Fusion of influenza virus membranes with liposomes at pH 7.5

(influenza virus entry/virus binding)

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ABSTRACT Influenza virus X-31 (H3N2) membranes fuse with liposomes containing ganglioside GD1a at pH 7.5. Fusion was demonstrated by electron microscopy and also can be measured by counting the labeled virus proteins incorporated into liposomes after bound virus has been removed. Liposomes composed of lipids that have no net charge behave as reported by other investigators and do not fuse with influenza X-31 membranes at neutral pH, but they do fuse at low pH. Therefore, the liposomal composition is a factor in whether liposomes fuse with influenza virus membranes at neutral pH, probably by determining whether binding occurs. The liposomal composition necessary for fusion at neutral pH needs to be individualized for each influenza subtype. To establish that a virus requires low pH for membrane fusion, it is first necessary to establish that fusion does not occur at neutral pH under conditions where adequate binding occurs.

Enveloped viruses enter cells by direct fusion of the viral membrane with the cellular plasma membrane, by endocytosis, or possibly by both methods (1–3). Fusion of the viral and host plasma membranes results in release of the viral nucleoprotein into the cell cytoplasm. On the other hand, if a virus is within an endocytic vacuole, the nucleoprotein is enclosed not only by the viral membrane but also by the membrane of the vacuole. One way the viral nucleoprotein could gain entry to the cytoplasm from an endocytic vacuole is by fusion of the membranes of the virus and endocytic vacuole.

The literature for many virus groups is replete with examples of entry both by direct fusion with the plasma membrane and by endocytosis. Influenza virus is no exception. Microscopic evidence for direct fusion of influenza virus membranes with cell membranes was first reported by Morgan and Rose (4), although they did not obtain unequivocal micrographs. Influenza WSN was reported to fuse with an isolated chicken embryo plasma membrane preparation in the presence of ATP and to be inhibited by NH_4Cl at 50 $\mu\text{mol/ml}$ (5). An H3N2 strain was reported to fuse with chicken embryo tracheal cells (6). Fidgen and Tisdale (7) showed micrographs of clearcut fusion of influenza A/NWS/33 (H0N1) and influenza A/Scotland/840/74 (H3N2) membranes with erythrocyte membranes at neutral pH by an "on grid" technique, although they noted that the fusion occurred very infrequently. Huang *et al.* (8) gave evidence that liposomes containing influenza proteins can fuse with cells at neutral pH. Others could only find evidence of entry of influenza at neutral pH by endocytosis (9–11). It has been suggested that entry of influenza is by endocytosis with subsequent acidification of an endocytic compartment and that the acidification causes the influenza hemagglutinin protein to undergo rearrangements that are required for fusion of the viral membrane with the membrane of the endocytic compartment (12–15). It has been possible to

demonstrate by electron microscopy the fusion of the membrane of an avian influenza A virus, fowl plague virus, with the plasma membrane of Madin–Darby canine kidney (MDCK) cells when the virus was allowed to bind and the pH was lowered (16). To our knowledge, however, direct microscopic evidence of fusion of a viral membrane with the membrane of an endocytic vacuole or its derivative has not been obtained. Hemolysis of erythrocytes by influenza, which involves a fusion event, has also been reported to require low pH (17, 18), although Laver (19) pointed out that some strains of influenza cause considerable hemolysis at neutral pH. Several influenza viruses have been reported to require acidic conditions to fuse with liposomes (12, 20). The conclusion that low pH is a requirement for fusion of influenza membranes with cellular membranes or with liposomes depends on the demonstration that the influenza membrane can fuse at low pH and also on the negative evidence that it cannot fuse at neutral pH. The latter has not been investigated as thoroughly as the former.

Influenza H3N2 was chosen to investigate fusion of influenza membranes at neutral pH, because it is one of the strains cited as fusing only at low pH and much of the protein chemistry related to fusion at low pH (13) has been done on this strain. For a virus to fuse its membrane with liposomes at neutral pH, the virus must first be able to bind to liposomes at neutral pH. Viral binding at neutral pH can be expected to depend on the liposomal composition. In this work, it is shown that influenza X-31 (H3N2) can fuse at both neutral and acid pH with liposomes containing ganglioside GD1a, but it does not fuse at neutral pH with liposomes bearing no net charge.

MATERIALS AND METHODS

Buffers. Buffer A is 137 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4 /1.5 mM KH_2PO_4 , with the pH adjusted to 7.5 unless stated otherwise. Buffer B is 3 mM Hepes [*N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid]/2.7 mM KCl/130 mM NaCl, with the pH adjusted to 7.5 unless stated otherwise. Buffer C is 20 mM Mes [2-(*N*-morpholino)ethanesulfonic acid]/130 mM NaCl, adjusted to pH 7.0 unless stated otherwise.

Virus Preparation. Influenza X-31 (H3N2) is a recombinant strain derived from influenza A/PR/8 and the Aichi strain of influenza A Hong Kong (21). Purified influenza X-31 in buffer A and 0.1% sodium azide was the kind gift of Robert Webster and was used in the experiment shown in Fig. 1. Seed virus was also obtained from R. Webster and grown in 11-day-old embryonated chicken eggs. The eggs were inoculated with 0.01 hemagglutinating units per egg and incubated for 48 hr at 33°C or 35°C. To label eggs, 0.15 mCi of [^{35}S]methionine (1 Ci = 37 GBq; Amersham) was injected per egg at 6, 12, or 22 hr after infection. (The 12-hr injection gave the highest specific activity.) The cell debris was removed by centrifugation at 4000 $\times$ g for 10 min. The viruses were concentrated by

centrifugation at $40,000 \times g$ (17,500 rpm) for 30 min in an SW28 rotor at 4°C. The pellet was resuspended in buffer A and layered over a 40–70% (wt/vol, in buffer A) sucrose gradient, and centrifuged at 20,000 rpm in an SW 27.1 rotor for 15.75 hr at 4°C. One-milliliter fractions were collected, and the peak fractions were pooled and frozen and later dialyzed against buffer A or B. The virus was then broken into small aliquots before freezing, so after dialysis it was thawed only once before use. A modification of the Lowry procedure (22) was used to determine the protein content of the virus preparations. The viruses used in Table 1 had a specific activity of 1951 cpm per μg of protein, and the viruses used in Tables 2 and 3 had a specific activity of 252 cpm per μg of protein. Some of the virus preparations that had been dialyzed against buffer A (Table 1 and Figs. 2 and 3) were found to have become aggregated after freezing and storage at -70°C . To disaggregate the virus, these preparations were sonicated for 15 sec at 4°C in the cup horn of a Heat System/Ultrasonics sonicator (model W-225R) at an output control setting of 9. That sonication caused only disaggregation, and no release of viral components was determined by velocity sedimentation and electron microscopy.

Lipids and Liposomes. The source and purity of the lipids and the formation of multilamellar liposomes were described (23). The liposomes were made within a few hours of use and incubated with viruses in a final vol of 0.3 ml.

Centrifugation of the Virus-Liposome Complexes. After incubation, the tubes containing viruses and liposomes were put in ice, and unless stated otherwise fetuin was added at a final concentration of 50 mg/ml for 15 min at 0°C. The samples were then mixed with a vol of 70% sucrose (wt/vol) to bring the final concentration to 40%. This was layered on 0.25 ml of 70% sucrose and under 2.6 ml of 30% sucrose and 0.3 ml of buffer A and centrifuged at 54,000 rpm for 40 min at 4°C in an SW60 rotor. The fraction at the interface between 30% sucrose and buffer A contained the liposomes, and the fraction at the interface between 70% sucrose and 30% sucrose contained the free virus.

Electron Microscopy. Negative staining was performed by one of two methods. In Fig. 1, the staining procedure was that described (23). In Figs. 2–4, samples (5 μl) were put on a grid for 5 min, and then the grid was put on three successive drops of 0.145 M ammonium acetate for a minute, and lastly put on a drop of 2% ammonium molybdate (pH 7.1) for 30 sec. The samples were examined in a Phillips EM 200 electron microscope. The virus was examined prior to the experiments to be certain of its purity and that pieces of infected chorioallantoic membrane (which can look like liposomes) were not present.

RESULTS AND DISCUSSION

Association of ^{35}S -Labeled Viral Proteins with Liposomes at 0°C and at 37°C. The most definitive method of determining whether fusion of a viral membrane with a liposome has occurred is electron microscopy. A more quantitative method for defining the conditions of fusion, however, is desirable. A simple method that has proved useful in studying fusion of Sendai virus membranes with liposomes is to count the amount of radioisotope-labeled virus protein incorporated into liposomes (23). This procedure depends on being able to remove the virus that is bound but not fused from the liposomes. Centrifugation at $300,000 \times g$ through a discontinuous sucrose gradient as described removes all but 3–4% of bound ^{35}S -labeled Sendai virus from liposomes. It is easy to demonstrate this for Sendai virus, since conditions exist where Sendai virus binds but is unable to fuse—i.e., at 0°C–4°C or when F_0 virus is used. When Sendai virus membranes fuse with liposomes, labeled viral proteins are incorporated into the liposomes and therefore cannot be removed. Influenza also binds at 0°C–4°C, and, although the possibility that entry can occur at 4°C has been raised (3),

entry and, hence, fusion are generally thought not to occur at 0°C–4°C. To determine whether a similar centrifugation procedure could be used to measure fusion between influenza virus membranes and liposomes, the counts in influenza protein remaining with liposomes after centrifugation were determined both when virus had been bound to the liposomes at 0°C and when virus had been incubated with liposomes at 37°C. Table 1 shows that, unlike Sendai viruses, influenza viruses that are bound to GD1a-containing liposomes are not easily removed by centrifugation. With influenza X-31, 78% of the virus added at 0–4°C remains with the liposomes after centrifugation. This is not significantly different from the counts (81%) that remain with liposomes when virus and liposomes have been incubated at 37°C for 1 hr. Thus, if any fusion has occurred, it is not detectable by this method.

Therefore, the method was modified. Sialoglycoproteins such as fetuin inhibit hemagglutination (24) and so could be expected to compete with ganglioside-containing liposomes for virus. Therefore, just prior to centrifugation, fetuin (final concentration, 50 mg/ml) was added to the virus-liposome mixture for 15 min at 0°C. After fetuin treatment and centrifugation, the amount of virus protein remaining associated with liposomes at 0°C was 11% of that added, and, after incubation at 37°C, the amount of viral protein remaining with the liposomes was 37%. A clear increase in the amount of viral protein associated with liposomes resulted from incubation at 37°C. This suggests that these counts represent viral proteins inserted into liposomes as a result of fusion during incubation at 37°C.

Electron Microscopy of Liposomes Fused with Influenza Virus at Neutral pH. Since fusion of viral membranes with liposomes results in insertion of viral membrane proteins into the liposomal membrane and deposition of the viral internal proteins inside the liposome, the absence of protein transfer to liposomes is evidence that fusion has not occurred. The association of viral protein with liposomes after incubation at 37°C could be due to membrane fusion or could result from tighter binding at 37°C, which is not released by addition of fetuin. Therefore, to determine whether the counts associated with liposomes after incubation at 37°C are indeed due to fusion of the viral and liposomal membranes, the liposomes were examined by electron microscopy.

Figs. 1–3 give examples of liposomes that have fused with influenza X-31 at pH 7.5. When fusion occurs between influenza virus membranes and liposomes, the viral membrane glycoproteins that have been transferred into the liposomal membrane are visible as spikes. Fig. 1 shows a liposome to which a very high multiplicity of virus was added. Multiple fusion events must have occurred with this liposome because there are viral glycoprotein spikes visible over its

Table 1. Effect of centrifugation with and without fetuin on the amount of ^{35}S -labeled influenza proteins associated with liposomes at pH 7.5 after binding at 0°C or after binding followed by incubation at 37°C

Temperature	Fetuin after incubation	% viral protein with liposomes
0°C 1 hr	—	78
0°C 1 hr, then 37°C 1 hr	—	81
0°C 1 hr	+	11
0°C 1 hr, then 37°C 1 hr	+	37

Liposomes were made from PtdCho/PtdEtn/cholesterol/GD1a (0.7 μmol /0.3 μmol /0.66 μmol /0.03 μmol) in buffer B. ^{35}S -labeled influenza virus (1.5 μg of protein) was added at the temperatures and for the times indicated. Where indicated, fetuin was added at a final concentration of 50 mg/ml and kept at 0°C for 15 min before layering the sample under a gradient, centrifuging, and determining the counts in the fraction containing liposomes as percentage of total counts.

FIG. 1. Fusion of liposomes with influenza virus at pH 7.5. Liposomes were made from 1 μ mol of PtdCho/0.05 μ mol of GD1a in buffer A. Influenza X-31 (117 μ g of protein) was bound to the liposomes at 0°C for 1 hr and then incubated at 37°C for 2 hr. ($\times 82,500$.)

complete surface. An unfused virus is seen next to the liposome. In Fig. 2, the viral glycoprotein spikes can clearly be seen spread over regions (arrows) that are part of a very large liposome (1.8 μ m in diameter). Viruses that are bound but not fused (arrowheads) are also seen.

To check whether the viral protein in the liposomal fraction after fetuin treatment and centrifugation represents protein inserted into liposomes and not protein in bound virus, the material in the interface between 30% sucrose and buffer A was examined under the electron microscope. Fig. 3 shows a large liposome from this fraction, and unfused viruses are absent. On one side of the liposome, a long sequence of viral glycoprotein spikes is visible; on the other side, a region containing the spikes has budded out to form a filament. Thus, electron microscopy clearly demonstrates that influenza X-31 can fuse with liposomes at neutral pH after incubation at 37°C. It also shows that the [35 S]methionine-labeled virus protein that remains with liposomes after incubation at 37°C in excess of that remaining after binding at 0°C is protein incorporated into liposomes as a result of fusion and, hence, is a measure of the amount of fusion.

Therefore, this assay measuring transfer of labeled viral protein into liposomes was used to investigate the effect of liposomal composition upon fusion of influenza virus membranes with liposomes at neutral pH.

Effect of Liposomal Composition on the Association of Influenza Proteins with Liposomes. The effect of different liposomal compositions on the association of viral proteins with liposomes was tested. As can be seen in Table 2, liposomes with no net charge did not fuse with viral membranes at neutral pH. Liposomes were made of the composition used by White *et al.* (12) in their studies on fowl plague virus—i.e., phosphatidylcholine/phosphatidylethanolamine/sphingomyelin/cholesterol (PtdCho/PtdEtn/Sph/cholesterol) in the molar ratio 1:1:1:1.5. The liposomes used by White *et al.* have no net negative charge at neutral pH, although they may have a charge at lower pH. [At neutral pH, PtdCho, PtdEtn, Sph, and cholesterol bear no net charge; whereas phosphatidylserine (PtdSer) and gangliosides are negatively charged (25).] As shown in Table 2, only 3% of viral protein was associated with these liposomes after binding at 0°C and only 6% after incubation at 37°C, which

indicates that they do not fuse significantly. This is consistent with the results of White *et al.* (12) with fowl plague virus. Even when fetuin was not used before centrifugation, only 6% of the viral protein was associated with liposomes after incubation at 37°C, which shows that viruses do not bind to these liposomes as they do to those containing GD1a. When 3 mol % GD1a is added to the composition of these liposomes, however, 30% of viral protein was associated with liposomes at neutral pH after incubation for 1 hr at 37°C, so the addition of GD1a gives these liposomes the ability to fuse at neutral pH. White *et al.* noted no effect on fusion of adding ganglioside to the liposome composition, but they only reported the effect at acid pH and not at neutral pH. A possible interpretation of these results is that influenza virus X-31 does not fuse at neutral pH with the liposomes of the composition used by White *et al.* (12) because it does not bind to these liposomes, but, when the liposomes contain GD1a, it is able to bind and therefore to fuse at neutral pH.

To further investigate the importance of adding a negative charge to liposomes, liposomes were made containing only PtdCho or PtdCho and a second lipid (Table 2). When liposomes containing only PtdCho were used, only 1% of the viral protein added was associated with the liposomes after binding at 0°C and only 3% was associated after incubation at 37°C. When the liposomes contained PtdCho and 3 mol % GD1a, however, the amount of viral protein remaining associated after binding at 0°C increase to 14% and after incubation for 1 hr at 37°C it increased to 39%. As shown in Fig. 1, fusion does occur with this composition. As also is shown in Table 2, when negatively charged PtdSer (5 mol %) was included with PtdCho in liposomes, the amount of viral proteins associated with the liposomes was greater than that associated with liposomes containing only PtdCho, but less than that associated with liposomes containing PtdCho and 3 mol % GD1a. Influenza viruses have been reported to bind to negatively charged surfaces (26). The fact that 3 mol % of GD1a seems to have a much greater effect than 5 mol % PtdSer on the transfer of viral proteins to liposomes raises the question of whether GD1a is merely contributing a negative charge or whether it has receptor activity for influenza X-31 (H3N2). These experiments were not intended to address this question, but it is of interest that preliminary experiments

FIG. 2. Fusion of liposomes with influenza virus at pH 7.5. Liposomes were made from 0.7 μ mol of PtdCho/0.3 μ mol of PtdEtn/0.66 μ mol of cholesterol/0.03 μ mol of GD1a. Influenza virus X-31 (15 μ g of protein) was bound at 0°C for 1 hr and then incubated at 37°C for 2 hr. ($\times 72,750$.)

FIG. 3. Fusion of liposomes with influenza virus at pH 7.5. Liposomes were made as described in Fig. 2, and after incubation, the virus and liposomes were treated with fetuin, centrifuged, and the liposome fraction was collected. ($\times 85,500$.)

with influenza A/PR/8/34 (H1N1) indicate that it does not interact at neutral pH with liposomes containing GD1a in the same manner as does influenza X-31 (H3N2). This is consistent with the fact that different influenza subtypes are known to vary in their binding to cells (27), and this has been related to their ability to bind to different sialic acid linkages (28). The liposomal composition that will allow binding at neutral pH, therefore, has to be individualized for each virus group, and in the case of influenza for each subtype.

Fusion of Influenza X-31 at pH 5.2. Since various subtypes of influenza virus including influenza X-31 have been reported to be able to fuse their membranes at pH 5.2 (12, 13, 16, 17), the ability of influenza X-31 to fuse at pH 5.2 with liposomes of the same composition used by White *et al.* (12) was measured by using the transfer of labeled viral protein as an assay. The procedure of White *et al.* was used; namely, viruses were adsorbed to the liposomes at 0°C and pH 7.0, the pH was adjusted to 5.2, and the mixture was kept at 37°C for 5 min before the pH was readjusted to 7.0 and the incubation at 37°C was continued for 15 min. The mixture was then chilled, fetuin was added for 15 min at 0°C, and the centrifugation was carried out. As can be seen in Table 3, when liposomes of the composition used by White *et al.* and influenza X-31 are brought to a pH of 5.2, 42% of the labeled viral protein remains with the liposomes after incubation with virus at 37°C but only 11% remains after binding at 0°C. This is in marked contrast to what happens when liposomes of this composition are incubated with virus at pH 7.5 (Table 2). Thus, when the liposome composition and procedure are

similar to those used by White *et al.*, it appears that influenza virus X-31, like fowl plague virus, can only fuse at low pH. The amount of viral protein remaining with liposomes was assayed after incubation at pH 5.5 and pH 6.0 at 37°C for 5 min, followed by neutralization to pH 7.0 and incubation at 37°C for another 15 min, and it was found to be 33%, and 4%, respectively. This is consistent with the reported pH dependence for membrane fusion for a number of strains of influenza (12, 13, 16, 17). Skehel *et al.* (13) have investigated the response to pH changes of bromelain-solubilized hemagglutinin of influenza X-31. They have found that bromelain-solubilized hemagglutinin binds vesicles of the composition used by White *et al.* (and some ^{32}P -labeled lipid) at pH 5.0 but not at pH 7.0. This is consistent with the data on the whole virus in Tables 2 and 3 and raises the possibility that the role of low pH is to cause virus to bind to surfaces to which it cannot bind at neutral pH (and as also noted by Skehel *et al.*, perhaps to cause rearrangement of proteins in the virus surface secondary to aggregation). The fact that fusion can occur at neutral pH indicates that acid-induced changes in the hemagglutinin are not a requirement for fusion to occur.

When liposomes of the same composition except that they also contain GD1a were tested at pH 5.2, 91% of the labeled viral protein was associated with liposomes after incubation at 37°C (Table 3). The amount of viral protein remaining with liposomes of this composition was also assayed after incubation at pH 5.5 and pH 6.0 at 37°C for 5 min, followed by neutralization to pH 7.0 and incubation at 37°C for another 15 min, and it was found to be 83% and 42%, respectively. The amount of viral protein remaining with GD1a-containing liposomes at low pH after incubation at 37°C cannot be

Table 2. Interactions of influenza virus with liposomes of different compositions at pH 7.5

Liposome composition, μmol	% viral protein with liposomes	
	0°C 1 hr	0°C 1 hr, then 37°C 1 hr
PtdCho/PtdEtn/Sph/cholesterol, 0.33/0.33/0.33/0.50	3	6
PtdCho/PtdEtn/Sph/cholesterol/GD1a, 0.33/0.33/0.33/0.50/0.03	14	30
PtdCho, 1.0	1	3
PtdCho/PtdSer, 0.95/0.05	7	15
PtdCho/GD1a, 1.0/0.03	14	39

Liposomes were made of the lipids indicated to make a total of 1 μmol of phospholipid. ^{35}S -labeled influenza virus (11.35 μg of protein) was added for the times and temperatures indicated. The percentage of counts remaining with liposomes after addition of fetuin and centrifugation through a discontinuous sucrose gradient was determined.

Table 3. Interactions of influenza virus with liposomes at pH 5.2

Liposome composition, μmol	% viral protein with liposomes	
	0°C	37°C
PtdCho/PtdEtn/Sph/cholesterol, 0.33/0.33/0.33/0.50	11	42
PtdCho/PtdEtn/Sph/cholesterol/GD1a, 0.33/0.33/0.33/0.50/0.03	86	91

Liposomes were made with or without 0.03 μmol of ganglioside GD1a in buffer C (pH 7.0). ^{35}S -labeled influenza virus (11.35 μg of protein) was added at 0°C for 1 hr. The pH was adjusted to 5.2 with acetic acid and the temperature was either held at 0°C or raised to 37°C. After 5 min, the pH was adjusted to 7.0 with NaOH, and the incubation was continued for another 15 min. The samples were all put at 0°C, and the percentage of counts remaining with liposomes after addition of fetuin and centrifugation through a discontinuous sucrose gradient was determined.

FIG. 4. Fusion of liposomes with influenza virus at pH 5.2. Liposomes were made from 0.167 μmol of PtdCho/0.167 μmol of PtdEtn/0.167 μmol of Sph/0.25 μmol of cholesterol/0.015 μmol of ganglioside GD1a in buffer C (pH 7.0). Influenza virus (13.8 μg of protein) was added at 0°C. The pH was adjusted to 5.2 with acetic acid, and the temperature was raised to 37°C. After 5 min, the pH was adjusted to 7.0 with NaOH and incubation at 37°C was continued for another 15 min. ($\times 68,250$.)

interpreted as measuring the amount of fusion, but it probably measures both fusion and binding because under these conditions the bound virus adheres to liposomes so tightly that most is not removed by fetuin and centrifugation. This is shown by the fact that 86% of virus added at 0°C to GD1a-containing liposomes at pH 5.2 remains bound to liposomes after fetuin treatment and centrifugation. This tight adhesion of virus to GD1a-containing liposomes at pH 5.2 could be because the viral binding to liposomes caused by lowering the pH and the viral binding caused by GD1a may be additive, or it could be because low pH may increase or change the nature of the binding to GD1a. To determine what occurs at 37°C under these conditions where there is very tight binding, virus and GD1a-containing liposomes after incubation at pH 5.2 and 37°C were examined under the electron microscope. Fig. 4 shows an aggregate of viruses and liposomes. Many areas of liposomal membrane contain viral glycoprotein spikes (arrows), so much of the virus fused. However, many viruses were not fused but bound (arrowheads) and often partially or completely enveloped by one or several liposomes. Many of the viruses that had not fused were beginning to disintegrate so that the ribonucleoprotein was visible and probably easily accessible to any contents that leaked from the liposome. This is consistent with previous reports that low pH makes influenza viruses lose their integrity, become permeable to negative stain, and aggregate (15). Since liposomes become leakier as the pH is lowered (29), it seems likely that at low pH the contents of the virus and liposomes can mix without fusion and may be protected from the external medium because of the very close apposition of the two membranes. Therefore, the phenomenon termed "leaky fusion" (12) may not represent fusion, and assays measuring mixing of virus and liposome contents become questionable at low pH.

In summary, influenza virus X-31 (H3N2) fuses with liposomes containing the ganglioside GD1a at pH 7.5, and it does not require low pH for fusion. Therefore, an acid-induced conformational change in the influenza HN protein is not a requirement for membrane fusion. Lowering the pH, however, does make influenza virus fuse with membranes with which it is otherwise unable to fuse. The simplest hypothesis is that low pH enables the virus to bind to these membranes. At present, the question of whether influenza virus enters cells by direct fusion at the plasma membrane or by endocytosis followed by fusion at either neutral or acid pH is unresolved.

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