

JUN 18 1991

Fusion of Influenza Virions with a Planar Lipid Membrane Detected by Video Fluorescence Microscopy

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ABSTRACT The fusion of individual influenza virions with a planar phospholipid membrane was detected by fluorescence video microscopy. Virion envelopes were loaded with the lipophilic fluorescent marker octadecylrhodamine B (R18) to a density at which the fluorescence of the probe was self-quenched. Labeled virions were ejected toward the planar membrane from a micropipette in a custom-built video fluorescence microscope. Once a virion fused with the planar membrane, the marker was free to diffuse, and its fluorescence became dequenched, producing a flash of light. This flash was detected as a transient spot of light which increased and then diminished in brightness. The diffusion constants calculated from the brightness profiles for the flashes are consistent with fusion of virus to the membrane with consequent free diffusion of probe within the planar membrane. Under conditions known to be fusigenic for influenza virus (low pH and 37°C), flashes appeared at a high rate and the planar membrane quickly became fluorescent. To further establish that these flashes were due to fusion, we showed that red blood cells, which normally do not attach to planar membranes, were able to bind to membranes that had been exposed to virus under fusigenic conditions. The amount of binding correlated with the amount of flashing. This indicates that flashes signaled the reconstitution of the hemagglutinin glycoprotein (HA) of influenza virus, a well-known erythrocyte receptor, into the planar membrane, as would be expected in a fusion process. The flash rate on ganglioside-containing asolectin membranes increased as the pH was lowered. This is also consistent with the known fusion behavior of influenza virus with cell membranes and with phospholipid vesicles. We conclude that the flashes result from the fusion of individual virions to the planar membrane.

INTRODUCTION

Since modern biology is concerned with fundamental cellular processes on the molecular level, considerable effort is currently directed toward identifying the cellular proteins that mediate fusion between membranes (Perrin et al., 1987; Trimble et al., 1988; Johnston et al., 1989; Perin et al., 1990). It is only in the case of

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fusion of lipid-enveloped virus to cell membranes, however, that the fusion proteins have been unambiguously identified (White et al., 1983).

In enveloped viruses, the envelope consists of a phospholipid bilayer with multiple copies of only a few types of integral membrane glycoproteins. These viruses initiate infection by attaching to the target cell via receptors on the cell surface, typically a glycoprotein or glycolipid (Karlsson, 1989). The attached virus then fuses (*a*) directly with the plasmalemma or (*b*) with the endosomal membrane after endocytosis when the medium of the vacuole surrounding the virus is acidified by H-ATPases (White et al., 1983; Ohnishi, 1988). Both routes result in the expulsion of the viral nucleocapsid into the cytosol.

Viral attachment and membrane fusion are mediated by the integral glycoproteins of the envelope. To explore the biophysical mechanisms by which these proteins effect membrane binding and fusion, we have developed a model system in which virions fuse with a planar phospholipid membrane. In this paper, using influenza virus, we describe the assay used to quantify fusion and establish that fusion is, in fact, the phenomenon being studied. In the companion paper (Niles and Cohen, 1991) we use the model system to study the consequences of viral binding to the planar membrane, determine the minimal requirements necessary for pH-dependent fusion to phospholipid membranes, and arrive at a working model for fusion.

Influenza virus enters cells via the endocytotic route and requires a low pH for fusion (White et al., 1983). Hemagglutinin (HA), expressed on the envelope in the form of homotrimeric spikes (Wilson et al., 1981), is responsible for attachment by binding to *N*-acetylneuraminic (sialic) acid, the only known determinant on host cell receptors (Wiley and Skehel, 1987). HA also causes fusion at low pH by undergoing a conformational change in which the hydrophobic NH₂-terminus of the envelope-anchored HA₂ subunit becomes exposed (Skehel et al., 1982; Daniels et al., 1983; Doms et al., 1985). The subunit is thought to trigger fusion by interaction with the target membrane (Harter et al., 1988). Influenza is a particularly useful virus with which to study fusion because the structure of the HA extraviral domain (ectodomain) at neutral pH is known (Wiley et al., 1981; Wilson et al., 1981; Caton et al., 1982) both in the presence and absence of sialic acid (Weis et al., 1988).

We use video fluorescence microscopy to detect the fusion of individual virions. As is illustrated in Fig. 1, we pack virion envelopes with a high concentration of the lipophilic fluorescent dye R18; this results in self-quenching of the dye fluorescence.¹ Virions are delivered to a planar membrane containing gangliosides, which mimic the cell surface receptors and allow the virions to bind. Upon fusion, the envelope becomes incorporated into the planar membrane and the R18 is free to diffuse throughout it. As the R18 is diluted by diffusion, its fluorescence becomes de-

¹ Concentration quenching of fluorescence arises dynamically by energy transfer from excited R18 dye molecules to nearby unexcited dye molecules in the virion envelope. As the electronic energy transfers to vibrational and rotational modes, the excitation is thermally dissipated, thereby decreasing the quantum yield of fluorescence. Quenching of R18 fluorescence also may arise statically by the formation of multimolecular aggregates with excitation spectra different from free R18 molecules. Regardless of the actual mechanism, surface densities of R18 in excess of 1% by mole fraction result in fluorescence quenching (Hoekstra et al., 1984).

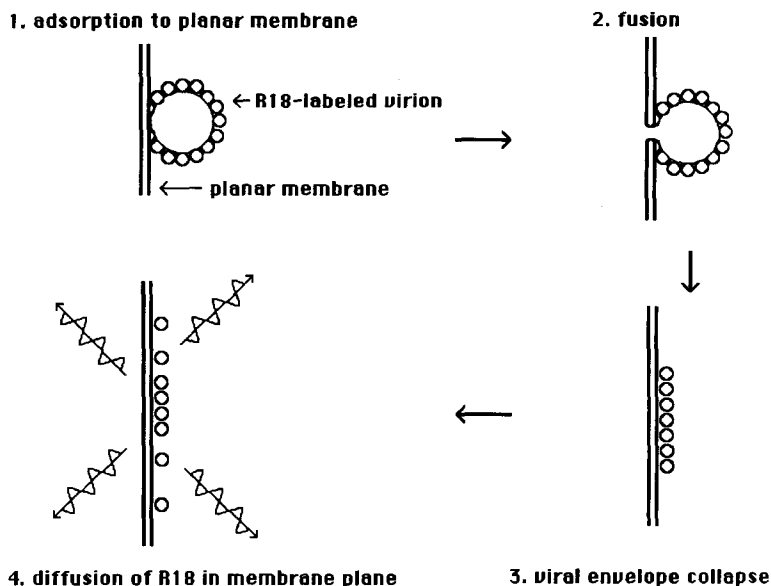


FIGURE 1. Detection of virion fusion with a planar phospholipid membrane as a flash of light emitted by dequenching of R18 fluorescence. (1) The lipophilic cationic dye R18 is incorporated into the virion envelope at a sufficient density for energy transfer between excited dye molecules to quench the fluorescence of the probe. The virion binds to the planar membrane by attachment of HA to gangliosides included in the planar membrane. (2) After maintenance at 37°C and low pH, which are fusigenic conditions for influenza virus, the envelope fuses with the planar membrane and (3) the R18 is liberated to diffuse in the much larger area. (4) Diffusion of R18 away from the site of fusion increases the distance between R18 molecules so that energy transfer between the excited probes decreases. This produces a flash of light as the fluorescence of the R18 is dequenched.

quenched and the fusion of the virion is detected as a flash of light. We have used this method to measure the pH dependence of influenza fusion to planar membranes.

MATERIALS AND METHODS

Labeling of Influenza Virion Envelopes

Virion envelopes were labeled with R18 according to the procedure of Hoekstra et al. (1984) with several modifications to optimize the visibility of flashes. Influenza virus, strain A/Puerto Rico/8/1934 (type H1), was grown in the allantoic fluid of chicken embryos, harvested, and purified by centrifugation in a sucrose gradient. Virions were labeled with R18 to achieve as high a surface density as possible without encountering detergent effects of the amphiphilic dye on the envelopes. 25 μ g (protein) of virus was added to 0.25 ml of the pH 7.4 planar bilayer chamber buffer consisting of 135 mM NaCl, 5 mM Na_2HPO_4 , 5 mM NaH_2PO_4 , and 5 mM citric acid, pH 7.4, in a screw-cap glass culture tube. 30 μ g of R18, from a 10 mg/ml ethanol stock solution, was slowly transferred from a glass pipette to the virus tube while vortexing for 15 s to increase R18 adsorption to the virus. The mixture was incubated at room temperature in the

dark for 1 h. The labeled virions were separated from the free dye by gel filtration in mini-chromatography columns (Pierce Chemical Co., Rockford, IL) containing either Sephadex G-25 medium (Pharmacia Fine Chemicals, Piscataway, NJ) or P-30 medium (Bio-Rad Laboratories, Richmond, CA). As determined visually, most of the dye associated with the virus. The virus-dye mixture was bath-sonicated briefly (5–10 s) to disaggregate virions before application to the column. The column was eluted with the buffer and the leading edge of the dye front as judged by eye was collected in 0.25-ml fractions. Labeled virions were stored on ice before use and prepared fresh daily.

Video Fluorescence Microscopy of the Planar Membrane

Virion fusion with a planar phospholipid bilayer membrane was detected in a bilayer chamber placed in a modified fluorescence microscope, previously described (Niles and Cohen, 1987). The planar membrane, formed across a 160- μm -diam hole through a black Teflon septum, separated two buffer-filled compartments. The front and rear walls of the chamber were glass coverslips. The planar membrane was viewed en face with a microscope that had been turned onto its back brace and had its stage removed. The chamber was situated with the planar membrane in the specimen plane of the microscope, and the image of the planar membrane was focused—with an 11-mm working distance infinity-corrected objective (L25X/N.A. 0.4; E. Leitz, Inc., Rockleigh, NJ) and zoom projection lens (#543 341; E. Leitz, Inc.)—onto the face of a low-light-sensitive video camera (SIT 66; Dage-MTI Co., Michigan City, IN). The video signal was observed on a monitor and recorded on tape (VO 5800H; Sony Communications Co., Parsippany, NJ). To measure the brightness values of fluorescence images, the video tape signal was digitized at 8-bit brightness resolution and 512 pixels/video line with an image processor (Series 151 Image Processor; Imaging Technology, Woburn, MA). The images were stored in 8-bit frame buffers and computations were performed in 16-bit frame buffers before truncation to minimize round-off error.

Virions were delivered to the planar membrane with an L-shaped pipette mounted to a micromanipulator and connected to a nitrogen supply through a button-activated electric valve (General Valve Corp., Fairfield, NJ). The pipette, fabricated by a procedure previously described (Niles and Cohen, 1987), held the virions in their pH 7.4 buffer. The pipette was immersed in the rear (*cis*) compartment and maneuvered to a position directly behind the hole in the partition. Virions were ejected at the planar membrane by briefly (3 ms) opening the electric valve. We typically delivered virions in a burst of squirts by opening the valve two to five times in rapid succession. The rear compartment also contained a small magnetic stirring bar to ensure vigorous mixing of the solution in the compartment. With a custom-made miniaturized heating coil (Control Products, Chicago, IL) and a thermistor (YSI-11; Yellow Springs Instruments, Yellow Springs, OH) immersed in the solution of the rear compartment, the temperature of the rear compartment was held at 37°C with a proportional controller (constructed by the Rush Electronics Facility). The pH of the rear compartment was monitored with a needle-encased micro-bulb electrode (SA4B; WPI, New Haven, CT). The pH of the virus-containing rear (*cis*) compartment was varied by the addition of 0.5 M H_3PO_4 . The pH of the virus-free front (*trans*) compartment was always 7.4.

Planar Membrane Composition

Membranes were formed by raising two phospholipid monolayers (Montal and Mueller, 1972) over a squalene-treated hole. The thinning of the membrane was monitored both electrically and optically (Niles et al., 1988). The monolayers were spread on the buffer in the two compartments of the chamber from a 1% solution of the lipid in hexane. The lipid-forming solutions were made of mixtures of lipid/ganglioside in molar ratios of 19:1 or 9:1. The lipid

consisted of 1:1 asolectin/cholesterol and the ganglioside consisted of 1:1 G_{D1a}/G_{T1b} . These gangliosides, consisting of sialic acid linked to a penultimate galactose, mimic the cell surface receptor for HA of the PR8 strain (Paulson, 1985). The ratios of lipid/ganglioside were chosen so that the calculated distances between the gangliosides in the planar membrane were 1–4 nm, which are the distances between the binding sites of the monomers within each trimer (Wilson et al., 1981). The gangliosides were solubilized in hexane by the addition of methanol to 5%. Ganglioside-free planar membranes were formed from 1:1 asolectin/cholesterol.

Erythrocytes

In experiments to assay reconstitution of the HA, a receptor for red cells, into the planar membrane, two pipettes were used in the rear compartment. The first pipette held labeled virus; the second contained red cells. Human red blood cells were obtained from the Rush-Presbyterian-St. Luke's Medical Center Blood Bank (Chicago, IL). The cells were washed three times in a pH 7.4 chamber buffer and resuspended to yield a density of 10^6 cells/ml. The cells, placed in a second L-shaped pipette and mounted adjacent to the first pipette, were directed at the planar membrane with a second pressure ejection system. This allowed delivery of the cells to the planar membrane within a short period of time after exposure to the virus.

RESULTS

Planar Membrane Appearance after Delivery of Virus

When an experiment was initiated, the aqueous compartments were maintained at 37°C and pH 7.4, as this pH is unfavorable for influenza virus fusion. A pipette containing R18-labeled virions was brought to within 10–20 μm of the planar membrane with the micromanipulator. Virions were squirted at the membrane and then the pipette was backed away. In our initial experiments we attempted to detect binding as a residual fluorescence following the delivery of the virions to the planar membrane. At ~ 15 s after virus delivery only a very dim fluorescence, uniform across the planar membrane, was observed (Fig. 2). This background fluorescence was associated with the planar membrane because it was unaffected by stirring the compartment, was in a single plane of focus, and disappeared when the planar membrane was broken. Images of planar membranes before and after exposure to virus were digitally subtracted from each other to obtain the background fluorescence intensities due to bound virus. This fluorescence intensity was very low (6 ± 3 brightness units/pixel \pm SD on a scale of 255, $n = 5$). We could not detect discrete, individually bound virions because they have diameters of 80–120 nm (Ruigrok et al., 1986), which are below the optical resolution of our setup (700 nm), and because the fluorescence of the probes in the virions was quenched. This low, uniform background fluorescence could, therefore, have been caused by individual bound virions and/or by residual free R18 that was not removed by gel filtration, partitioning into the planar membrane. Thus we could not reliably quantify virus binding.

In some labeled virus preparations, irregularly shaped aggregates of virions, large enough to be resolved and brighter than the background fluorescence, were observed. An aggregate is seen above the scale bar in Fig. 2. The aggregates were often only very slowly removed by prolonged stirring, but they disappeared when the membrane was broken. Their velocity of movement, 1–10 $\mu\text{m}/\text{s}$, was much less than that of the out-of-focus particles unassociated with the planar membrane but within

the “unstirred layer,” which moved at velocities approaching 100 $\mu\text{m/s}$ because of the vigorous stirring. Aggregates were not consistently obtained and their binding was not rigorously studied.

Detection of Flashes

Nature of flashes. Propelling virus toward the planar membrane in a burst of pressure ejections under conditions known to be favorable for influenza virus infection (37°C

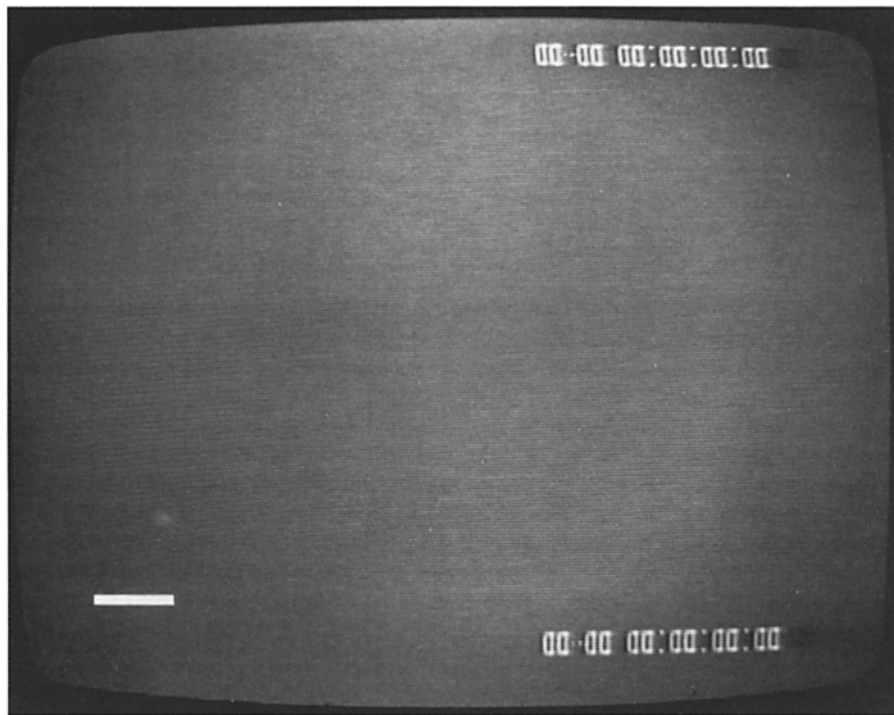


FIGURE 2. Background fluorescence of a planar membrane exposed at pH 7.4 and 37°C to R18-labeled influenza virus by ejection from a pipette. The membrane, (asolectin/cholesterol 1:1)/($G_{D1a} + G_{T1b}$ 1:1) 9:1, exhibits a slightly higher fluorescence than the black Teflon partition surrounding the hole. The fluorescence originates from virions bound to the planar membrane as well as from free R18, not removed by the labeling procedure, that has partitioned into the membrane.

and low pH, e.g., pH 5, within the *cis* compartment) led to the rapid appearance of small, brief flashes of light. The rate of flashes was greatest during the first few seconds after delivery and then declined rapidly. This is expected for particles not bound tightly to the planar membrane as the detached virions were rapidly swept away by the vigorous stirring. The flashes typically ceased by 30 s (usually sooner), but sometimes isolated flashes were observed as long as a few minutes after applying virus.

Each flash initially appeared as a small, spatially localized, bright spot of light, which grew to a larger diameter as its brightness gradually decreased. The flash duration was variable and ranged from 100 ms to 10 s. This variability occurred mostly between different preparations of labeled virus despite ostensibly identical labeling conditions. Brighter flashes always lasted longer. In the beginning of an experiment flashes were easy to see against the background fluorescence of the planar membrane. With time, the flashes imparted so much R18 to the planar membrane that flashes could no longer be reliably observed against the high background. The high background brightness was reached with relatively few bright flashes or, correspondingly, with a greater number of dull flashes.

Fig. 3 illustrates several sequential video frames of a single flash. As shown in the frame obtained immediately before the eruption of fluorescence (Fig. 3A), we did not detect bound or fusing virions before flashes. By four video frames (133 ms) later (Fig. 3B), the bright disk of R18 fluorescence has grown to 6 μm in diameter. A virion deposited its content of R18 into the planar membrane at a point location and the dye diffused for some distance in the planar membrane during the time between the two video frames. We will argue in subsequent sections that the R18 is deposited via fusion of the viral envelope with the planar membrane. As the R18 diffused (free diffusion of R18 is demonstrated below), its concentration at the fusion site decreased, which relieved the self-quenching of fluorescence and produced a bright emission of light from a small area. As the diffusing R18 fanned out over time and its concentration increased at distances far from the fusion site, the diameter of the fluorescence area increased as its brightness decreased (Fig. 3, C and D). This sudden high brightness in a small area followed by spreading of the fluorescence with decreasing intensity over a larger area was the characteristic profile of a flash.

Flash characterized by free diffusion of R18. The spatial and temporal evolution of brightness in each flash was consistent with free diffusion of R18 in a lipid bilayer and the consequent relief of quenching of the dye's fluorescence. This can be seen from the spatial distributions of brightness along diameters of flash images. Fig. 4A shows the brightness profile of the 28th video frame (933 ms) after the initiation of the flash. The brightness profile is reasonably fit by the equation for radial diffusion in a plane from a point source (Crank, 1975, Eq. 3.4) with a diffusion constant (D) of 10^{-8} cm^2/s as illustrated by the solid curve. This D can be estimated by noting that the brightness decreases to 37% of its peak value 3–4 μm away from the center, consistent with the two-dimensional diffusion equation $r^2 = 4Dt$. The spatial profile of this flash in the 38th video frame (1.267 s) is shown in Fig. 4B. Again, the profile is well approximated with $D = 10^{-8}$ cm^2/s (solid curve). The peak brightness, however, is the same as in Fig. 4A, although for free diffusion the peak concentration of dye should fall by 27% in the latter frame. This illustrates that the fluorescence of the dye is still self-quenched, even after diffusing for 1 s.

We also estimated D by tracking the position of a threshold brightness at two times within a flash. The procedure is illustrated by Fig. 4C, where the flash brightness profile from the 5th video frame after the start of a flash is shown overlaid with the brightness profile obtained in the 10th frame. Note that the flash has shifted to the right. This is due to the direction of the stirring within the *cis* compartment which swept the fluid mosaic of the lipid bilayer rightward. The precise shifts were also

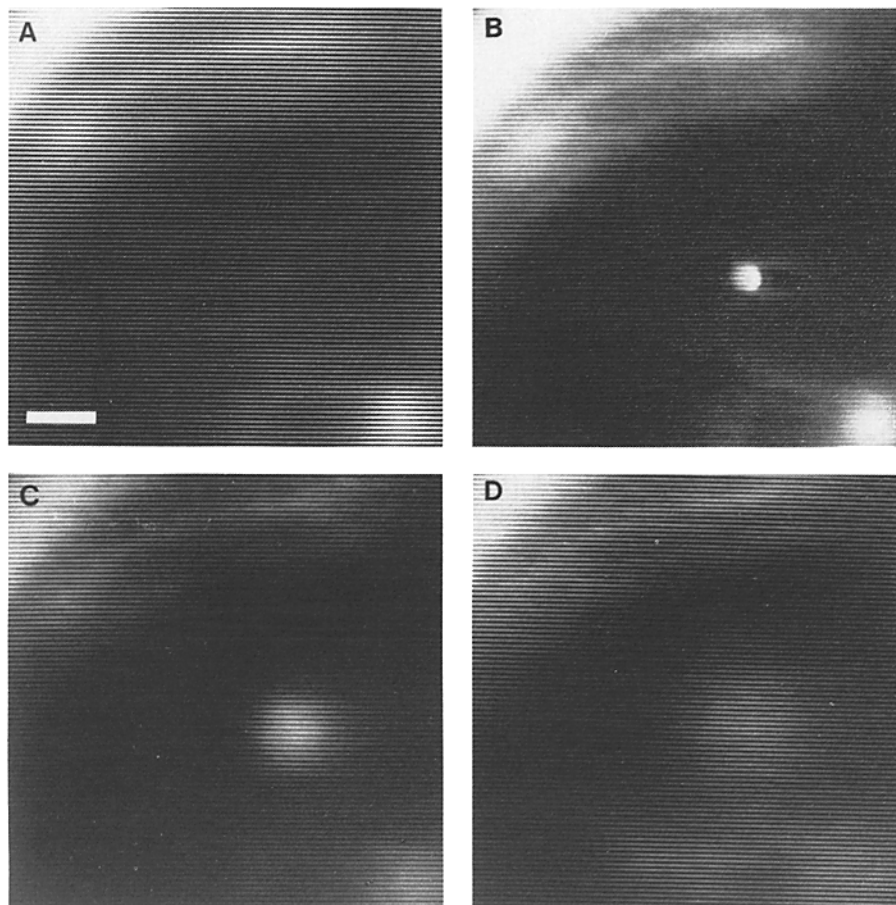


FIGURE 3. Video frames of a single flash resulting from the fusion of an influenza virion with a planar membrane at pH 5.2. (A) The image of a small area of planar membrane obtained in the video frame immediately before the eruption of fluorescence. The bright curved region at the upper left is the torus. The less intense circular region is the remnant of an earlier flash. Before flashes, no fluorescent particles are detected because (a) the R18-labeled influenza virions are smaller than the resolution of the video microscope and (b) the fluorescence of the R18 in the virion envelope is sufficiently quenched. The scale bar denotes 10 μm . (B) The image of the same area of planar membrane as in A, but obtained four video frames (133 ms) later. The R18 has diffused sufficiently far in the planar membrane from the site of virion fusion for the fluorescence to become dequenched. The flash appears as a very small intense spot of brightness. (C) The same area 2.1 s (63 video frames) later. The R18 continues to diffuse to greater distances from the site of fusion in the planar membrane and its fluorescence continues to become dequenched. The diameter of the intensely bright region of the flash has increased over its size in B, and the brightness has decreased steeply at the edge of the flash. (D) 5.9 s (177 video frames) after C. The R18 has diffused very far from the site of virion fusion. The brightness of the flash, although covering a large area, has diminished.

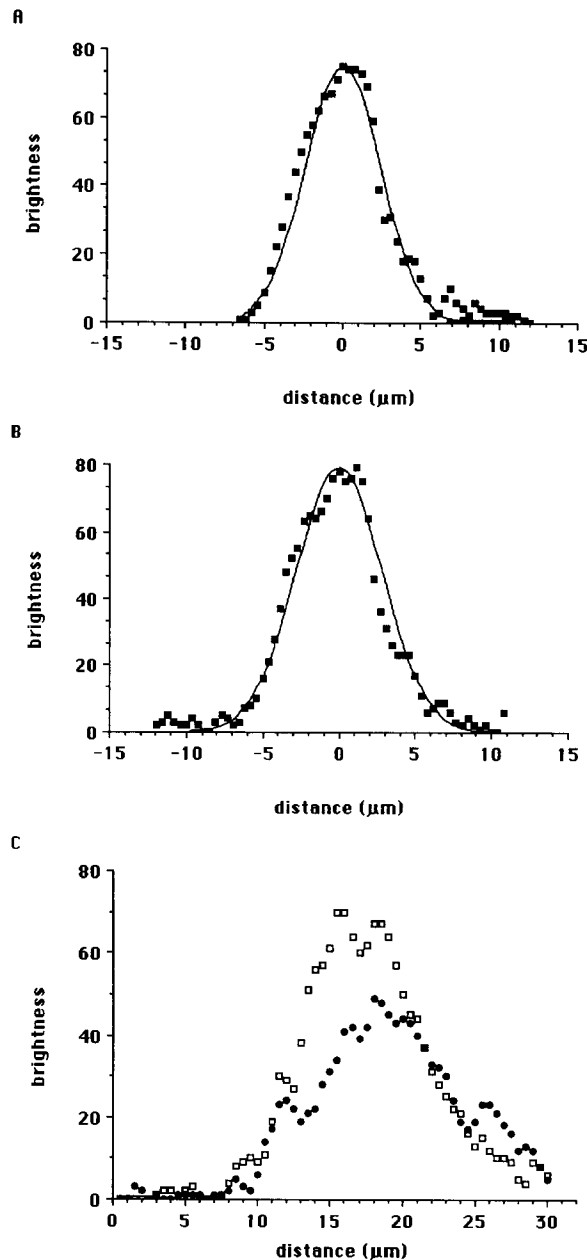


FIGURE 4. Brightness distributions along diameters of flash images. Each brightness profile was determined from the digitized video frame containing the image of the flash after subtracting the video frame containing the image of the planar membrane immediately before the flash (as in Fig. 3 A). A line segment cursor was overlaid on the diameter of the flash in the resulting image and the brightness values of the pixels were read. The distance scale shown corresponds to the line segment distance of 60 pixels (30 μm). (A) The brightness profile of a flash diameter 28 video frames (933 ms) after the eruption of fluorescence. The observed pixel brightnesses (*filled symbols*) are plotted together with the theoretical distribution of brightness (continuous curve) for diffusion of R18 in the planar membrane with a diffusion constant D of 10^{-8} cm^2/s and the peak concentration at the center scaled to the maximum brightness of the observed distribution (see text). The theoretical curve for free diffusion of R18 in the membrane fits the experimental brightness points well. (B) The brightness distribution of the diameter of the same flash in the 38th frame, 1.267 s after the onset of the flash. (C) Brightness profiles from a flash obtained 5 (*open squares*) and 10 (*filled circles*) video frames (115 and 330 ms)

after eruption. The rightward shift in the center of the distribution in the latter frame results from convection of the chamber solution near the planar membrane. The diffusion constants measured from the brightness profiles in these two frames, after correction for the shift in the center, are 1.6×10^{-7} cm^2/s for diffusion to the left and 7.9×10^{-8} cm^2/s for diffusion to the right.

affected by motions in the bilayer induced by pressure-ejecting virus toward the membrane. To determine the diffusion constant of the probe, we shifted the brightness distributions so as to colocalize their maxima. Frames between 33 and 333 ms apart and threshold brightnesses between 10 and 40% of the maximum brightness in the first video frame were used. Solving the diffusion equation for a point source in an infinite plane (Crank, 1975, Eq. 3.4) yields the positions of a given concentration of diffusing substance at two different times: $D=(r_1^2/t_1 - r_2^2/t_2)/4\ln(t_2/t_1)$, where r_1 and r_2 are the radial positions of the threshold from the center of the brightness profiles at times t_1 and t_2 after the beginning of the flash. The diffusion constants for the flash brightness profile, as measured by this temporal method, were $4.41 \pm 1.10 \times 10^{-7}$ cm²/s to the left (mean \pm SE, six frame pairs) and $1.41 \pm 0.68 \times 10^{-7}$ cm²/s to the right. These values are greater than the diffusion constant necessary to fit the flash brightness profile in single frames (Fig. 4, *A* and *B*) and exceed measured values of D ($\sim 10^{-8}$ cm²/s) for lipid probes in phospholipid bilayer membranes (Fahey and Webb, 1978). The overestimated diffusion constants reflect the dequenching of R18 fluorescence far from the site of viral fusion (see Discussion). Nevertheless, the brightness profiles are consistent with free diffusion of the alkyl probe R18 deposited into the lipid bilayer at a point source by virion fusion.

Flashes Are Due to Fusion

R18 flashes only occur under fusion conditions. Control experiments with R18 argue that flashes were due to fusion. Micelles of R18 were formed by following the virion labeling procedure described in Materials and Methods except that it was done in the absence of virus. Ejecting these micelles toward the planar membrane did not result in flashes, although the planar membrane gradually increased in brightness over a 10-min period. Thus, flashes did not arise by the rapid diffusion of R18 into the planar membrane from the aqueous medium. Similarly, flashes did not occur when phospholipid vesicles (formed by the sonication-freeze-thaw procedure and labeled with R18 by the same protocol used to label virions) were ejected toward the planar membrane. Nor did binding these vesicles to planar membranes with calcium (Akabas et al., 1984; Niles and Cohen, 1987) lead to flashes. Flashes were observed and the planar membrane became brighter when the vesicles were osmotically swollen, a condition known to produce membrane fusion (Cohen et al., 1980; Zimmerberg et al., 1980). These control experiments strongly indicate that R18 flashes occur only as a result of membrane fusion and do not originate from rapid transfer of R18 from the lipid envelope of the virions in the absence of fusion.

Reconstitution of red cell receptor and correlation with brightness. For the flashes to have resulted from virion fusion, the hemagglutinin glycoprotein of the viral envelope, HA, must have been reconstituted into the planar membrane. We assayed for the presence of HA in the planar membrane by utilizing its well-known hemagglutinating (red cell-binding) properties (Salk, 1944). We determined whether the flashes were correlated with red cell binding to the membrane at various pH values.

After the planar membrane was exposed to virus, a second pipette filled with erythrocytes was maneuvered toward the membrane and the red cells were ejected.

Loosely associated red cells were removed by vigorous stirring. We counted the number of cells that remained motionless and in focus at the plane of the membrane during stirring as viewed in bright-field microscopy. These cells were considered to be bound to the planar membrane since they were in a single plane of focus, were virtually immobile, and could not be removed even with vigorous stirring. At all pH values cells did not bind to planar membranes that had not been exposed to virus.

Consistent with the idea that flashes are due to fusion, red cell binding was directly related to the R18 fluorescence of the planar membrane resulting from the flashes. As described below, when ganglioside-containing membranes were used, flashes were observed at low pH. Subsequent ejection of red cells from a second pipette resulted in their binding to the planar membrane. A planar membrane exposed to virus and red cells is illustrated in Fig. 5. As seen in the upper right panel, which is a view in bright-field illumination, very few red cells are bound after virions are ejected into pH 7.4 buffer. The fluorescence of the same membrane is dull (upper left panel) because very few flashes are observed at neutral pH (few virions fuse). While some cells that did bind may have been attached to unfused virions that remain adsorbed to the planar membrane, virions were not tightly bound to planar membranes.² The lower panels show the same membrane exposed to virus at pH 5. A large number of red cells are bound (lower right) and the membrane is much brighter (lower left) due to the greater number of flashes. In four experiments in which erythrocytes were ejected at the planar membrane after exposure to virus, the average R18 brightness at pH 7.4 was 6 ± 2 brightness units/pixel and an average of 3 ± 2 red cells were bound, while in the range of pH 5.3 to 4.8 the brightness averaged 59 ± 18 brightness units/pixel and 37 ± 11 cells were bound. We conclude that the flashes resulted from the fusion of individual virions with the planar membrane as these flashes reconstituted HA into the planar membrane.

Measurement of the Flash Rate and Its pH Dependence

Because it is well known that low pH promotes fusion of influenza virus with its host membrane (Huang et al., 1981; Stegmann et al., 1985), we measured the pH dependence of the rate of flashes. Initially, the solution in the *cis* compartment was maintained at pH 7.4, virions were ejected from the pipette, and the rate of flashes was measured. After two to five squirts of virus (with less than 1 s between squirts) at the planar membrane, flashes appeared immediately and, when high rates of flashes occurred, started before the cessation of the series of squirts. Flashes were observed over a period of time (the observation time) lasting from 5 s to 3 min after each burst of squirts. The flash rate for each burst was calculated by dividing the number of flashes counted during this period by the observation time. We refer to this as the

² Delivering virions to a membrane bathed by pH 7.4 buffer did not result in flashes. Subsequent acidification to pH 5.0 also did not result in flashes, indicating that any virions bound at pH 7.4 did not remain attached during the acidification. When fresh virions were then delivered to the membrane at pH 5.0 flashes were observed. We also wish to point out that the force of the solution ejected from the pipette toward the bilayer was large enough to cleanse the bilayer of particles that may have still been bound from prior ejections. Hence, red cells were not tightly bound to the membrane via intermediary bridging by unfused virions (see Discussion).

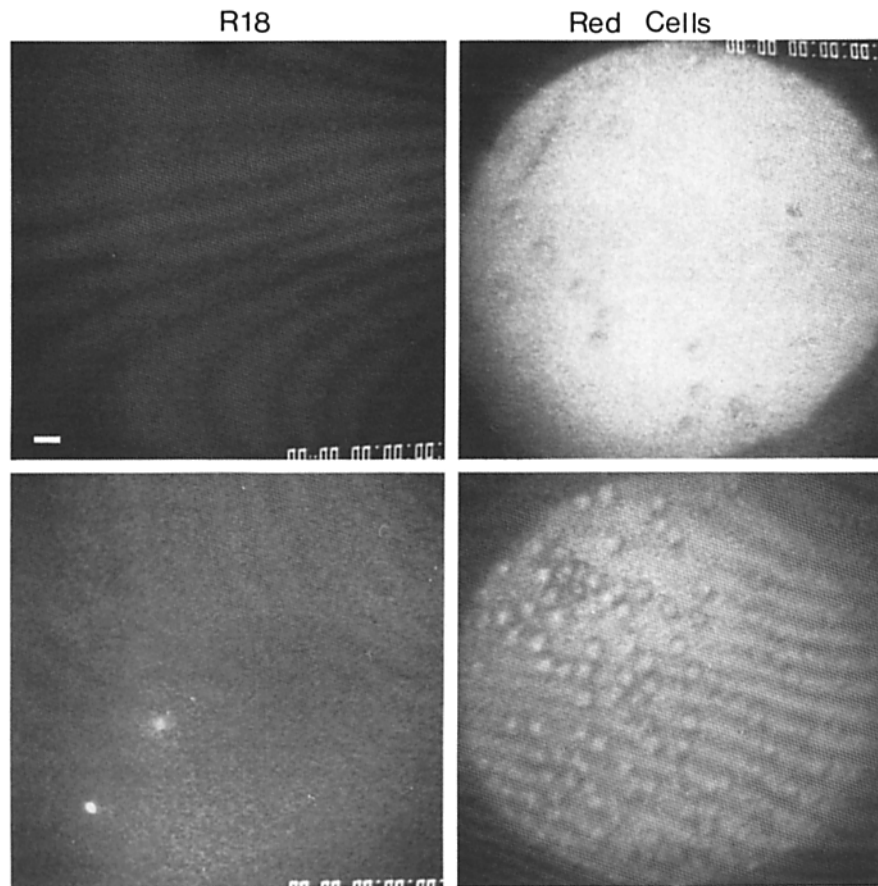


FIGURE 5. Red cells are bound to HA reconstituted into a planar membrane by virion fusion. The left panels are fluorescence images of a planar membrane exposed to R18-labeled influenza virus at pH 7.4 (*upper left*) and the same planar bilayer after exposure to virus at pH 5.0 (*lower left*). The fluorescence of the membrane has been increased at the lower pH due to the incorporation of R18 by the flashes. After the planar membrane was exposed to virus at each pH, a second pipette was maneuvered to the hole and red cells were ejected toward the membrane. Unbound cells were stirred away. The right panels are bright-field images of the red cells that remained adherent. At pH 7.4 (*upper right*) few cells are bound. The planar membrane does not contain an endogenous receptor for erythrocytes, and the negatively charged gangliosides on the surfaces of the planar membrane and the erythrocytes repel one another. The numerous red cells bound at pH 5.0 (*lower right*), however, adhere to HA incorporated into the planar membrane by virion fusion. Both the fluorescence brightness and the number of red cells bound to the planar membrane increase by an order of magnitude at low pH. As a result of fusion the HA and R18 incorporate into the planar membrane in the same ratio as they exist in the viral envelope. The scale bar denotes 10 μm .

“measured flash rate.”³ The observation time was varied to include all flash events: it was longer when flash rates were low than when the rates were high. The number of squirts was varied to compensate for flash rate differences between different preparations of labeled virus (e.g., differing amounts of labeled virus recovered from the column). Independent of pH and temperature, more squirts were applied when the preparation exhibited a low flash rate. As elaborated in the companion paper (Niles and Cohen, 1991), these procedures allowed small differences between flash rates under different conditions to be determined.

After a series of bursts at pH 7.4 and measurements of the flash rates, the pH of the *cis* compartment was lowered to an acidic level ranging from 6.5 to 2.0 by the addition of H₃PO₄. Virions, held in the pipette at pH 7.4, were again delivered to the membrane and the new flash rates were determined as above. The pH of the *cis* compartment was lowered further and the measurements were repeated. The experiment was terminated when the brightness of the planar membrane became too large for the visual detection of flashes. We could measure fusion rates for each membrane at at least two pH values.

Averaged flash rates. Our method of determining the flash rates would be strictly correct if the flashes were distributed uniformly over the observation time. This is obviously not the case because the material near the planar membrane surface and the planar membrane itself are continually swept by the stirring and pipette ejections (Niles and Cohen, 1987). Diffusion and convection continually deplete the planar membrane of bound virions. Ideally, to quantify flash rates, the number of bound virions and the exact time (on a video frame basis) that each flash is initiated would be measured. The resulting distribution of flash times would yield the “true” flash rate and knowing the time-varying number of bound virions would yield the likelihood that a bound virion fuses. Each of our measured flash rates is the mean value of the true flash rate averaged over the observation period.

To estimate the flash rate at each pH, we averaged the measured flash rates (referred to as the averaged flash rate) for all of the bursts of squirts obtained at the same pH. An alternate method to estimate the flash rate at each pH would be to compute an ensemble mean at each pH by summing all the numbers of flashes and dividing by the sum of the observation times. This ensemble mean is the rate expected if the flash events were truly distributed uniformly over the observation times. We found that the two estimates were not significantly different as determined by a *t* test.

pH dependence of flash rate. The averaged flash rates, pooled for all membranes bathed in the same pH buffer, are shown in Fig. 6. The rate of virion fusion to asolectin-ganglioside planar membranes varied greatly with pH. At pH 7.4 the averaged rate of flashes was $1.6 \pm 0.6/\text{min}$ ($0.027 \pm 0.010/\text{s}$, mean \pm SE) with 19:1 asolectin/ganglioside and $1.8 \pm 0.7/\text{min}$ ($0.027 \pm 0.010/\text{s}$) with 9:1 asolectin/ganglioside. Typically, zero to five flashes were observed during the first 1-min period after

³ As an experimental test that our flash rates reflected virion fusion activity, we measured rates with two different ratios of labeled to unlabeled virions. When labeled virions were diluted 10-fold with unlabeled ones, the flash rate at pH 4.8 on ganglioside-containing planar membranes decreased by about 20. With a fivefold dilution of labeled virions, the flash rate decreased by about a factor of two. Thus, the flash rates varied roughly in accord with the ratio of labeled to unlabeled virions.

the cessation of the series of squirts. In many preparations, no flashes were observed at pH 7.4. Although the rates were relatively constant over different membranes of the same composition for a given labeled batch of virions, the rates did vary between different lots of virus and between preparations of labeled virions from the same lot. Thus, there are relatively large errors associated with both the ensemble means and the averaged flash rates. Nevertheless, the ensemble mean rates, 1.32/min ($0.022 \pm 0.011/s$) for the 19:1 and 1.92/min ($0.032 \pm 0.011/s$) for the 9:1 lipid mixtures, were fairly close to the averaged rates. This again illustrates that the rates were estimated with reasonable precision by our estimation procedure.

At pH less than 6.0, the flash rates were greatly increased. Over the range of pH 5.7–5.4, the rate increased from 0.06 to 0.5 flashes/s, while between pH 5.4 and 5.0 the flash rate approached 1/s. The deviation of the averaged rates from the ensemble

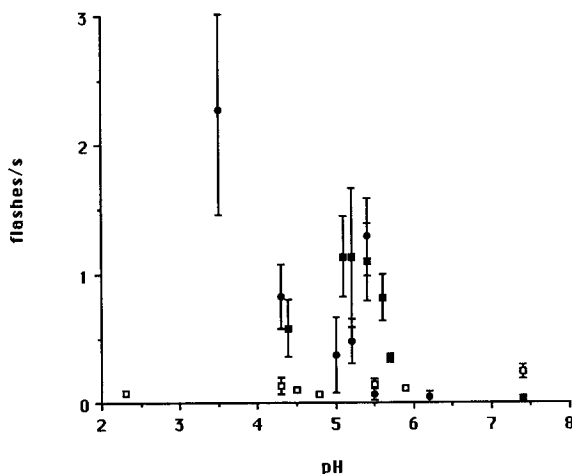


FIGURE 6. pH dependence of the flash rate of R18-labeled influenza virions to asolectin/cholesterol (1:1) planar membranes with and without gangliosides. Averaged flash rates were obtained from the rates measured at the same pH for different membranes. The gangliosides were G_{D1a} and G_{T1b} (1:1). The lipid/ganglioside ratios were 9:1 (filled squares) and 19:1 (filled circles) in different planar membranes. Flash rates obtained on asolectin/cholesterol planar membranes without gangliosides are also

plotted (open squares). The flash rate increased steeply at low pH when gangliosides were present. In the absence of gangliosides, the flash rate was independent of pH.

rates was greatest in this pH 5.7–5.0 range (Fig. 6), reflecting the steep increase in the rate of virion fusion. Below pH 5.0 the rates continued to increase but the deviations of the averaged flash rates from the ensemble mean were less. We were unable to decrease the pH to less than 2.0 because the planar membranes became unstable below this value. As seen in Fig. 6, the flash rates were not affected by the precise mole fraction of the gangliosides within the membranes. When gangliosides were omitted from the lipid mixture, however, the flash rate was independent of pH (Fig. 6). The dependence of the flash rate on the type of phospholipid and on the presence of HA receptor determinants in the planar membrane is the subject of the companion paper (Niles and Cohen, 1991).

DISCUSSION

We have shown that if R18 is packed into the virion envelope so that its fluorescence is self-quenched, a flash of light is detected in the video fluorescence microscope when the virion fuses and liberates its R18 to diffuse in the planar membrane.

Red Cell Binding to Membranes

We confirmed that flashes result from fusion by demonstrating that HA is reconstituted into the membrane, as measured by red cell binding to planar membranes. The number of red cells bound is correlated with the amount of R18 fluorescence due to the flashes. Since the virions were weakly bound to the planar membrane at pH 7.4 (see footnote 2), they would not be expected to provide a bridge for red cells to adhere to the planar membrane. Consistent with this, we found that red cells bound more tightly to planar membranes containing reconstituted HA rather than virus to planar membranes. This is in part because the glycoprotein of erythrocytes has a higher affinity for the binding site of HA than do gangliosides (Paulson, 1985). Additionally, each virion is very small and is anchored to the planar membrane via few HA molecules, whereas the large red cells can form bonds with relatively large numbers of HA molecules embedded in the planar membrane.

Utility of R18

R18 is a useful probe in studying virion fusion. It transfers only slowly into the planar membrane from micelles dispersed in the aqueous phase and does not spontaneously transfer from phospholipid vesicles. The brightness background of the membrane, which determines the ease with which flashes are observed, increases when R18 is incorporated by fusion, but is not significantly increased by the nonfusional slow transfer of R18 from virions to the planar membrane. Moreover, the flash assay is unambiguous; only flashes are scored to indicate fusion and they are easily resolved. Therefore, the rate of virion fusion is directly measured in video microscopy by counting the number of flashes occurring within a given period of time after the delivery of the virions to the planar membrane. While R18 is a useful probe, it is by no means ideal. Each virion within a batch does not become labeled with the same density of R18; therefore, virions produce flashes of different durations and intensities. However, as individual fusion events are scored in the planar membrane (but not the vesicle) system, nonuniformities in labeling are not severe because each event is weighted equally, independently of the contribution that a particular flash makes to the total brightness. Also problematic is that the degree of labeling is not quantitatively reproducible from batch to batch.

Diffusion of R18 within the Planar Membrane

The self-quenching of R18, which varies with concentration, decreases the brightness profile. Only the concentration of probe obeys the diffusion equation; brightness due to the probe does not. Because of quenching, the brightness of probe can be greater at lower concentrations than at higher concentrations. For the same reason, the brightness of a flash can be greater in later frames than in earlier ones. (If this were

not the case, fusion would not result in flashes.) Therefore, we do not place great significance on the precise numerical values we obtained for diffusion constants based on brightness rather than concentration profiles. But we infer that, once deposited in the bilayer, the dye essentially diffuses freely. This should be contrasted with the extremely slow diffusion of R18 that occurs when labeled influenza virus fuses with erythrocytes (Georgiou et al., 1989; Lowy et al., 1990).

The R18 diffusion constant is inaccurately estimated when calculated from brightness profiles. As the dye diffuses from the site of fusion (center of a flash) into surrounding regions, self-quenching is relieved and the brightness profile more closely approximates the concentration profile of the R18. At distances far from the center of the flash, the brightness increases due to both diffusion and dequenching. When the position of an arbitrary threshold brightness near the edge of the profile is determined at two times as in Fig. 4 C, the threshold brightness in the first frame underestimates the dye concentration to a greater extent than in the latter frame. Therefore, as the probe dequenches, the spatial profile of brightness moves faster than it would if only diffusion were occurring (i.e., the threshold brightness moves further than would be expected from only diffusion of the dye). This leads to an overestimate of the diffusion constant.⁴

The movement of R18 due to the lateral convection of the planar membrane (resulting from stirring the *cis* compartment bathing the membrane) did not significantly contaminate our estimates of the diffusion constants. After correcting for convective motions, the diffusion constants evaluated from R18 movement to the left and right of the flash center were not significantly different (Fig. 4 C). Any small differences were probably due to incomplete correction for convective motions of the bilayer affecting the brightness profiles.

Ejection of Virions and pH Changes

Virions were ejected from a pipette and delivered in a bolus of pH 7.4 buffer to the planar membrane. The solution of the *cis* compartment was set at the desired pH (e.g., low pH). The volume of each bolus ($>0.1 \mu\text{l}$) was sufficient to replace the volume of the low pH bathing solution within the $10 \mu\text{m}$ between the pipette and the planar membrane ($\sim 2 \times 10^{-4} \mu\text{l}$) many times over. The bolus was quickly replaced by the low pH bathing solution near the planar membrane through the vigorous stirring of the solution. Hydrogen ions had to diffuse over only a relatively short

⁴ That the diffusion constant is incorrectly estimated in the presence of fluorescence dequenching can be shown quantitatively. In an early frame, time t_1 , the radial distance of a brightness from the center of the flash is x_1 , and in the latter frame, time t_2 , this brightness has moved to a radial distance x_2 . We let $c(x, t)$ be the concentration of the R18 at a radial distance x at time t and set $c_1 = c(x_1, t_1)$ and $c_2 = c(x_2, t_2)$. We define q_1 and q_2 to be the quantum yields of an R18 molecule at concentrations c_1 and c_2 , respectively. If the threshold brightness is the same in the two frames, $q_1 c_1 = q_2 c_2$. When the diffusion equation is used to describe brightness profiles, $(q_1/4\pi D t_1) \exp(-x_1^2/4D t_1) = (q_2/4\pi D t_2) \exp(-x_2^2/4D t_2)$ or $D = (x_1^2/t_1 - x_2^2/t_2)/4 \ln(q_1 t_2/q_2 t_1)$, where D is the diffusion constant of the probe. Letting D_e be the diffusion constant estimated by assuming that $q_1 = q_2 = 1$, $D_e/D = [\ln(t_2/t_1) + \ln(q_1/q_2)]/\ln(t_2/t_1)$. Since the R18 diffuses from a point source in an infinite medium, at the center the concentration always decreases, $q_1 < q_2$, $\ln(q_1/q_2)$ is negative, and D_e/D is always less than 1. Away from the center, however, the dye concentration can transiently increase, in which case $q_1 > q_2$ and D_e is an overestimate of D .

distance for the virions that remained bound to the planar membrane (unattached virions were swept away) to become acidified. Although the pH was quickly lowered from 7.4 to that of the bath, the virions were obviously exposed to a continuum of intermediate pH values. While we refer to the bath pH, each of our flash rates at $\text{pH} < 7.4$ reflects the time dependence of the pH of the solution surrounding each virion, as well as the pH dependence for the conformational changes of HA that trigger fusion. That the flash rates continually increased as the pH was lowered indicates that the pH gradient in the bolus rapidly collapsed. Direct visualization of the contrast change of phenol red ejected from a pipette showed that the bolus was dispersed by the bathing solution within 100 ms (three video frames).

pH Dependence

The pH-dependent rate of virion fusion to the ganglioside-containing membrane is consistent with the known activity of influenza: pH-dependent fusion to cells (Huang et al., 1981) and phospholipid vesicles (Stegmann et al., 1986; Wharton et al., 1986), and pH-dependent fusion of erythrocytes to transfected fibroblasts expressing surface HA (Morris et al., 1989; Sarkar et al., 1989). The virus-planar membrane system offers a purely biophysical system in which pH-dependent influenza virus fusion can be studied on a direct kinetic basis at the level of single events. A systematic study of this pH dependence is the subject of the companion paper (Niles and Cohen, 1991).

We wish to thank Dr. Mark E. Peeples for the generous gift of virus inoculum and the use of his laboratory. We also thank Dr. Peeples and Dr. Eduardo Rios for careful readings of the manuscript.

This work was supported by NIH grants R01 GM-27367 and BRSO S07 RR05477-02.

Original version received 12 October 1990 and accepted version received 8 January 1991.

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