# The Role of *N*-Acetylneuraminic (Sialic) Acid in the pH Dependence of Influenza Virion Fusion with Planar Phospholipid Membranes

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ABSTRACT It is known that fusion of influenza virus to host cell membranes is strongly promoted by acidic pH. We have determined conditions required to obtain pH-dependent fusion of influenza virus to planar bilayer membranes. The rate of viral fusion was determined from the flash rate of R18-labeled virions delivered to the surface of the planar membrane by pressure-ejection from a pipette. For a bilayer formed only of phospholipids and cholesterol, the fusion rate was independent of pH and unaffected by the phospholipid composition. When the gangliosides $G_{D1a}$ + $G_{T1b}$ were included in the planar membrane, however, the fusion rate varied steeply with pH. The rate at pH 7.4 in the presence of the gangliosides was about an order of magnitude less than in their absence. At pH less than $\approx$5.5, the rate was about an order of magnitude greater in the presence of gangliosides than in their absence. The fusion rate with planar membranes containing globoside, a ceramide-backboned glycolipid, was also independent of pH, indicating that the pH dependence required sialic acid on the carbohydrate moiety of the glycolipid. The gangliosides $G_{M1a}$ and $G_{M3}$, both of which possess sialic acid, produced the same pH-dependent fusion rate as seen with $G_{D1a}$ + $G_{T1b}$, indicating that the presence, but not the location, of terminal sialic acids is critical. Incubating virus with soluble sialyllactose blocked fusion to both ganglioside-free and ganglioside-containing planar membranes. These results show that the pH dependence of influenza virion fusion arises from the interaction of the sialic acid receptor with the influenza hemagglutinin. A model for sialic acid–hemagglutinin interactions accounting for pH-dependent fusion is presented.

## INTRODUCTION

Lipid-enveloped viruses utilize membrane fusion to initiate cell infection. After virion attachment to receptors on the host cell surface, the virus deposits its nucleocapsid into the cytosol of the cell by fusing its lipid bilayer envelope either directly with the plasma membrane of the cell or, after endocytosis, with membranes of intracellular vesicles (White et al., 1983; Ohnishi, 1988). For influenza virus, the hemagglutinin

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(HA) glycoproteins of the envelope control both attachment and fusion. Each HA is expressed as a homotrimer, with each monomer consisting of single $HA_1$ and $HA_2$ subunits linked by a disulfide bridge. In the globular domain (head region) of the trimer, each $HA_1$ subunit contains a binding site specific for *N*-acetylneuraminic acid (sialic acid). Sialic acids are present as terminal sugar moieties on glycolipids and glycoproteins of the cell surface and serve as receptors for HA (Paulson, 1985; Karlsson, 1989). At acidic pH (such as in an endosome or lysosome), HA unfolds and regions of the stalk of the trimer, including the hydrophobic $NH_2$ terminus of the $HA_2$ subunit (the so-called fusion peptide), are exposed (Wiley and Skehel, 1987). The pK of influenza-to-host fusion coincides with the acidic pH at which the conformational change in HA occurs, and this conformational change is believed to underlie HA-induced fusion (Skehel et al., 1982; Daniels et al., 1983; Wiley and Skehel, 1987). HA is an attractive molecule in which to study the molecular basis of membrane fusion because its crystal structure is known (Wilson et al., 1981), a large number of monoclonal antibodies against it are available (Yewdell et al., 1983; White and Wilson, 1987), and there are many systems to assay its function (Stegmann et al., 1989*a*).

In the preceding paper we described a new method for studying the fusion of individual influenza virions with a planar lipid membrane (Niles and Cohen, 1991). Viral envelopes were labeled with a self-quenched concentration of the lipophilic fluorescent marker octadecylrhodamine B (R18). Virion fusion with the planar membrane resulted in release of the R18 into the membrane and the concentration quenching of the R18 was relieved. A fusion event was detected as a brief emission of light, which we termed a flash. Flashes occurred only as a result of fusion. A relative measure of fusion activity, the flash rate, was determined by counting the number of flashes after delivery of virus to the planar membrane.

In this paper we use the flash assay to probe the function of HA and its receptor in mediating viral fusion activity. For type A strains of influenza virus, the only known essential determinant for the cell surface receptor is sialic acid (Paulson, 1985; Suzuki et al., 1986; Pritchett et al., 1987). Sialic acid is present as the terminal residue of numerous gangliosides, which are glycolipids built around a ceramide backbone. Gangliosides included in the planar membrane, therefore, serve as cell surface receptors for the influenza HA. pH-dependent fusion occurs only when gangliosides are included in the planar membrane, independent of the phospholipid composition. We argue from our results that the receptor of the target membrane not only allows proximity of the virus with host membrane, but also regulates the fusion activity of influenza HA. This regulation is discussed in terms of the known structure of HA.

## MATERIALS AND METHODS

### *Video Fluorescence Microscopy*

A video fluorescence microscope was used to detect the fusion of R18-labeled influenza virions with a planar lipid membrane as described in the previous paper (Niles and Cohen, 1991). Virions were delivered to the planar membrane by pressure-ejection from an L-shaped pipette. The flashes due to dequenching of R18 fluorescence after fusion of individual virions with the planar membrane were observed on a monitor at a total magnification of 1,000×. The planar

membrane was voltage clamped either to a constant 50 mV or a −5 mV square wave, and the membrane current was converted to voltage and displayed on a chart recorder and/or digitized and displayed on the video monitor. The video signal was recorded on ¾-in. U-matic tape.

### *Planar Membranes*

*Composition.* Planar membranes were formed from both purified phospholipids (Avanti Polar Lipids, Birmingham, AL) and asolectin as described in the previous paper. The purified phospholipids were bacterial phosphatidylethanolamine (PE), diphytanoyl phosphatidylcholine (DPhPC), and bovine phosphatidylserine (PS). Cholesterol (Sigma Chemical Co., St. Louis, MO), recrystallized once from ethanol, was added to the phospholipids to increase planar membrane stability (see Results). The base mixture for each membrane-forming solution (10 mg/ml in hexane) was phospholipid/cholesterol in a molar ratio of 1:1.

*Gangliosides and other glycolipids.* Gangliosides or globosides were included in planar membranes to provide virion receptors. The glycolipid was added to the 1:1 phospholipid/cholesterol mixture to yield a final ratio of 9:1 lipid/glycolipid. The gangliosides $G_{D1a}$ and $G_{T1b}$ (Sigma Chemical Co.) were purified by HPLC and checked by thin layer chromatography; the gangliosides $G_{M1a}$ and $G_{M3}$ (Matreya Inc., Pleasant Gap, PA) were used without further purification. Gangliosides were solubilized in the hexane of the membrane-forming solution by the addition of 5% methanol. We selected these gangliosides for their α2-3 disaccharide links between the terminal sialic acid residues and galactose residues of the carbohydrate head groups. The binding site in the HA of the strain of influenza virus we use, A/Puerto Rico/8/34 (type H1), prefers this α2-3 anomeric configuration (Winter et al., 1981; Paulson, 1985; Weis et al., 1988). Globoside (Matreya Inc.) was selected to provide a glycolipid without terminal sialate. The carbohydrate sequences of these gangliosides and glycolipids are: $G_{M3}$, NeuAcα2-3Galβ1-4Glcβ1-Cer; $G_{M1a}$, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer; $G_{D1a}$, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer; $G_{T1b}$, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer; and globoside, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer.

Sialyllactose was used as obtained (Sigma Chemical Co.); its anomeric composition was 85% α2-3, 15% α2-6.

*pH variation.* The membranes were initially bathed by symmetrical solutions of 135 mM NaCl, 5mM $Na_2HPO_4$, 5 mM $NaH_2PO_4$, and 5 mM citric acid, pH 7.4. Flash rates as a function of pH were obtained by lowering the pH of the virus-containing solution by adding pretitrated quantities of 0.5 M $H_3PO_4$. This pH, monitored with a microelectrode (SM4A; WPI, New Haven, CT), is the pH referred to throughout the text. The pH of the virus-free compartment was maintained at pH 7.4.

*Measurement of flash rates.* Rates of fusion of R18-labeled influenza virions to planar membranes of different phospholipid compositions were measured for different pH's of the solutions bathing the membrane as described in the previous paper (Niles and Cohen, 1991). The temperature was maintained at 37°C by the immersed heating coil. (In the absence of applied heat the planar bilayer was typically at a temperature of 30–32°C, due to heating by the fluorescence light source. We have made only preliminary observations of the flash rate as a function of temperature.) Labeled virus, contained within a pH 7.4 solution held in a pipette, was brought up to the planar membrane bathed by a solution of the desired pH. The virus was pressure-ejected at the planar membrane in two to five releases delivered ~1 s apart, with each release 3 ms in duration. After a round of releases, the flashes detected by eye above the fluorescence background brightness of the planar membrane were counted for a period of time lasting from the start of the first release until detection of the final flash or until ejection was resumed. The duration of the counting interval ranged from 5 s to 3 min as discussed previously (Niles and Cohen, 1991). The flash rate was taken as the ratio of the number of flashes to the duration of the counting interval. The flash rate measured after each series of

releases was averaged for all releases obtained on different planar membranes of the same composition at the same pH.

## RESULTS

### *Temporal Flash Pattern*

A temporal record of the flash events after two releases in conditions favorable for viral fusion, pH 4.8, 37°C, is shown in Fig. 1. The initiation of each flash event is represented as a vertical line at the time of its occurrence after the start of the ejections. In this record, the flashes were counted until the ninth flash was observed. In all records, flashes scored after a burst of releases were assigned to that burst. After each burst we waited for flashes to subside before applying new virus to the

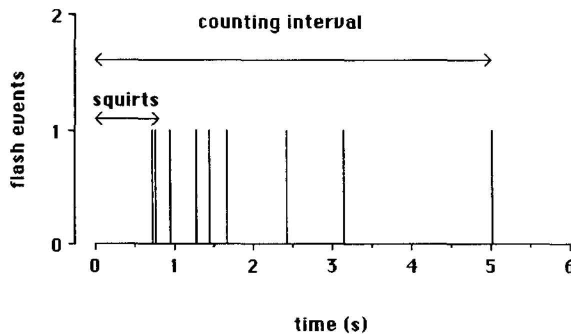


FIGURE 1. A representative sample of the temporal sequence of flash events after delivery of virions to a planar membrane. Starting at time 0, virions were delivered in two rapid releases from the pipette over the period of time denoted by the short horizontal line (0.8 s). The initiation of each subsequent flash event is denoted by a vertical line at the time of its occurrence. As detected by eye, nine flashes were observed. The longer horizontal line depicts the counting interval selected for this set of releases. The counting interval extends from the start of the first release (0 s) to the initiation of the ninth and final flash (5 s). The flash rate is determined as the number of flash events divided by the counting interval, and for this set of releases, was 1.8 flashes/s. Because the flash events occur at a nonuniform rate throughout the duration of the counting interval, this measured flash rate provides a mean value for the rate of flashes after the set of releases. Flash rates were measured in this way after each series of releases as described in the Results.

membrane. In this way we avoided assigning flashes from a previous burst to a subsequent burst, although such misassignments would balance each other out. To calculate the rate, the time from the initiation of the burst to the last flash was used, not the time between bursts.

As seen in Fig. 1, under good fusion conditions flahses appeared in a cluster or group, with the first few flashes beginning during the burst, and several more flashes occurring within the next few seconds. The flash events then abruptly ceased and the counting was stopped. To ensure the accurate determination of the final flash we waited for several more seconds (5–15) before applying more virus. Under conditions of low fusion activity (e.g., pH 7.4, membranes with gangliosides), however, especially with preparations of labeled virus with intrinsically low flash activity, the first flash event often did not occur until 60 s (and in one instance 3 min) after delivery of

virions to the membrane. In these cases, we waited for appropriately longer intervals before applying more virus. Allowing the flash rate to determine the counting interval tends to decrease the number of flash rates estimated to be zero, whereas a fixed counting interval of short duration would miss flash events occurring at a low rate. Counting flashes until the last event, rather than calculating rates during the cluster of flashes, tends to lower the measure of high fusion rates. As this method of counting tends to underestimate high rates and overestimate low rates, differences in rates determined by this procedure are significant.

Delivering the virus to the planar membrane by the pipette ejection technique was more reproducible than by adding virus to the entire chamber (Niles and Cohen, 1987). Because of the inherent hydrodynamic nature of pressure ejection, however, the flash rates are expected to vary due to different amounts of virus delivered with each release. As the bolus containing the ejected virions is acidified by diffusion of hydrogen ions from the low-pH bath, the time course for acidification varies with the volume of the bolus (Niles and Cohen, 1991). In addition, the number of virions in contact with the planar membrane and hence available for fusion continually declined after delivery, because virions bound to the planar membrane were able to dissociate, diffuse, and be swept away by the stirring of the solution. The rate at which virions were lost from the planar membrane could not be assumed to be uniform between each series of releases, since the loss was affected by the volume of the ejected bolus and variable velocity of convection near the planar membrane. While we assume that the flash rate was related to the number of virions bound to the planar membrane at any given time, we did not have a measure of virion binding. However, counting until the last flash assured that some virus was always associated with the membrane during the counting interval. Therefore, the flash rate provides an estimate of only the mean value of the virion fusion rate. Variation of the duration of counting interval compensates for the variabilities better than a fixed counting period.

A more refined analysis of flash rates could be obtained from the time series of the instants of initiation of flash events after each burst. But this laborious task requires computer recognition of flashes. Also, a method to detect bound virions is clearly needed. We are currently developing dynamic pattern recognition algorithms to automate detection of flashes and exploring new fluorescent probes to study binding.

### *The Use of Cholesterol*

We began our studies using only phospholipids to form the planar membranes. The action of the influenza virus on these membranes, however, caused them to become unstable, particularly at lower pH. Including cholesterol in the planar membranes led to more stable, longer lasting membranes, allowing the multiple manipulations needed to complete experiments. The cholesterol did not affect the flash rate of R18-labeled virions at pH 7.4. The flash rate was 13 ± 3/min for two bursts each delivered to three asolectin membranes in the absence of sterol, while for three asolectin/cholesterol (1:1) planar membranes at the same conditions with the same virus preparation, the rate was 12 ± 3/min. Because the cholesterol-free membranes broke when the pH was lowered, we could not investigate whether cholesterol had an effect on the flash rate at lower pH. In addition, we found that cholesterol aided in

the solvation of the gangliosides in the membrane-forming solutions and was required for the stability of ganglioside-containing films at acidic pH.

### *Flash Rate Independent of pH with Ganglioside-free Membranes*

With planar membranes composed of phospholipid/cholesterol (1:1) but containing no gangliosides, flashes were observed to occur at a relatively low rate of ~0.1–0.3/s (or 6–18 flashes/min), which was independent of pH. This is illustrated in Fig. 2, in which the averaged flash rates for planar membranes made from asolectin, DPhPC, PS, and PE are plotted against pH. We obtained the averaged flash rates and standard errors at pH 7.4 and at an acidic pH (5.4–5.1 as given in Table I) where the strain of influenza virus we used (A/PR/8/34) is most active (Huang et al., 1981). For all ganglioside-free membranes, the greatest flash rate was observed at pH 7.4. The flash rate decreased at lower pH for these membranes, perhaps reflecting the low pH–induced inactivation of fusion activity (see below). We never observed an increase in the flash rate on these membranes in the range of pH 5.6–4.8, a pH range over which most strains of influenza virus exhibit a steep increase in the rate of fusion to cell membranes (Huang et al., 1981; Wiley and Skehel, 1987).

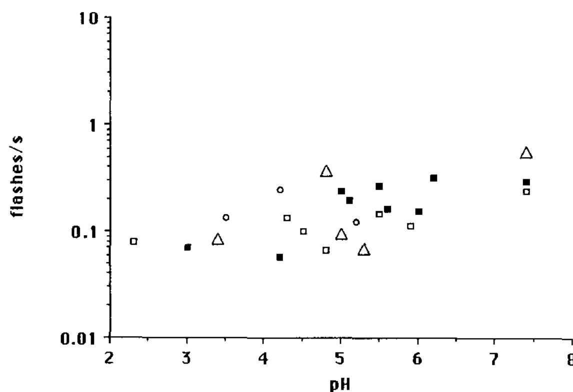


FIGURE 2. pH independence of the flash rate of R18-labeled influenza virions with phospholipid/cholesterol (1:1) planar membranes. A flash rate was measured by counting the number of flashes appearing during an interval of time after several pressure-ejections of virus at a planar membrane. The flash rates obtained on different membranes of the same composition at the same pH were averaged. The averaged flash rates are plotted against pH. The various phospholipids present in the planar membranes were asolectin (*open squares*), DPhPC (*filled squares*), bacterial PE (*open triangles*), and bovine PS (*open circles*). In the absence of gangliosides, the flash rate is independent of pH. The following total numbers of flash events were detected with planar membranes of the indicated phospholipid composition: asolectin, 207; DPhPC, 541; bovine PS, 41; bacterial PE, 66.

The flash rate was relatively independent of the phospholipid composition of the bilayer. Even asolectin, which contains the zwitterionic phospholipids PC and PE, and the anionic lipids PS, PI, PG, and PA among its over 20 components (Erdahl et al., 1973), yielded the same rates as monocomponent phospholipid bilayers. At pH 7.4 the coefficients of variation (standard error/mean) ranged from 17 to 21% among the various phospholipids, with the exception of bacterial PE (42%). There were no statistically significant differences in fusion between phospholipids.

### *pH Dependence of Flash Rate with Ganglioside-containing Planar Membranes*

When the planar membranes contained gangliosides, the flash rate was dependent on pH. This is shown in Fig. 3, where the averaged flash rates are plotted as a function of pH for planar membranes composed of different phospholipids all containing $G_{D1a}$ + $G_{T1b}$ (1:1). The ratio of lipid/ganglioside was 9:1 for the purified phospholipid membranes.[1] We have also replotted the data for asolectin/$G_{D1a}$ + $G_{T1b}$, both 19:1 and 9:1, from Niles and Cohen (1991) for comparison. For all ganglioside-containing planar membranes at pH 7.4, the flash rates were less than for the corresponding ganglioside-free membranes (Fig. 2) by about a factor of 10. As seen in Table I, the rate at pH 7.4 was ~0.03–0.05 flashes/s (or ~2–3/min) for all the lipid mixtures containing gangliosides. The averaged flash rates for the purified phospholipids (DPhPC, PS, PE) with gangliosides at pH 7.4 are not significantly different than for asolectin with gangliosides. The flash rate of R18-labeled influenza virus with ganglioside-containing planar membranes at pH 7.4 was the smallest rate observed for all conditions.

TABLE I

*Rates of R18-labeled Influenza Virion Flashes on Phospholipid Planar Membranes*

| Phospholipid | Averaged flashes/s (±SEM) | | |
|---|---|---|---|
| | at pH 7.4 | at low pH | (pH) |
| Asolectin | 0.24 ± 0.05 | 0.14 ± 0.04 | (5.4) |
| DPhPC | 0.30 ± 0.05 | 0.16 ± 0.03 | (5.2) |
| PS | 0.24 ± 0.06 | 0.12 ± 0.05 | (5.2) |
| PE | 0.57 ± 0.24 | 0.14 ± 0.06 | (5.3) |
| Asolectin:$G_{D1a}$+$G_{T1b}$ (19:1) | 0.03 ± 0.01 | 1.29 ± 0.30 | (5.4) |
| Asolectin:$G_{D1a}$+$G_{T1b}$ (9:1) | 0.03 ± 0.01 | 1.13 ± 0.31 | (5.1) |
| DPhPC:$G_{D1a}$+$G_{T1b}$ (9:1) | 0.05 ± 0.01 | 0.47 ± 0.11 | (5.1) |
| PS:$G_{D1a}$+$G_{T1b}$ (9:1) | 0.04 ± 0.03 | 0.38 ± 0.09 | (5.1) |
| PE:$G_{D1a}$+$G_{T1b}$ (9:1) | 0.05 ± 0.02 | 0.76 ± 0.15 | (5.2) |

With ganglioside-containing planar membranes, the flash rate increased substantially as the pH was lowered. As seen in Fig. 3, when the pH was dropped from 7.4 to as low as 5.6, the rate increased by only a small percentage. At a pH value in the range of 5.5–5.1, a threshold for flash activity was reached and the flash rate increased by a factor of ~10 for DPhPC and PS, 15 for PE, and 40 for asolectin over the rate at pH 7.4 (Table I). At pH values $<5.1$ the flash rate either plateaued (PC, PE, and PS) or continued to increase (asolectin). Therefore, in contrast to the results without gangliosides, here we found some degree of influence of the phospholipid

[1] This combination of gangliosides was used for most experiments because it dissolved more readily in the membrane-forming solutions than either of these gangliosides alone or any other glycolipid used in this study. Also, planar membranes could be formed with them without undue aggravation, and they were more stable than membranes formed with any of the single glycolipids. At the other extreme, $G_{M3}$ and globoside proved the most difficult; planar membranes made with these glycolipids broke after very few releases.

composition on the flash rate. Whether the greater flash rates observed with asolectin reflect some property of its combination of diverse lipids or some additional constituent has not yet been determined.

### *Inactivation of Flash Activity by Pre-exposure of Virus to Low pH*

As seen in Fig. 1 and described above, the flashes observed at acidic pH with ganglioside-containing planar membranes occurred quickly after delivery of virus to the membrane and often began before the cessation of virion release from the pipette. It is clear that the initial high fusion rate correlated with the application of virus freshly dispensed from a pH 7.4 solution. It is known that the fusion ability of influenza virus inactivates when exposed to low pH in the absence of a target membrane (Doms et al., 1985; Stegmann et al., 1985, 1986). To determine whether fusion in the model system parallels the pH-dependent inactivation observed under

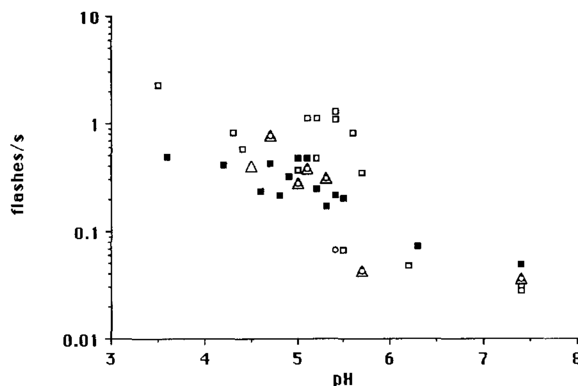


FIGURE 3. pH dependence of the flash rate with phospholipid/cholesterol (1:1) planar membranes containing the gangliosides $G_{D1a}$ and $G_{T1b}$. Flash rates averaged for each pH are shown. The ganglioside mixture, 1:1 $G_{D1a}/G_{T1b}$, comprised 10% of the planar membrane composition for DPhPC (*filled squares*), bacterial PE (*open triangles*), and bovine PS (*open circles*). For asolectin (*open squares*), flash rates obtained on membranes composed of 9:1 and 19:1 lipid/ganglioside are shown. The flash rate is low at pH 7.4 and is increased over the pH range of 5.6–5.1, consistent with the known fusion activity of PR8 influenza virus. The following is the number of flash events observed with planar membranes of the denoted phospholipid composition (at all values of pH): asolectin, 659; DPhPC, 1069; bovine PS, 72; bacterial PE, 96.

biological conditions (Stegmann et al., 1989*a*), we incubated R18-labeled virus at 37°C in pH 5.0 buffer for 5 min before placing a small aliquot in the pH 7.4 solution of the pipette. When these virions were ejected at three asolectin/$G_{D1a}$ + $G_{T1b}$ (9:1) planar membranes bathed in pH 7.4 or pH 5.0 buffer, no flashes were observed. This lack of flashes also shows that any virions that remained bound to the planar membranes from prior releases, and hence exposed to acidic pH for a relatively long period of time, did not reactivate by the transient exposure to released pH 7.4 solution and therefore did not contribute to our measurements of the flash rate.

### *pH Dependence of Flash Rate Requires Sialic Acid*

The pH dependence of the flash activity in the presence, but not in the absence, of gangliosides suggests that the pH-dependent fusion requires specific interaction of the sialic acid residues of the gangliosides with the viral HA. To determine whether

this is in fact the case or if some nonspecific effect of the carbohydrate headgroup on the virus is causative, we measured the pH dependence of fusion rates of virions to globoside-containing planar membranes. Because the glycolipid globoside, with its terminal *N*-acetylgalactosamine, does not contain sialic acid, it was used to ascertain whether the sialic acid was the crucial determinant of the pH dependence. In Fig. 4 the flash rate is plotted against pH for planar membranes made of 9:1 DPhPC (+cholesterol)/globoside. (Globoside-containing planar membranes formed with other phospholipids were not sufficiently stable to conduct flash rate measurements.) The greatest flash rate was observed at pH 7.4; the rate was not increased at low pH. The rate at pH 7.4 was similar to the rates at pH 7.4 obtained with ganglioside-free planar membranes. In fact, the flash activity resembled that in ganglioside-free conditions at all pH values. Thus, the sialic acid appears to be necessary for pH-dependent fusion.

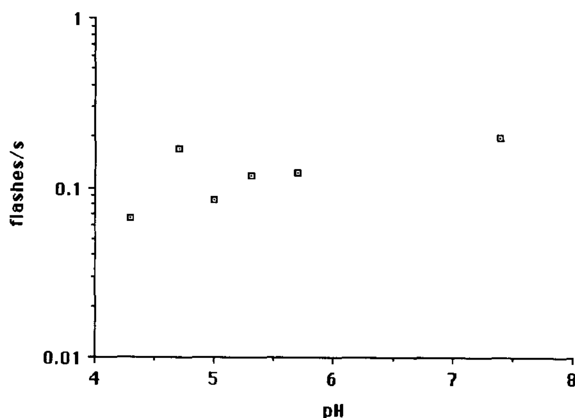


FIGURE 4. pH independence of the flash rate with globoside-containing planar membranes. The planar membrane composition was 9:1 [1:1 DPhPC/cholesterol]/globoside. The flash rate of R18-labeled influenza virions is not increased at low pH with globoside present in the planar membrane. This indicates that the pH dependence observed in the presence of gangliosides is due to the terminal sialic residues of the ganglioside carbohydrate moiety and is not the result of a nonspecific interaction of the virus with carbohydrates on the planar membrane surface.

### *pH Dependence of the Flash Rate with Other Gangliosides*

The headgroups of different gangliosides vary in the number and position of sialates and the degree of branching. We tested whether these factors affect the pH dependence of the flash rate. The flash rates for asolectin planar membranes containing $G_{M1a}$ or $G_{M3}$ (9:1 lipid/ganglioside) are presented in Fig. 5. We have replotted the data for asolectin/$G_{D1a}$ + $G_{T1b}$ to aid comparison. For both $G_{M1a}$ and $G_{M3}$ the flash rate is low at pH 7.4 and similar to the rate observed with $G_{D1a}$ + $G_{T1b}$. Moreover, at low pH the flash rate is increased with these gangliosides, and the pH dependence resembles that for $G_{D1a}$ + $G_{T1b}$. We found no statistically significant differences in the flash rates among planar membranes containing these gangliosides. We conclude that while the pH dependence of the fusion rate requires

sialate-containing gangliosides, the exact position of the sialate is of relatively little importance.

### *Inhibition of Flashes by Free Sialyllactose*

Having established that the sialic acids of the gangliosides were interacting with the binding pockets of HAs, we investigated the effects of sialyllactose (a soluble, free receptor for HA) on fusion. We preincubated R18-labeled virions with 10 mg/ml sialyllactose at 4°C, pH 7.4 for 15–30 min, which is known to saturate the binding of HA (Pritchett et al., 1987). No flashes were detected with ganglioside-containing planar membranes at either neutral or acidic pH. More surprisingly, fusion to ganglioside-free membranes was also abolished by the sialyllactose at all pH values. It is clear that sialate binding to HA profoundly affects influenza fusion activity (see Discussion).

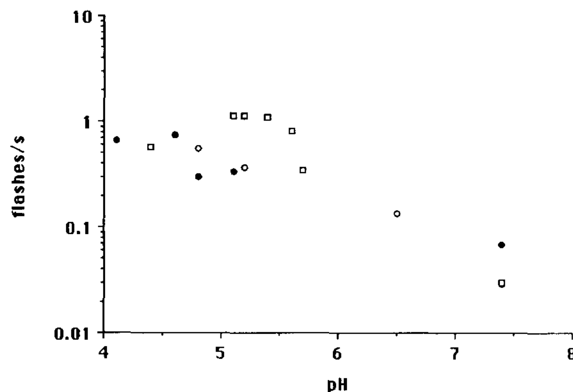


FIGURE 5. pH dependence of the flash rate with planar membranes containing the gangliosides $G_{M1a}$ and $G_{M3}$. The base phospholipid mixture was 1:1 asolectin/cholesterol and the ganglioside was added to yield a final ratio of 9:1 lipid/ganglioside. For both $G_{M1a}$ (*filled circles*) and $G_{M3}$ (*open circles*) the flash rate is increased at low pH. The flash rates with these gangliosides are essentially the same as those obtained with $G_{D1a}$ + $G_{T1b}$ (*open squares*).

### *Electrical Events Not Associated with Virion Fusion*

Rates of fusion were independent of whether the voltage-clamped membrane was held at 50, 0, or −50 mV for ganglioside-free and ganglioside-containing membranes at both neutral and low pH. Ejecting virus at planar membranes could cause nonspecific conductance increases, or destabilization and breakage. However, within a bandwidth of ~50 Hz and a current noise level of 0.1 pA rms, current increases were not observed to be correlated with flashes. Viral fusion was not accompanied by reliable increases in planar membrane conductance.

## DISCUSSION

### *Sialic Acid Activates pH-dependent Fusion*

It is well established that the fusion activity of influenza virus to host cell membranes (Huang et al., 1981; Skehel et al., 1982; White et al., 1982) and the activity of the HA glycoprotein expressed on the surfaces of cells in culture (White et al.,1983; Morris et al., 1987; Sarkar et al., 1989) is strongly promoted by low pH. Our study of influenza

virus fusion to planar membranes shows that pH-dependent fusion occurs only when gangliosides are present in the planar membranes.

Gangliosides and other sialyloligosaccharides are the cellular receptors for influenza virus (Paulson, 1985). Their addition restores infection of sialidase-treated cells (Suzuki et al., 1986). As sialic acid is the receptor determinant for the HAs from the A strains of influenza (Paulson, 1985),[2] gangliosides are functional receptors in both the model and biological systems. It is well recognized that surface sialic acid allows proximity of influenza virus to the target membrane surface through interaction between the cell surface receptor (the sialate) and the binding pocket of each HA monomer (Wiley and Skehel, 1987; Weis et al., 1988). Our findings indicate that an additional, and previously unsuspected, critical function of sialic acid is to confer pH dependence to the fusion process.

At pH 7.4, the fusion rate with ganglioside-containing planar membranes is about a factor of 10 less than the rate in the absence of gangliosides. Therefore, at neutral pH the interaction between the surface sialate and the binding site of HA stabilizes the HA molecule in a set of nonfusigenic conformations, while without surface sialate the HA molecule is able to adopt fusigenic conformations. (Whether these latter conformations are at least partially protonated forms of HA is unknown.) That free sialyllactose inhibits fusion also shows that sialate locks HA into nonfusigenic conformations.

It is perhaps not surprising that free sialate blocks fusion between virus and ganglioside-containing membranes since occupation of the sialate binding site prevents virus binding to the sialyloligosaccharides on the planar membrane surface. It is most unexpected, however, that fusion is abolished, at all pH's, between virus incubated with sialyllactose and phospholipid planar membranes. With virion fusion to phospholipid membranes, the binding site on the HA is presumably unoccupied and hence this binding site would be expected to be unimportant. Yet, occupying this site with sialyllactose totally blocks fusion! In other words, the binding and fusion domains of HA are functionally coupled. This view is contrary to the commonly held assumption that HA binding is independent of low pH–induced conformational changes responsible for fusion (Wharton et al., 1986; Wiley and Skehel, 1987; Stegmann et al., 1989*a*). For some viruses, independent functioning is certainly the case: in paramyxoviruses, such as Sendai or Newcastle disease viruses, attachment and fusion are uncoupled because they are governed by different envelope proteins (White et al., 1983; Ohnishi, 1988).

### *Sialate May Make HA Less Mechanically Flexible*

Each monomer of the homotrimeric HA glycoprotein is long (~13.5 nm), with a globular head at the end of an extended stalk (Wilson et al., 1981) and is illustrated

[2] In addition to HA, the viral envelope also contains the neuroaminidase (NA) glycoprotein which also binds sialic acid. However, cultured cells expressing only HA fuse with erythrocytes and phospholipid vesicles at low pH (Morris et al., 1987; Ellens et al., 1989; Sarkar et al., 1989), and virosomes reconstituted with HA but not NA also exhibit normal fusogenic activity (Nussbaum et al., 1987). We conclude that the virus–ganglioside interactions underlying pH-dependent fusion occur between the surface sialates and HA, rather than with NA. (NA is not involved in fusion [Wiley and Skehel, 1987]. It is needed for proper viral assembly [Palese et al., 1974]).

very schematically in Fig. 6. The binding sites for sialate, one per monomer, reside within the globular head; each site is formed by amino acids of only the $HA_1$ subunit (Weis et al., 1988). The fusion peptide, composed of 10 nonpolar amino acids of the $NH_2$ terminus of $HA_2$, is ~10 nm from the sialate binding site and the surface of the host membrane. The $HA_1$ and $HA_2$ subunits interact with each other all along the stalk region via salt bridges, Van der Waals interactions, and a covalent disulfide bridge (Wiley and Skehel, 1987).

Experiments suggest that conformational changes occur throughout the HA rather than being localized to discrete domains. Lowering the pH causes conformational changes over an extended range of the HA: a different pattern of exposed epitopes to monoclonal antibody binding throughout the globular head (Yewdell et al., 1983; Doms and Helenius, 1986; White and Wilson, 1987), exposure of proteolytic digestion sites within the stalk of the trimer (Skehel et al., 1982; Doms et al., 1985),

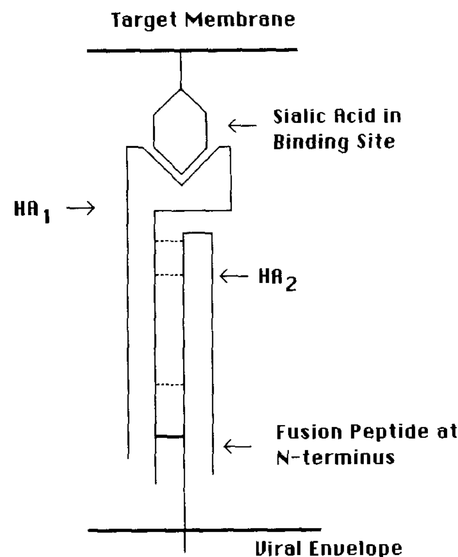


FIGURE 6. Schematic diagram of HA monomer. The molecule is anchored to the viral envelope at the COOH terminus of $HA_2$. The fusion peptide, located at the $NH_2$ terminus, is tucked between $HA_2$ subunits of the trimer (not shown). The sialic acid binding site is located entirely in the $HA_1$ subunit that forms the head of the monomer and faces the target membrane. The $HA_1$ and $HA_2$ subunits are linked by a single disulfide bond (*solid line*) and multiple noncovalent interactions (*dotted lines*) along the stalk.

and exposure of the hydrophobic fusion peptide $NH_2$ terminus of $HA_2$ (Wilson et al., 1981; Doms et al., 1985).

We suggest that upon binding sialate at neutral pH, the resulting reduction in motional (e.g., translational, rotational, vibrational) energy within the globular head propagates through the $HA_1$ to the $HA_2$ via $HA_1$-$HA_2$ interactions, thus stiffening $HA_2$ as well. This restriction in the possible conformations of HA upon binding sialate would result in a mechanically less flexible molecule, thereby making transitions to fusigenic conformations less likely. With its binding site unoccupied at neutral pH, the mechanically more flexible HA trimer is looser, increasing the likelihood that fusigenic conformations (e.g., exposure of the fusion peptide to the aqueous phase) can occur. Surface phospholipid head groups may perturb the globular head of HA, leading to exposure of the fusion peptide in the absence of protonation, which can

then interact with the planar membrane. This would account for the higher fusion rate at neutral pH without gangliosides.

When virions are bound to membranes via gangliosides, HAs are positioned sufficiently near the membrane surfaces for fusion to take place when transitions to fusigenic conformations do occur. When HA becomes protonated, salt bridges are presumably broken (Wiley and Skehel, 1987; Stegmann et al., 1989*a*), and the HA becomes more flexible (Yewdell et al., 1983; Doms et al., 1985; White and Wilson, 1987). We envision that when sialate is bound to HA, protonation of HA favors adoption of those conformations that are intrinsic to fusion. When the binding site of HA is occupied by sialyllactose, the virion is unable to associate with the planar membrane and fusion does not occur. When not bound by sialate, HA is associated too weakly with the planar membrane for protonation to enhance the fusion rate.

The above description is experimentally testable. The low pH–induced conformational changes in HA reported by the investigators cited were performed in the absence of sialate. We predict that if these experiments were performed in the presence of sialyllactose, a different fingerprint of changes would be observed.

### *A State Model for HA-mediated Fusion*

In Fig. 7, X and HX denote the sets of initial, nonfusigenic conformations of the unprotonated and fully protonated forms of HA, respectively. For simplicity, we ignore intermediate states of protonation. X* and HX* denote activated, fusigenic conformations of the glycoprotein; both activated species can either fuse with the target membrane or inactivate to a fusion-incompetent form (Skehel et al., 1982). In the absence of gangliosides, both activated forms are equally effective in promoting fusion, and the rate constants of activation (from X and HX states) are equal (i.e., $k_0 = k_1$). When HA is bound to gangliosides, the unprotonated form, X, is stabilized in its initial state. The protonated form, HX, however, is able to become activated, and so $k_1 > k_0$. As protonation is rapid, we assume that X and X* are in equilibrium with HX and HX*, and the scheme reduces to that shown in the bottom row of Fig. 7. This is redrawn in Fig. 8 *A* with the transition rate constants shown; the equivalent free energy diagram is shown in Fig. 8 *B*.

In Fig. 8, HA denotes hemagglutinin associated with the planar membrane either via binding to surface sialic acid (Fig. 8 *B*, *right*) or through the postulated weaker interaction with the phospholipid head group (Fig. 8 *B*, *left*). HA represents both the unprotonated (X) and protonated (HX) forms of the macromolecule restricted to nonfusigenic conformations. HA* represents activated protein, i.e., HA that has adopted fusigenic conformations. These fusigenic conformations are the result of structural destabilization of the trimer, either by interactions with the planar membrane surface or by protonation. The rate constant for activation, HA → HA*, is denoted by $k_a$. Because activation can occur through either of two independent routes, $k_a$ is the sum of the rate constants for the two transitions, $k_b + k_h$, where $k_b$ is the rate constant of activation through encounter with the planar membrane (X → X*), and $k_h$ is the activation rate constant for the protonated entity (HX → HX*). (To the extent that protonation reactions are instantaneous, $k_0 = k_b$, and $k_1 = k_h$.)

The stabilization of HA conformation at pH 7.4 is illustrated in the free energy

diagram of Fig. 8 *B*. HA bound to ganglioside at pH 7.4 has a lower free energy than does the macromolecule when interacting with ganglioside-free membranes. As the activation energy is larger with ganglioside-containing membranes, $k_h$ is smaller and fusion rates are lower ($k_h$ is small at pH 7.4). At low pH, the free energy of HA bound to ganglioside is increased, decreasing the free energy difference between HA and HA* (which increases $k_h$), resulting in a large rate of fusion.

We show the activated state having the same free energy under all conditions in Fig. 8 *B*. The interaction of HA with sialate lowers the free energy of HA at neutral pH. pH dependence of fusion is conferred by the degree of stabilization in the sialate-bound state. It is this feature, based on our data, that distinguishes this model from others previously proposed (Blumenthal, 1988), which ascribe pH dependence

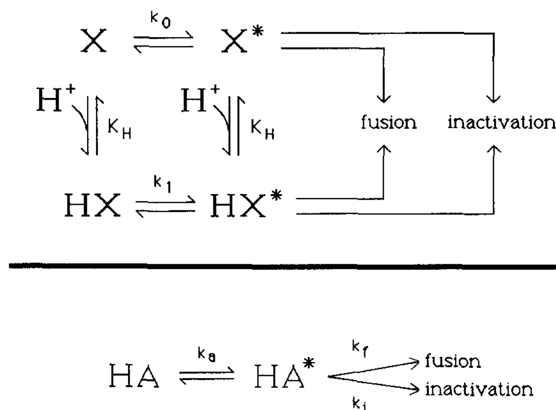


FIGURE 7. State model for the activation of the influenza HA glycoprotein. X and HX denote the unprotonated and protonated conformations, respectively, of the macromolecule and include only those conformations that are unable to cause fusion. For simplicity, our scheme shows protonation of the macromolecule by only a single proton, with the equilibrium dissociation constant denoted as $K_H$. We assume that protonation and deprotonation are very rapid compared with the rates of conformational change. *X** and *HX** represent the activated unprotonated and protonated conformations of HA, which are able to cause membrane fusion. The transition rate constants for activation are $k_0$ for the unprotonated species (X → X*) and $k_1$ for the protonated species (HX →HX*). Below the line, we show the collapsed state model. HA denotes both the unprotonated and protonated conformational states of HA that are unable to trigger membrane fusion. HA* denotes both unprotonated and protonated conformations of HA that cause fusion. The transition rate constant between stable and fusigenic states is denoted as $k_a$. The HA* either triggers fusion with rate constant $k_f$ or inactivates to a nonfusigenic set of conformations with rate constant $k_i$.

of fusion to rates that vary as a function of the degree of protonation. Our model treats HA as switching between unactivated and activated states or transitions between sets of nonfusigenic and fusigenic conformations. The pH dependence is due solely to varying amounts of activated HA. If HA were an ideal switch, the fusion rates of Figs. 3 and 5 would jump as a step function at a transition pH. But as only a finite number of acidic residues need to become protonated for HA to switch to its fusigenic state, the fusion rate increases over a narrow range of pH. We place little mechanistic significance on the precise form of this rise, while other models place importance upon the exact Hill coefficient (Blumenthal, 1988).

To illustrate the effects of pH, we consider a simple single-site protonation scheme of HA. For a $pK_a$ of 5.3, the amount of protonated HA increases by a factor of about

200 over the pH range of 7.4–5.0. For fusion to be independent of pH in the absence of gangliosides, $k_b$ must be greater than or equal to $k_h$ for all pH values. Equivalently, the increase in the amount of HA* due to protonation must be insignificant compared with the much larger pool of unprotonated HA* arising from interaction with the phospholipid membrane. After binding gangliosides at pH 7.4, $k_b$ is lowered and $k_h$ is increased, so that $k_a$ is lowered by about a factor of 10 (Figs. 2 and 3). In this

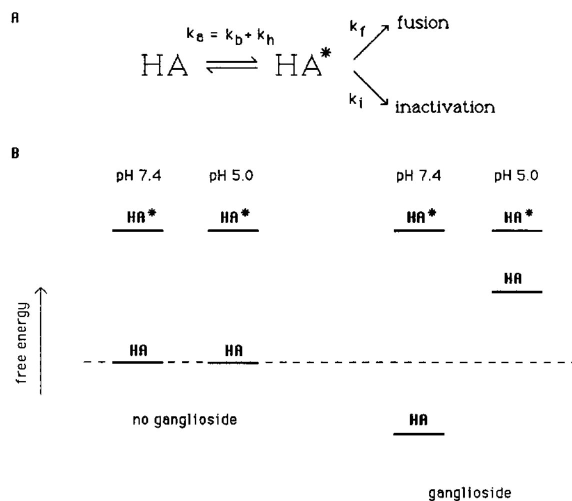


FIGURE 8. A state model and free energy diagram for the activation of influenza HA (*A*) The collapsed state model is recapitulated from Fig. 7. The transition between nonfusigenic (*HA*) and fusigenic (*HA**) conformations is a manifold of two independent routes. In one route, HA* is formed by the destabilization of the protonated macromolecule with a transition rate constant of $k_h$. In the other route, HA* is generated by the destabilization of unprotonated HA through the interaction of the macromolecule with the surface of thetarget membrane with a transition rate constant of $k_b$. Since the HA can adopt a fusigenic conformation through either of these routes, the transition rate constant for activation of the molecule, $k_a$, equals $k_b + k_h$. (*B*) Free energies of the stable and fusigenic conformations of HA in the presence and absence of sialic acid binding at neutral and acidic pH. For clarity, we show the activated, fusigenic conformations (HA*) as having the same free energy under all conditions. Only the energy levels of the initial, nonfusigenic conformations (*HA*) are varied. In the left panel, the energy levels are shown in the absence of gangliosides, while those on the right are for HA bound to sialic acid on the target membrane surface. Without bound sialate, the initial free energy of HA is the same at both neutral and acidic pH and thus pH-independent fusion is observed. In the right panel, sialic acid is bound to HA, the HA is stabilized by a little over 2kT with respect to the ganglioside-free case, and therefore fusion is less likely at neutral pH in the presence of gangliosides. At acidic pH protonation causes significant destabilization of the sialate-bound HA's structure, and the free energy difference between HA and HA* is decreased by ~4kT with respect to the free energy difference at neutral pH. Thus, with sialate-bound HA fusion is dependent on pH.

case, protonation will be the primary route to activate HA. Since only the protonated form has a significant rate constant of activation when sialate is bound ($k_b \ll k_h$), the HA* state will only become populated as the pH is lowered, and the fusion rate will be pH dependent.

When HA is activated, the virus either fuses with the planar membrane, with rate constant $k_f$, or inactivates (becomes fusion incompetent) with rate constant $k_i$.

Whereas the pH dependence for the rate of fusion is a function of $k_f$ and $k_i$ in addition to $k_a$ (Fig. 8 *A*), the total extent of fusion is controlled solely by $k_f$ and $k_i$. The fraction of HA* that triggers fusion vs. the fraction that inactivates will also be affected by ganglioside binding. Binding may orient the molecule into a position that favors fusion with the target membrane, perhaps by holding the hydrophobic portions of the molecule close to the target phospholipids. We expect that $k_f/k_i$ is greater for ganglioside-containing target membranes. Inactivation may be the result of exposure of the hydrophobic domains of HA at a location too far away from the target membrane to effect fusion. In the absence of sialate on the target membrane surface, HA may futilely adopt "fusigenic" conformations, only to inactivate.

### *Comparison with Other Systems*

*Differences with pH dependence of virus–vesicle fusion.* The conventional view that pH-dependent, fusigenic conformational changes in HA occur without binding to membrane–surface terminal sialate acids, and that gangliosides have little if any effect on fusion, is almost solely based on studies of virus fusion to vesicles (Nir et al., 1986; Wharton et al., 1986; Stegmann et al., 1989*b*). (Haywood and Boyer [1985], however, do report that gangliosides significantly affect virus–vesicle fusion.) The coupling of functions may depend on the strain of virus, as we have used the PR8 strain while vesicle studies have typically used X31 and X47. The strain differences may be significant because PR8 prefers α2-3 anomeric linkages of sialate, whereas X31 and X47 prefer α2-6 linkages (Rogers et al., 1983; Webster et al., 1983). As gangliosides have α2-3 linkages, X31 and X47 may not bind as strongly to gangliosides as does PR8 (Pritchett et al., 1987). Although there are only small differences in the binding constants of these anomers to single HA sites (Sauter et al., 1989), cooperative binding, as involved in hemagglutination, shows large differences (Pritchett et al., 1987).

We note that there are differences between vesicle and planar bilayer fusion systems. Conditions that promote binding between vesicles often directly lead to fusion, whereas these same conditions in the vesicle–planar membrane system will cause binding but not fusion (i.e., bound vesicles are stable and do not spontaneously fuse). In the latter, bound vesicles must be independently stressed, notably by generating intravesicular pressures, to cause fusion (Niles et al., 1989). We have previously summarized our view of the basis of these differences (Niles and Cohen, 1987). We do not, however, understand the reasons for differences in virus–planar membrane and virus–vesicle systems. We wish to emphasize that, independent of how differences between various fusion systems are eventually understood, fusion of influenza virus to ganglioside-containing planar membranes is pH dependent. The planar membrane–virus system is devoid of biochemistry and therefore in this case a purely physical mechanism must be responsible for fusion.

*Fusion to planar membranes is fast at low pH.* Fusion is triggered within seconds after exposure of the virions to pH less than 5.6 in the presence of a ganglioside-containing membrane. During the short time between the ejection of virions (held at pH 7.4 within the pipette) and the earliest flashes, around 1 s, the virions must bind

to the planar membrane, protons (hydronium ions) must move from the bath to surround the virions (as discussed in Niles and Cohen, 1991), HA must become protonated, a sufficient number of HA trimers must change conformation, the envelope must fuse, and the R18 must diffuse a sufficient distance in the planar membrane to relieve the self-quenching in order to produce a flash. Because diffusion of R18 in the planar membrane is relatively slow ($D = 10^{-8}$ cm$^2$/s; Niles and Cohen, 1991), we conclude that protonation and the resulting conformational change occur very quickly. Experiments that do not detect individual fusion events and therefore rely on the integration of signals from a large number of events yield much slower rates. For example, as measured spectrofluorimetrically, the time course of fusion of R18-labeled virus with phospholipid vesicles is ~30 s (Stegmann et al., 1986) and the virus-induced fusion of phospholipid vesicles with each other occurs with a time course of 40–60 s (Wharton et al., 1986). Similarly, the exposure of the $NH_2$ terminus of the $HA_2$ subunit (the fusion peptide) occurs over a 30-s time course as measured by the binding of radiolabeled and anti–fusion peptide antibodies (White and Wilson, 1987). These differences between the kinetics of HA activation probably reflect the fact that with video microscopy single virion fusion events are detected, whereas the other assays are macroscopic so that many events must occur to produce a detectable signal. Rapid activation kinetics of HA ($< 1$ s) have also been inferred from single influenza virion fusion with red cells studied with the R18 assay (Lowy et al., 1990).

*Permeability increases are not associated with fusion to planar membranes.* Viral fusion does not induce conductance increases simultaneously with flashes in phospholipid membranes (see Results). This direct experimental result argues against the conjecture put forth by Young et al. (1983) that fusion was marked by conductance increases observed when virus was added to aqueous phases bathing planar membranes. Although virus fusion to protein-free planar membranes does not reliably lead to permeability increases, virus–cell fusion does lead to increases in cell membrane permeability (Impraim et al., 1980; Knutton and Bachi, 1980).

A final point: Viral entry is conventionally described from the viewpoint of the cell; the nomenclature reflects this focus. It would, perhaps, be useful to consider fusion from the perspective of the virion. Just as cellular receptors are activated by agonists, one can consider the influenza HA to be the macromolecular receptor activated by the small ligand sialate (Paulson, 1985). In the conventional view, the gangliosides/glycoproteins with terminal sialates are considered to be the receptors. As our data indicate that sialic acid activates the receptor HA into its pH-dependent, fusion-competent form, the cell surface-bound sialic acid may be regarded not as the receptor, but as the agonist.

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