4 | 40±4 | 73 |
| 5 | 34±5 | 30 |
| ≥6 | 59±7 | 35 |

vesicles from the pipette. After each spurt, the objective was focused on the vesicles nearest to the planar membrane and the ensuing flashes were recorded on video tape. For each flash, we measured the initial diameter of the vesicle producing the flash, and the number of video fields in which the fluorescence of the released calcein saturated the video camera. $P(U \mid t)$ and $P(Q \mid t)$ were obtained for each flash from the look-up tables of the conditional probabilities of flash duration. These conditional probabilities obtained for individual flashes were pooled into groups having the same initial vesicle diameter, the same side on

which cobalt was present, and the same method of swelling. The average conditional probability of any group determined the likelihood of fusion for each vesicle in each group. The data are summarized in Table I.

The likelihood that dye release was due to vesicles fusing to the planar membrane was ~50%, the exact number depending on the details of the conditions. When swelling was done by dissipating the KCl gradient with nystatin and cobalt was in the *trans* compartment, 51 ± 4% (SEM for 199 flashes) of the vesicle ruptures were due to fusion. For the same method of swelling, the likelihood of fusion was 40 ± 7% (103 flashes) with cobalt on the *cis* side of the membrane. (There was no statistically significant difference between these likelihoods as determined by a two-sample $t$ test [$P < 0.02$]. This indicates that cobalt, at the concentrations used, did not affect the likelihood of fusion.) When swelling was induced by adding urea to the *cis* side, thus making this compartment hyperosmotic, the probability that flashes were due to fusion was 55 ± 4% (276 flashes) with cobalt *cis* and 51 ± 4% (314 flashes) with cobalt *trans*. It is striking

TABLE II

*Percentage of Vesicles Fusing with the Planar Membrane in the Presence of 100 mM Calcium*

| Diameter | Percent of vesicles fusing | Number of vesicles |
|---|---|---|
| *μm* | *±SEM* | |
| Cobalt *trans*/swelling with urea | | |
| 2 | 71±2 | 154 |
| 3 | 75±3 | 69 |
| 4 | 52±7 | 23 |
| 5 | 63±13 | 8 |
| ≥6 | 0 | 3 |

that for the two different methods of swelling vesicles, approximately the same percentage of flashes of light were due to fusion.

*Divalent cations increase the likelihood of fusion.* We determined that calcium affects the likelihood that vesicle rupture results in fusion. Experiments were performed with sucrose buffer containing 40 mM cobalt citrate on the *trans* side of the planar membrane and 550 mM sucrose buffer containing 100 mM calcium chloride in the *cis* compartment. Vesicles were swollen by making the *cis* compartment hyperosmotic with 400 mM urea. The results are shown in Table II, in which the likelihood of fusion was averaged for each vesicle diameter. Although no dependence of fusion on vesicle diameter was apparent, the three vesicles at least 6 μm in diameter did not fuse with the planar membrane but lysed. The average likelihood of fusion for all vesicles is 69 ± 2% (257 flashes). Thus, the larger divalent cation concentration results in an increased likelihood of fusion, even though 20 and 100 mM calcium lead to the same number of vesicles bound to the planar membrane.

## DISCUSSION

Video fluorescence microscopy greatly expands the ability to study the fusion of phospholipid vesicles with a planar phospholipid bilayer membrane. This method allowed us to study the individual steps in the fusion process—approach, binding, and fusion—on the quantal level of single vesicles. We have used large vesicles in this study because of the obvious technical advantages they have for microscopic observation. But the methodologies of this article can be applied to smaller vesicles, as we have detected both binding of, and dye release from, sonication–freeze-thaw vesicles that are <1 μm in diameter.

### *Approach of Vesicles to Planar Membrane*

The approach of large vesicles to the planar membrane is limited by diffusion. From the Stokes-Einstein relation, which states that the diffusion constant of a particle varies inversely with the radius, we calculate a diffusion coefficient, $D$, of $8 \times 10^{-10}$ cm$^2$/s for a 5-μm-diam vesicle. From the diffusion equation $\langle x^2 \rangle = 2Dt$, it follows that it takes $\sim 10^3$ min for such vesicles to diffuse through a 100-μm unstirred layer. To overcome this theoretical 16-h diffusion time, we pressure-ejected the vesicles toward the membrane from a pipette that was brought to within <10 μm of the membrane. In addition to obviating diffusion of vesicles through the unstirred layer, this procedure has other advantages: far fewer vesicles need to be added than are required with bath application to obtain binding and fusion, and the small number of vesicles added does not markedly increase the background level of fluorescence.

### *Binding of Vesicles and Nature of Region of Contact*

Whereas binding between vesicles has been studied as a function of divalent concentration (cf. Wilschut et al., 1981), quantitative studies of binding between vesicles and planar bilayers have not been previously performed. The methods traditionally used with planar membranes, conductance and flux measurements, are not suitable for the study of binding. Fluorimetric detection of vesicles bound to planar membranes has been previously reported (Niles and Eisenberg, 1985) using a photon-counting photomultiplier tube to detect fluorescence. With photomultiplier tubes, large vesicles dominate the signal; vesicles that are in the light path but are not bound to the bilayer contaminate the signal. With video recording, spatial localization is achieved, the above problems are avoided, and quantitative studies are possible.

The binding of asolectin vesicles to planar asolectin membranes increased steeply as the calcium concentration increased from 0 to 30 mM, with half-maximal binding at ~15 mM. This is similar to the findings of previous studies with membranes containing anionic phospholipids (Cohen et al., 1980, 1984; Akabas et al., 1984). We decided not to fit our data with a theoretical relation. Because we shot a small number of vesicles at the membrane, binding could only occur during the initial encounters of the vesicles with the membrane, whereas classical binding theories assume that an equilibrium is reached between the bound and unbound species. In our system, this equilibrium was not reached. In fact, binding was irreversible: once bound, the vesicles remained bound. (Even

if equilibrium binding were reached, the differences between the vesicle-calcium-planar membrane binding scheme and classical ligand-receptor binding should be noted. In the latter case, binding can be described with a single association constant [and the total number of binding sites] between ligand and receptor. In the vesicle-bilayer system, the association constant also varies with the calcium concentration in a manner that does not at present have either experimental or theoretical underpinnings.)

It is noteworthy that at 30 mM calcium, ~70% of the vesicles that were accessible to the planar membrane adsorbed to it. This 70% figure is a minimum for the percentage of accessible vesicles that bind. Some of the vesicles within the depth of focus (~10 $\mu$m) may have never approached sufficiently close to the planar membrane to have made actual contact. We can therefore think of the bilayer, at high enough calcium concentrations, as a sticky surface for vesicles: when vesicles strike the planar membrane, they usually adhere to it.

While calcium promotes adhesion, it does not lead to semi-fusion. This was shown by the inability of nystatin, when added *trans*, to cause flashes from adsorbed vesicles. For our membranes with a diameter of 160 $\mu$m and a conductance of $10^{-4}$ S after adding nystatin *trans*, there were $2.5 \times 10^3$ channels/$\mu m^2$, assuming a single channel conductance of 2 pS for nystatin (Kleinberg and Finkelstein, 1984). Therefore, if semifusion had occurred, it would have been detected because the number of nystatin channels was orders of magnitude in excess of that needed to be certain that channels formed in the contact region.

We define the region of contact as the area of the vesicular membrane in which osmotically induced rupture leads to fusion with the planar membrane. Our finding that 50% of the vesicle ruptures result in fusion has implications regarding the relative size and properties of the contact region. If osmotically induced rupture is equally likely at all points on a vesicle's surface, our finding indicates that, on the average, 50% of the surface area of each vesicle is in contact with the planar membrane. Alternatively, if <50% of each vesicle is in contact with the planar membrane, then this region must be preferentially weakened. Direct measurements of the area of contact would establish whether this region preferentially ruptures when the vesicle is osmotically swollen.

Although increasing the calcium concentration above 20 mM did not lead to binding of more vesicles (Fig. 4), it did result in significant increases in the likelihood that vesicles rupture in the contact region. According to standard models of divalent cation-mediated interactions between phospholipid bilayers (Portis et al., 1979), each divalent cation bridges two monovalent anionic moieties on the phospholipid head groups of two opposing monolayers. It has been shown experimentally that divalent cation binding dehydrates the headgroups (Portis et al., 1979; Parsegian and Rand, 1983). Our data indicate that vesicle-membrane binding is initiated and maintained without divalent cations saturating the monovalent anionic sites. The calcium concentration needed for half-maximal binding of the vesicles, ~15 mM, was approximately fivefold less than the concentration of calcium needed for half-maximal occupancy of phosphatidylserine (PS), as predicted from the intrinsic association constant of 12 $M^{-1}$ for calcium and PS (McLaughlin et al., 1981). Thus, maximal vesicle-membrane binding can be

achieved without saturation of the calcium-binding sites. (Dissociation constants in the micromolar range have been measured for calcium binding between PS lamellae [Feigenson, 1986]. If such binding occurred in our experiments, all anionic sites on the phospholipids would have bound calcium in the region of contact. If this were the case, it would be curious that vesicle binding was most sensitive to calcium concentrations in the 10–20-mM range.)

While the percentage of applied vesicles that bind is saturated by 30 mM calcium, the contact region between the vesicles and planar membrane was affected by higher calcium concentrations. This was experimentally illustrated by our finding that the percentage of flashes that were due to fusion was higher for 100 than for 20 mM calcium. This could have simply been the result of calcium increasing the area of contact. Alternatively, some of the anionic sites within the region of contact might not have been spanned at 20 mM calcium and could have retained their waters of hydration. These undisplaced water molecules would have prevented the intermingling of headgroups from apposing monolayers and maintained a space or corridor between the vesicle and planar membranes within the region of contact (Akabas et al., 1984). Increasing the divalent cation concentration (to 100 mM) could have resulted in additional cross-bridges between the apposed monolayers, an increased area of contact, further dehydration of the headgroups, and a decreased space between the vesicle and planar membranes. This would favor fusion at the expense of lysis when the vesicles built up a hydrostatic pressure as a result of swelling, as was observed experimentally.

While there may be some water molecules in the adhesion region, this study suggests that the vesicle and planar membranes are sufficiently close that the region of contact can be considered to be dehydrated. Dissipation of a KCl gradient causes rupture of all osmotically active vesicles, regardless of the thickness of the corridor separating the two membranes. On the other hand, when water flows across the planar membrane, only the vesicles directly abutted against the membrane (i.e., dehydrated) will swell (Akabas et al., 1984). Two sets of numbers indicate that the thickness of the corridor is small. First, because the likelihood of fusion was the same for the two methods of swelling (see Table I), vesicles rupturing in KCl were as close to the membrane as those swollen with the osmotic gradient. Second, roughly the same percentage (within a factor of three) of adsorbed vesicles ruptured for the two methods of swelling.

### *Fusion Events*

The techniques used in this article have the advantage that they allow detection of single fusion and single lysis events. For vesicle-vesicle fusion studies in a cuvette, only a large number of events are detected, and distinguishing fusion from leakage is difficult (Kendall and MacDonald, 1982; Wilschut et al., 1983). With video microscopy, the basis for release of vesicular contents can be determined for each event.

*Histograms.* We investigated strategies to determine whether a flash of light resulting from dye release was due to fusion or lysis based on spatial and/or temporal distributions of light intensity. Without measures of the light intensities at every point within a flash, little was gained by combining the spatial and

temporal information. We further concluded that the duration of the flash is a more reliable indicator of quenching by cobalt than is the diameter of the flash. In essence, this choice of strategy follows from the nature of sampling the flash with the video camera. Each video field is scanned from top to bottom in ~16.7 ms. Therefore, all vesicles that are observed to last the same number of video fields had flash durations within 33.3 ms of each other. The observed maximum diameter is, however, a relatively poor measure of the true maximum diameter. The diameter of a flash varies within a video field, and this variation can be large. We therefore used duration histograms to derive the probability of a flash being quenched. These ideas are discussed in detail in the Appendix.

*Development of hydrostatic pressures.* Our hypothesis is that a hydrostatic pressure in the vesicle leads to fusion or lysis. Because water is incompressible, a net influx of water generates a pressure after the vesicle membrane becomes taut. We have used two methods to obtain a net influx of water.

The case where swelling occurs via dissipating a KCl gradient is illustrated in Fig. 8*A*. The internal calcein and external KCl are osmotically balanced, and the vesicular membrane is initially impermeable to both. There is no solute flow and therefore no net water flow. When nystatin channels form in the vesicle membrane, KCl enters, whereas calcein remains impermeant. Water follows, maintaining isotonicity, the vesicle swells, and an intravesicular hydrostatic pressure develops.

The case where vesicles are swollen with an osmotic gradient across the planar membrane is illustrated in Fig. 8, *B–D*. When an osmoticant (a solute used to raise osmolarity) is added to the *cis* compartment, vesicles initially shrink and water begins to flow from the *trans* to *cis* compartments and into adsorbed vesicles. If the osmoticant is membrane impermeant (Fig. 8*B*), then clearly the vesicle can only reswell, at most, to its initial volume. If osmoticant-permeable channels are present (Fig. 8*C*), in the absence of a hydrostatic pressure, the only way for a steady state to be reached is for the concentration of the osmoticant in the vesicle to be equal to that in the *cis* compartment. However, such a steady state is impossible, because if the concentrations were equal, water would flow into the vesicle from the *trans* compartment, dilute the osmoticant, and cause further water flow into the vesicle. The vesicle would swell indefinitely. Hence, the only way to reach a steady state is to develop an intravesicular hydrostatic pressure. In the case where the osmoticant is permeant through bare bilayer (Fig. 8*D*), it can be shown, again, that a hydrostatic pressure develops only if channels are present in the vesicle. (More precisely, if the ratio of the water and solute permeabilities is the same in the region of contact as in the noncontact region, then a hydrostatic pressure does not develop. With channels, this ratio is not equal in the two regions and a pressure develops.) These arguments have been rigorously developed (Niles, W. D., F. S. Cohen, A. Finkelstein, and M. H. Akabas, manuscript in preparation). Woodbury (1986) has arrived at similar conclusions.

### *Relation to Vesicle-Vesicle Fusion*

There are numerous independent reports that calcium alone promotes vesicle-vesicle fusion, whereas it is clear that, in the vesicle-planar membrane system,

calcium promotes adhesion, but not fusion. One would expect similar mechanisms to account for fusion in the two systems because, while they have different geometries, their membranes have the same molecular composition and structures. Therefore, correct models for fusion must account for experimental differences between the two systems on the basis of the different geometries.

We propose that vesicle-vesicle fusion is also driven by intravesicular hydrostatic pressures. It has been shown that fusion between vesicles is stimulated by intravesicular pressures (Miller et al., 1976). Further, it has been demonstrated that for large vesicles, which can be easily resolved with video-enhanced differ-

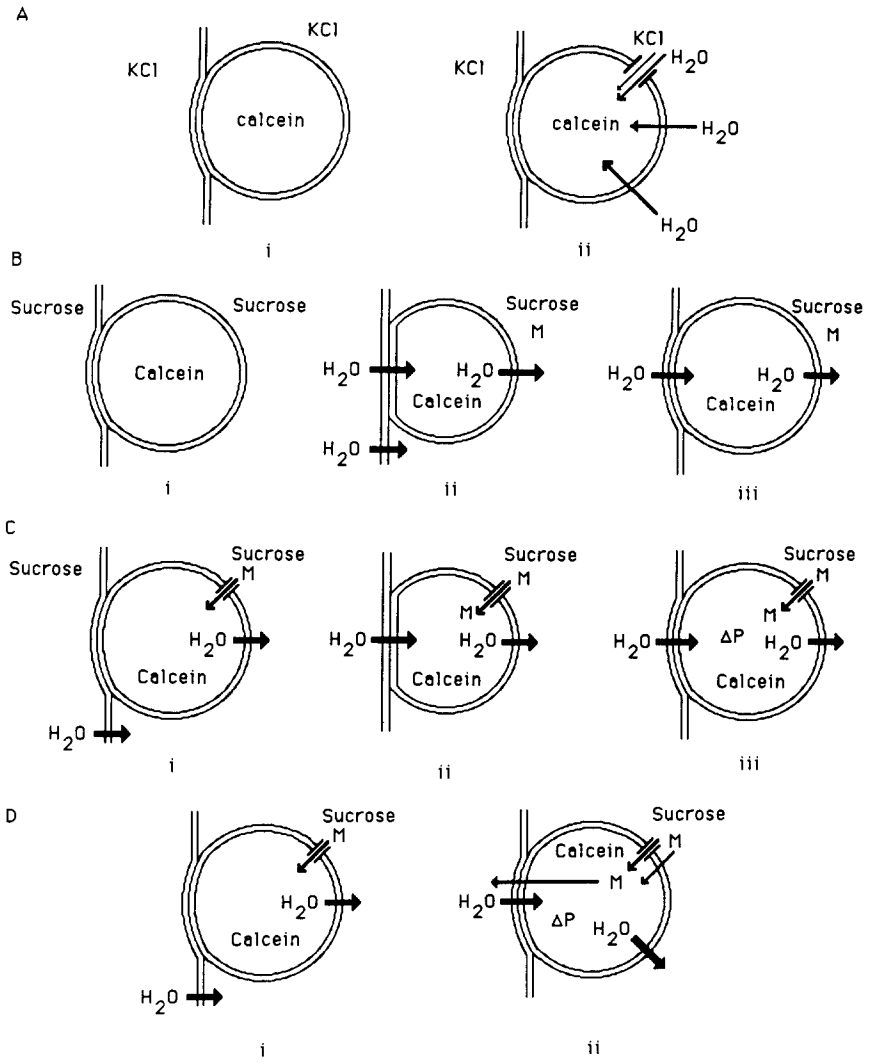


FIGURE 8.

ential interference microscopy, the calcium-induced adhesion of vesicles results in a flattening of the vesicles against each other (Rand et al., 1985; Kachar et al., 1986). This flattening causes intravesicular pressures to develop, which could cause fusion, lysis, or volume loss (Kachar et al., 1986). It may be noteworthy that these investigators also found that 50% of the adhered, flattened vesicles fused.

*Possible Relevance to Biological Fusion*

There is abundant evidence that granule swelling is associated with secretion in many biological systems (reviewed in Finkelstein et al., 1986). It has therefore been suggested that swelling of biological granules is the driving force for fusion (Ferris et al., 1970; Pollard et al., 1979; Cohen et al., 1980; Niles and Smith, 1982). However, evidence has been presented that exocytosis occurs in chromaffin cells with flaccid chromaffin granules (Holz and Senter, 1986). Furthermore, recent studies of mast cells from beige mice showed that the capacitance increase of the plasma membrane expected from fusion preceded the swelling of granules and that flaccid granules fused (Zimmerberg et al., 1987; Breckenridge and Almers, 1987).

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FIGURE 8. (*opposite*) Osmotic swelling of vesicles bound to planar membrane. (*A*) Swelling is induced by dissipating a KCl gradient. (*i*) Calcein and KCl are osmotically balanced. (*ii*) KCl enters the vesicle via the channels, water follows, and the vesicle swells. (*B*) An osmotic gradient formed across the planar membrane, *cis* side hyperosmotic, with no channels in the vesicle membrane. (*i*) Calcein and sucrose are osmotically balanced. (*ii*) A membrane-impermeant osmoticant, M, is added to the *cis* side. Water flows from the interior of the vesicle into the *cis* compartment and the vesicle shrinks, raising the concentration of calcein in the vesicle interior. Water therefore enters the vesicle from the *trans* compartment. (*iii*) A steady state is established; the rate of water entry into the vesicle from the *trans* side equals the rate of water exiting the vesicle to the *cis* side. The vesicle will be shrunken compared with panel *i*. If 100% of the vesicle were in contact with the planar membrane, the vesicle would reswell to its initial volume, but a hydrostatic pressure would not develop. Even if M were permeable, a hydrostatic pressure would not develop. (*C*) Case *B* with channels. (*i*) Addition of the osmoticant, M, results in entry of M into the vesicle via the channels and exit of water from the vesicle and into the *cis* compartment. Water flows from the *trans* to *cis* compartments. (*ii*) The vesicle has shrunk. M equilibrates between the *cis* compartment and vesicle interior. Note that the osmoticant cannot leave the vesicle through the region of contact. Water flows across the region of contact from the *trans* compartment into the vesicle interior. (*iii*) A steady state is reached. If a hydrostatic pressure did not develop, the vesicle would swell indefinitely (see text), which is a physical impossibility. In steady state, the vesicle fully swells and the hydrostatic pressure, $\Delta P$, that develops drives water out of the vesicle through the channels and opposes water entry into the vesicles from the *trans* compartment. (*D*) Swelling with channels and the bare bilayer is permeable to the osmoticant. (*i*) Upon addition of the osmoticant, water leaves the vesicle and M enters. (*ii*) In steady state, a hydrostatic pressure develops. The rate of water entry equals its rate of exit, and the rate at which M enters the vesicle equals the rate at which M leaves the vesicle, through the region of contact, into the *trans* compartment.

An influx of fluid into the interior of a vesicle results in an intravesicular hydrostatic pressure, which causes stretching of the vesicular membrane. The membrane is thus stressed in the region of contact as well as in the remainder of the vesicle. A procedure to induce stresses only in the adhesion region should result in fusion without any lysis. The possibility that locally induced stresses in the region of contact (e.g., via proteins) drive biological fusion should be considered (cf. Parsegian and Rand, 1983).

*Relevance to Reconstitution of Proteins into Planar Membranes*

The techniques developed for this study can be used to improve reconstitution of channels into planar membranes by fusion techniques. Traditionally, dissecting microscopes have been used for visual observation of planar membranes because of their long working distance. However, the bilayer can be observed in far greater detail with an inexpensive upright microscope mounted on its back brace, a long-working-distance objective, a video camera, and a monitor. This allows pressure-driven delivery of vesicles via a pipette brought to within 10 $\mu$m of the planar membrane. This method uses approximately seven orders of magnitude less material than is required with the more traditional technique of adding the vesicles to the bath. Furthermore, the improved visual observation allows smaller membranes to be used, with their better noise-bandwidth properties as compared with larger membranes, since clogged holes (which are not rare occurrences when small solvent-free membranes are made) are readily apparent.

Nystatin can also be used to achieve greater fusion. When native vesicles (those derived directly from biological tissue) are used, there are usually a variety of channels present and fusion is easily generated. However, when purified channels are reconstituted into phospholipid vesicles, fusion has, in practice, proven to be more difficult. This is in large part because open channels are needed to develop intravesicular pressures. If ion-selective channels are reconstituted, these channels must be opened and a counterion must be able to enter the vesicles before fusion can proceed. By adding nystatin to the *cis* compartment, nonselective channels will readily form in the vesicular membranes if they contain sterols. These channels will dissociate into nonconducting nystatin monomers in a planar membrane that does not contain sterols. If it is undesirable to include sterols in the vesicular membrane, large concentrations of nystatin can be tried. The nystatin can, in general, be perfused away, as has been demonstrated by its use in reversibly permeabilizing a wide variety of cells. Also, as one-sided nystatin channels form in solvent-free, but not in solvent-containing, membranes, the nystatin need not even be perfused out when solvent-containing membranes are used.

Native vesicles often contain trapped sucrose after centrifugation in sucrose gradients. Nystatin channels will allow entry of salts into the vesicles, with water following (sucrose is impermeant) in exactly the same manner used in this study. Therefore, nystatin can be beneficial in inducing fusion with native vesicles.

Reconstitution of channels via fusion methods has usually been into decane-containing membranes because vesicles fuse more readily to them (Cohen et al., 1984; Cohen, 1986). With the techniques outlined here, fusion with solvent-free

membranes can be obtained more readily than before, and proteins can be studied in a solvent-free environment.

## APPENDIX

In this appendix, we present a theoretical description of the spatial concentration profile of calcein as a function of time for quenched and unquenched flashes. The unquenched flash was modeled as dye diffusing away from the ruptured vesicle. The profile of a quenched flash was evaluated by allowing the diffusing calcein to react with cobalt. The concentration profile of the free, unreacted dye was calculated. These continuous functions of distance and time were then sampled at time points analogous to discrete video fields. We show that the flash duration is a more reliable measure of fluorescence quenching than is the diameter determined by a reference intensity. We present the calculated concentration profiles in space and time without transforming them into fluorescence intensities. This avoids having to correct for self-quenching, dequenching, and inner filter effects.

We described the vesicle as dye initially constrained and distributed uniformly within a sphere of radius $a$. The initial concentration of dye in this spherical source was 200 mM. We modeled an unquenched flash as dye diffusing, starting at time $t = 0$, into an infinite medium. The equation describing diffusion in general is

$$dC/dt = \nabla \cdot (D \nabla C),$$

where $C$ is the concentration at a point at a particular time and $D$ is the diffusion constant. Diffusion of dye is spherically symmetric in this case, and the solution of the diffusion equation is

$$C(r, t) = C_0 \cdot \left( \frac{1}{2} \left[ erf\left( \frac{a + r}{2\sqrt{Dt}} \right) + erf\left( \frac{a - r}{2\sqrt{Dt}} \right) \right] - \frac{1}{r} \sqrt{(Dt/\pi)} \left\{ \exp\left[ -\frac{(a - r)^2}{4Dt} \right] - \exp\left[ -\frac{(a + r)^2}{4Dt} \right] \right\} \right), \quad \text{(A1)}$$

where $r$ is the distance from the center of the spherical source (Crank, 1975). For numerical evaluation, the diffusion constant $D$ was assumed to be $5 \times 10^{-6}$ cm$^2$/s.

We modeled a quenched flash as dye, at $t = 0$, diffusing into an infinite medium containing 5 mM free cobalt, which is the concentration of free cobalt expected for 40 mM cobalt citrate. We calculated the concentration of free calcein subject to the assumption that the reaction between cobalt and calcein was first-order and irreversible. The concentration of unreacted calcein, $C_u(r, t)$, is given by the equation

$$\partial C_u/\partial t = D\partial^2 C_u/\partial r^2 - kC_u, \quad \text{(A2)}$$

where $k$ is the unidirectional formation rate for the reaction of calcein and cobalt. We solved Eq. A2 subject to the initial condition that the concentration of calcein was 0 for $r > a$ at $t = 0$, and the boundary condition that the concentration gradient, measured in an outward direction along the normal to the vesicular surface, $\partial C/\partial N$, was proportional to the dye concentration (i.e., $\partial C/\partial N = \alpha C$ where $\alpha$ is a constant) at all points on the surface for $t > 0$. The solution is obtained by Danckwerts' method and is given by

$$C_u(r, t) = k \int_0^t C(r, t) e^{-kt'} dt' + C(r, t) e^{-kt}, \quad \text{(A3)}$$

where $C(r, t)$ is the solution in the case of diffusion alone (Eq. A1) (Crank, 1975). The

unidirectional formation rate $k$ was taken to be $k_1[Co^{2+}]$, where $k_1$, $6 \times 10^6$ $M^{-1}s^{-1}$, is the forward rate constant for the association of cobalt and calcein and $[Co^{2+}]$ is the concentration of free cobalt. (The forward rate constant for the association of cobalt and calcein was estimated from the known association constant of calcein for cobalt [Martell and Smith, 1977] and the dissociation rate of cobalt from EDTA. Calcein is a fluorescein derivative with two chelating moieties. Each moiety is one-half of an EDTA arm; i.e., each chelating arm of calcein is iminodiacetate.) Eq. A3 was solved numerically with a modified midpoint algorithm, in which the number of times the integrand is evaluated increases proportionally with the integration interval (Press et al., 1986). Diffusion of cobalt into the vesicle was ignored and therefore the solutions are valid only at distances, $r$, greater than or equal to the initial vesicle radius, $a$.

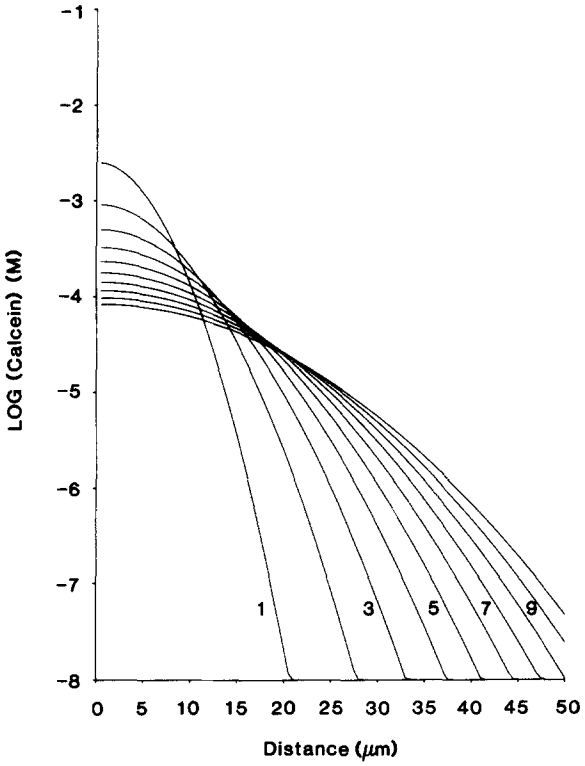


FIGURE A1. Calculated concentration profile for unquenched dye released from a 3-μm-diam vesicle. The concentration of calcein is plotted as a function of distance from the center of the vesicle. The profiles were calculated for the first 10 video fields, as indicated, after vesicle rupture.

Concentration profiles for both the free diffusion (unquenched) and diffusion with reaction (quenched) cases were calculated for vesicles with initial radii of 1–5 μm at distances between 0.5 and 100 μm away from the center of each vesicle. In order to mimic video sampling, integral multiples of the video field time, 16.7 ms, were used as time points. Because the time at which a vesicle ruptures, which corresponds to $t = 0$ in the solutions to the equations, does not coincide with the onset of any particular video field or video scan line, we also calculated concentration profiles at times 3, 6, 9, 12, and 15 ms after vesicle rupture. This mimics the effect of sampling a flash at some arbitrary time after its onset. Note that because of the nature of video sampling, a flash that actually lasts $(n + \epsilon)$ fields, where $n$ is an integer and $0 < \epsilon < 1$, will be observed as lasting either $n$ or $(n + 1)$ fields; a flash that lasts $(n - \epsilon)$ fields will be observed to last $n$ or $(n - 1)$

fields. The observed flash duration depends on the time between the video camera scanning the vesicle and the precise moments at which the flash began and ceased. Because the image of a vesicle and subsequent flash subtended a small number of video scan lines, we neglected the small changes in concentration that occur during the short period of time necessary to scan the image within a single video field.

The concentration profile of calcein changed greatly between each video field. This is shown in Figs. A1 and A2, where the concentration of calcein released by a 3-$\mu$m-diam vesicle is plotted as a function of distance. Concentration profiles obtained for the first 10 video fields, with the assumption that the vesicle ruptured 16.7 ms before the first field, are shown for diffusion alone (Fig. A1) and diffusion with reaction with cobalt (Fig. A2). In both cases, within one field the calcein concentration decreased from 200 to <10 mM at the boundary of the vesicle.

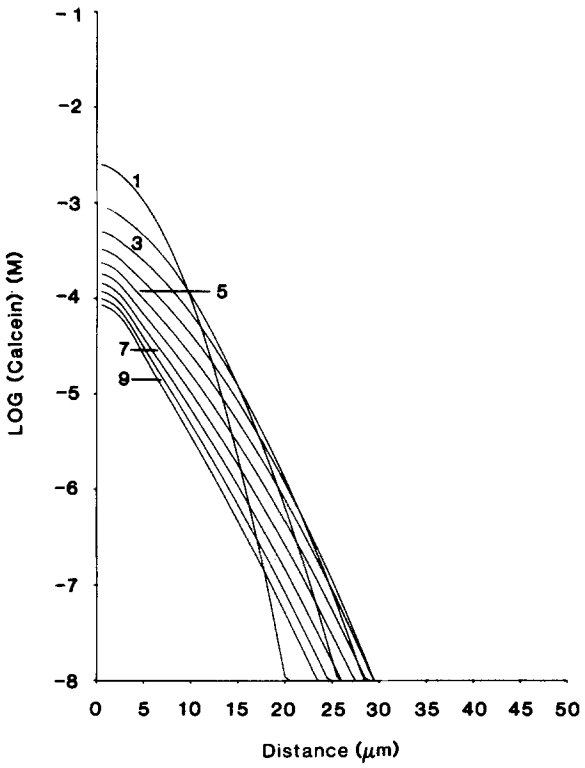


FIGURE A2. Same as Fig. A1 but profiles for a flash quenched in 40 mM cobalt citrate.

To compare our computed profiles with the diameters and durations of the saturated images obtained in experiments, we needed to determine the minimum concentration of dye that produced a fluorescence intensity sufficient to saturate the video camera. We estimated this value from the maximum radii of flash profiles in the first video field after ruptures. We determined the theoretical concentration from the computed profiles that corresponded to these radii. This value was 0.1 mM.

From Fig. A1, a 3-$\mu$m-diam vesicle is expected to produce, 16.7 ms after rupture, a flash diameter of 22 $\mu$m, which, after 33.4 ms, increases to 26 $\mu$m in the absence of quenching. However, the sampling of this flash by the first video field will occur between 0 and 16.7 ms after the occurrence of the rupture. Thus, the diameter of the saturated

image in the first field is expected to be distributed between 3 and 22 μm. Similarly, the expected diameter of the flash in the second video field is distributed between 22 and 26 μm. In the presence of quenching by cobalt (Fig. A2), the saturated-image diameter ranges between 3 and 20 μm in the first video field and between 20 and 22 μm in the second field. This overlap of the theoretical distributions of saturated-image diameters was observed in our measurements of quenched and unquenched flashes. Thus, the diameters of saturated images were unreliable in distinguishing between quenched and unquenched flashes.

With the saturated-image approach, we found that the histograms for the duration of the experimental quenched and unquenched flashes were significantly different. The theoretically derived concentration profiles also predict that the durations of the quenched flashes should be distinctly shorter than the durations of unquenched flashes. This is discernible from Figs. A1 and A2. For example, in a quenched flash, the concentration of dye falls to 0.1 mM at a distance of 2.5 μm from the center of the flash within six fields, whereas it takes eight fields for an unquenched flash to reach this level. It is worth noting that in the unquenched profile, the concentration falls gradually with distance from the original boundary of the vesicle. In the quenched case, however, the concentration falls appreciably faster with distance near the original boundary of the vesicle. This helps to ensure that unquenched flashes will stay saturated for longer times than quenched flashes. Thus, while the theoretical difference in field durations between the quenched and unquenched cases is not large, it exists and in fact is experimentally observed.

There are algorithms suggested by the diffusion model for flashes for distinguishing quenched from unquenched flashes. For instance, an inspection of Fig. A1 shows that, theoretically, the diameter of the saturated image remains equal to or greater than its diameter in the first video field during the next five fields, when the flash is unquenched. For the quenched flash, the diameter of the saturated image decreases to its value in the first field by the second field. However, we found that our procedure of comparing individual flash durations with duration histograms generated from control data was statistically as effective in differentiating quenched from unquenched flashes as any other procedure tested.

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