Kinetics of pH-Dependent Fusion between Influenza Virus and Liposomes†

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ABSTRACT: The pH-dependent fusion between influenza virus and liposomes (large unilamellar vesicles) has been investigated as a model for the fusion step in the infectious entry of the virus into cells. Fusion was monitored continuously, with a fluorescence assay based on resonance energy transfer (RET) [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099], which allows an accurate quantitation of the fusion process. Evidence is presented indicating that the dilution of the RET probes from the liposomal bilayer into the viral membrane is not due to transfer of individual lipid molecules. The initial rate and final extent of the fusion reaction increase dramatically with decreasing pH, fusion being virtually complete within 1 min at pH 4.5–5.0. From experiments in which the ratio of virus to liposomes was varied, it is concluded that virus–liposome fusion products continue to fuse with liposomes, but not with virus. Fusion is most efficient with liposomes consisting of negatively charged phospholipids, while phosphatidylcholine and sphingomyelin are inhibitory. The reaction is completely blocked by an antiserum against the virus and inhibited by pretreatment of the virus with trypsin. The effect of proteolytic pretreatment at pH 7.4 is enhanced after preincubation of the virus at pH 5.0, consistent with the occurrence of a low pH induced, irreversible, conformational change in the viral fusion protein, the hemagglutinin (HA), exposing trypsin cleavage sites. When, after initiation of the fusion reaction at pH 5.0, the pH is readjusted to neutral, the process is arrested instantaneously, indicating that the low pH induced conformational change in the HA protein, in itself, is not sufficient to trigger fusion activity.

Several enveloped viruses enter cells by a process of receptor-mediated endocytosis, routing the viral particles into the endosomal/lysosomal compartment of the cell (Helenius et al., 1980; Matlin et al., 1981; White et al., 1983). Subsequently, induced by the low pH in this compartment, the viral membrane fuses "from within" with the endosomal/lysosomal membrane, thereby releasing the nucleocapsid into the cytoplasm. Low pH induced fusion activity has been reported for the alphavirus group of togaviruses (e.g., Semliki Forest virus; Helenius et al., 1980), for rhabdoviruses (e.g., vesicular stomatitis virus; Matlin et al., 1982), and for orthomyxoviruses (e.g., influenza virus; Matlin et al., 1981; White et al., 1982a). The initial adhesion of the virus to the membrane and the fusion reaction are mediated by viral spike glycoproteins. For influenza virus, both activities are confined to the hemagglutinin (HA)[1] glycoprotein, which in its active form consists of two subunits, HA_1 and HA_2. The receptor binding site is located on HA_1, while fusion activity is thought to reside on the HA_2 subunit. It has been demonstrated that low pH induces an irreversible conformational change in the HA molecule, exposing a hydrophobic sequence of amino acid residues on the HA_2 subunit (Skehel et al., 1982; Yewdell et al., 1983). The exposure of this apolar segment has been proposed to trigger the fusion activity (Sato et al., 1983). However, little is known about the mechanism by which the actual merging of the membranes occurs.

In order to gain more insight into the characteristics of the process, low pH induced fusion of influenza virus has been investigated, with various target membranes such as plasma membranes (Matlin et al., White et al., 1981, 1982b), erythrocytes (Huang et al., 1980, 1981; Väänänen & Kääriänen, 1980; Lenard & Miller, 1981; Hosaka et al., 1983; Sato et al., 1983), or planar lipid bilayers (Young et al., 1983). Liposomes (phospholipid vesicles) have also been used as target membranes for the virus. White et al. (1982a) encapsulated trypsin in liposomes and determined the degradation of internal viral proteins after fusion of the liposomes with the virus. Maeda et al. (1981) used virus, containing a spin-labeled lipid, and monitored the dilution of the spin-label into the liposomal membrane. Although the above approaches do reveal the pH dependence of the fusion reaction, the assays do allow neither a continuous kinetic characterization of the initial stages nor an accurate quantitation of the final extent of the process.

We have investigated fusion of influenza virus with liposomes, using a fluorescence assay based on resonance energy transfer (RET) (Struck et al., 1981; Hoekstra, 1982). In this assay two fluorescent lipid derivatives, N-NBD-PE and N-Rh-PE, are incorporated in the liposomal bilayer. The efficiency of energy transfer between N-NBD-PE, the energy donor, and N-Rh-PE, the energy acceptor, is dependent on the surface densities of the probes in the membrane. Upon fusion of the labeled liposomes with the virus, the density of the fluorophores decreases, thus causing a decrease in transfer efficiency and, hence, an increase of the fluorescence intensity of the donor lipid, N-NBD-PE. This increase can be moni-

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[1] Abbreviations: Chol, cholesterol; CL, cardiolipin; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; HA, hemagglutinin protein (of influenza virus); HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; LUV, large unilamellar vesicles; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PnAPC, 1-palmitoyl-2-parinaroylphosphatidylcholine; PS, phosphatidylserine; RET, resonance energy transfer; SM, sphingomyelin; SUV, small unilamellar vesicles.

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tored continuously and allows an accurate quantitation of the fusion process.

Materials and Methods

Chemicals. *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE), cardiolipin (CL), phosphatidylserine (PS), dioleoylphosphatidylethanolamine (DOPE), and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Sphingomyelin (SM), cholesterol (Chol), and calcein were from Sigma Chemical Co. (St. Louis, MO); 1-palmitoyl-2-parinaroylphosphatidylcholine (PnAPC) was a generous gift from Dr. K. W. A. Wirtz (Laboratory of Biochemistry, University of Utrecht, The Netherlands). Trypsin was from Boehringer (Mannheim, FRG).

Virus: Propagation and Handling. The X-47 recombinant influenza strain, carrying the hemagglutinin of influenza A/Victoria/3/75, and a rabbit antiserum against this strain were gifts from Dr. J. C. de Jong (Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands). The influenza strain was originally obtained from Dr. J. J. Skehel (World Influenza Center, London). Virus was grown for 3 days in the allantoic cavity of 10-day-old embryonated eggs. Harvested allantoic fluid was centrifuged at 1000*g* for 15 min to remove debris. Virus was sedimented from the supernatant at 75000*g* for 90 min, washed with 0.25 M sucrose, 5 mM HEPES, 0.1 M EDTA (pH 7.4), and stored in the same buffer at −80 °C. Viral phospholipid content was determined, after extraction of the membrane lipids (Bligh & Dyer, 1959), by phosphate analysis (Bartlett et al., 1959). Protein was determined according to Lowry et al. (1951). The phospholipid to protein ratio of the virus was 175 nmol of phospholipid/mg of protein.

Preparation of Liposomes. Large unilamellar vesicles (LUV) were prepared, as described before (Wilschut et al., 1980), in 150 mM NaCl, 5 mM HEPES, and 0.1 mM EDTA (pH 7.4) by reverse-phase evaporation (Szoka & Papahadjopoulos, 1980) and sized by extrusion through Unipore polycarbonate membranes (Bio-Rad, Richmond, CA) with a pore size of 0.2 μm (Olson et al., 1979). Small unilamellar vesicles (SUV) were prepared from LUV by sonication in a bath-type sonicator (Lab Supplies Inc., Hicksville, NY) for 1 h. The concentration of the liposome preparations was determined by measuring phospholipid phosphorus according to Bartlett et al. (1959).

Fusion Assays. In the resonance energy transfer (RET) fusion assay, 0.6 mol % each of N-NBD-PE and N-Rh-PE was incorporated in the bilayer of the liposomes. Measurements were carried out in a final volume of 1.4 mL of 140 mM NaCl, 10 mM sodium citrate, 10 mM HEPES, and 0.1 mM EDTA, adjusted to the desired pH. The buffer in the cuvette was maintained at 37 °C (unless stated otherwise) and stirred continuously. After addition of the liposomes to the cuvette, the reaction was initiated by injection of a small volume (70 μL) of virus suspension, appropriately diluted in 150 mM NaCl, 5 mM HEPES, and 0.1 mM EDTA (pH 7.4), with a Hamilton syringe, and the increase of N-NBD-PE fluorescence, due to dilution of the fluorophores into the viral membrane, was recorded continuously. Fluorescence was measured in an SLM-8000 fluorometer (SLM/Aminco, Urbana, IL) equipped with a double-excitation monochromator and a stripchart recorder. The excitation and emission wavelengths were 465 and 530 nm, respectively. A cutoff filter (<515 nm) was placed between sample and emission monochromator.

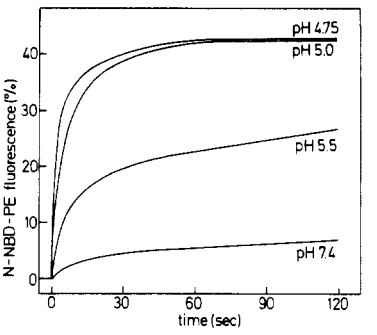

FIGURE 1: Interaction between influenza virus and CL LUV, labeled with N-NBD-PE and N-Rh-PE, at different pH values. A small volume of a concentrated virus suspension was injected into a cuvette, containing the liposomes in a medium adjusted to the pH values indicated, and the increase of the N-NBD-PE fluorescence was recorded continuously. The medium in the cuvette was magnetically stirred, and mixing of the viral suspension with the medium was complete within 1 s of injection. The ratio of the final liposomal to viral phospholipid phosphorus concentration was 1:1, and the total phospholipid phosphorus concentration was 50 μM.

For calibration of the fluorescence scale, the initial residual fluorescence of the liposomes was taken as the zero level and the fluorescence at infinite probe dilution as 100%. The latter value was determined by addition of Triton X-100 (0.5% v/v) to the liposomes and subsequent correction of the fluorescence intensity for the sample dilution and for the effect of Triton on the quantum yield of the N-NBD-PE (Struck et al., 1981).

Alternatively, fusion was measured by monitoring the relief of self-quenching of PnAPC (Somerharju et al., 1981) during its dilution from the liposomal bilayer into the viral membrane. PnAPC was incorporated in the liposomes at a concentration of 10 mol %. Measurements were carried out as described for the RET assay, with excitation and emission wavelengths of 325 and 420 nm, respectively, without the use of a cutoff filter. A narrow excitation slit was used to prevent photodegradation of the probe. The fluorescence scale was calibrated such that the residual fluorescence of the liposomes was taken as the zero level. Since the fluorescence intensity of PnAPC does not increase linearly with dilution, the fluorescence intensity of liposomes, containing 5 mol % PnAPC, at a concentration 2-fold higher than the liposome concentration used in the fusion assay, was taken as the maximal level. This level corresponds to the intensity that would be obtained after complete lipid mixing in a 1:1 mixture of virus and liposomes, containing 10 mol % PnAPC.

Release of Liposomal Contents. To monitor release of aqueous liposomal contents, 100 mM calcein in 5 mM HEPES (pH 7.4) was encapsulated in the liposomes. Nonencapsulated fluorophore was removed by gel filtration on Sephadex G-50. Measurements were carried out under conditions as described above for the RET assay, with excitation and emission wavelengths of 490 and 515 nm, respectively, and a cutoff filter (<495 nm) between sample and emission monochromator. Residual fluorescence of the liposomes was taken as the zero level and the fluorescence intensity after lysis of the liposomes with Triton X-100 (0.5 % v/v) as the maximal value.

Electron Microscopy. For electron microscopic examination of the fusion reaction, LUV containing colloidal gold were prepared according to the procedure of Hong et al. (1983). Samples were mounted on Formvar/carbon-coated copper grids, stained with 1% uranyl acetate, and examined in a Philips 300 electron microscope, operating at 80 kV.

Results

pH-Dependent Lipid Mixing during Interaction of Influenza Virus with Liposomes: Figure 1 shows the fluorescence de-

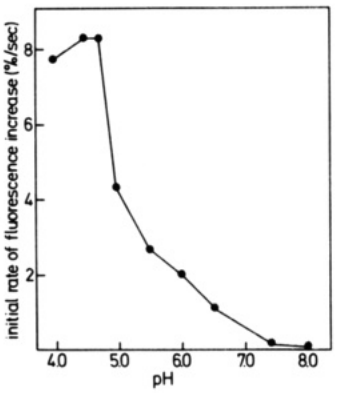

FIGURE 2: Initial rate of N-NBD-PE fluorescence increase upon interaction between influenza virus and fluorescently labeled CL LUV as a function of pH. Values were calculated from tangents drawn at $t = 0$ to fluorescence tracings as presented in Figure 1. At the lower pH values, the fluorescence increase was recorded at very high chart speeds.

velopment upon addition of influenza virus to CL liposomes, containing N-NBD-PE and N-Rh-PE, at different pH values. At neutral pH a slow increase in fluorescence intensity was observed. With decreasing pH the rate and extent of fluorescence development increased dramatically. The initial rate of fluorescence increase as a function of pH is shown in Figure 2. The rate increased steeply below pH 5.5–6.0, reaching a maximum at pH 4.5–4.8. Upon a further lowering of the pH (<4.5), a slight relative decrease of the rate was observed, probably due to inactivation of the virus. However, no inactivation of virus occurred during prolonged (up to 1 h) preincubation at pH 5.0. The kinetics and extent of probe dilution were the same irrespective of the order of addition of liposomes and virus.

The fluorescence scale in the above experiments was calibrated such that 100% fluorescence corresponds to the intensity at infinite probe dilution. At a 1:1 ratio of labeled to unlabeled membrane vesicles of equal size, complete mixing of the lipids in the system would result in a 2-fold dilution of the fluorophores. Since, starting at the initial probe concentrations used, the donor fluorescence increases linearly with a decreasing surface density of the probes (Struck et al., 1981), a 2-fold dilution would result in a fluorescence increase of 50%. At pH 5.0 and below, an approximately 45% increase in N-NBD-PE fluorescence was reached, indicating that virtually complete mixing of the lipids in the system had occurred. At pH 4.5 this extent of lipid mixing was reached in ~40 s (Figure 1). The extent of lipid mixing at higher pH values was relatively low, and the time required to reach the final level increased progressively with increasing pH. For example, at pH 7.4 a level corresponding to 15% N-NBD-PE fluorescence increase was reached after 30 min.

The above results demonstrate the fast and extensive pH-dependent dilution of N-NBD-PE and N-Rh-PE from liposomes into the viral membrane, consistent with the well-known pH-dependent fusion activity of influenza virus (Matlin et al., 1981; White et al., 1981, 1982a). Rather than by membrane fusion, dilution of the fluorophores could take place through transfer of individual molecules. This is an unlikely possibility, however, since N-NBD-PE and N-Rh-PE have been shown to be nonexchangeable (Struck et al., 1981; Hoekstra, 1982; Kumar et al., 1982).

Further evidence against transfer of individual lipid molecules was obtained from an experiment in which the interaction between influenza virus and liposomes, labeled with PnAPC, was studied. PnAPC, when incorporated in liposomal bilayers

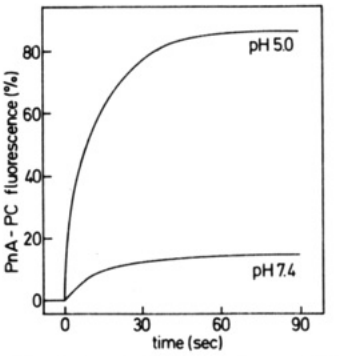

FIGURE 3: Interaction between influenza virus and CL LUV, labeled with 10 mol % PnAPC, at pH 7.4 and pH 5.0. Conditions of the experiment were as described in the legend to Figure 1. The increase of PnA fluorescence was recorded continuously.

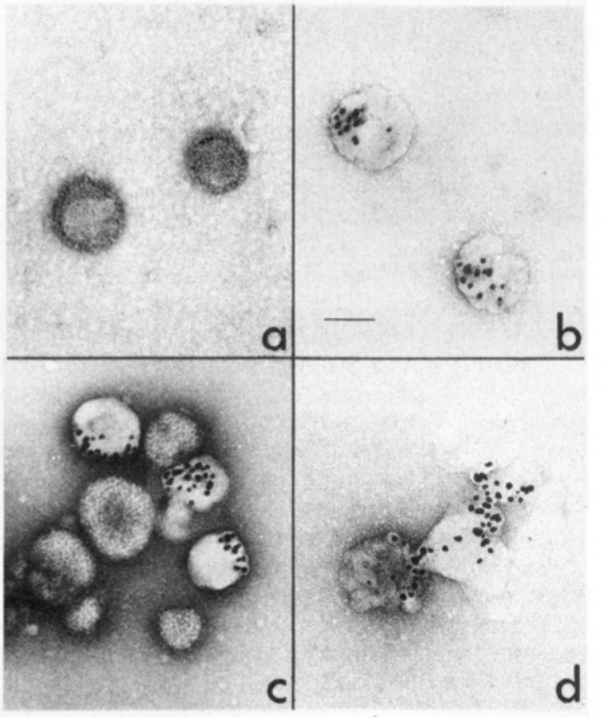

FIGURE 4: Interaction between influenza virus and CL LUV, containing colloidal gold, examined by negative-stain electron microscopy. Samples were preincubated at 37 °C for 1 min at different pH values and stained subsequently with 1% uranyl acetate. (A) Virus, pH 5.0; (B) liposomes, pH 5.0; (C) virus and liposomes, pH 7.4; (D) virus and liposomes, pH 5.0. The bar represents 0.1 μm.

at a sufficiently high concentration, is self-quenched, and its dilution into an unlabeled membrane can be monitored continuously (Somerharju et al., 1981). Figure 3 shows the fluorescence development after addition of virus to CL liposomes, containing 10 mol % PnAPC, at neutral and low pH. The pH dependence of PnAPC dilution was very similar to that seen with the RET assay. Moreover, the extent and kinetics of dilution of the probe at pH 5.0 were essentially the same as those observed for fusion between influenza virus and CL liposomes, containing 10 mol % DOPC, utilizing the RET assay (results not shown).

Transfer of Liposome-Encapsulated Colloidal Gold. In order to determine whether or not communication between the internal space of the liposomes and the virus particles is established during the fusion reaction, we incubated CL LUV containing colloidal gold (Hong et al., 1983) with the virus at different pH values and examined the interaction products by negative-stain electron microscopy. Panels a and b of

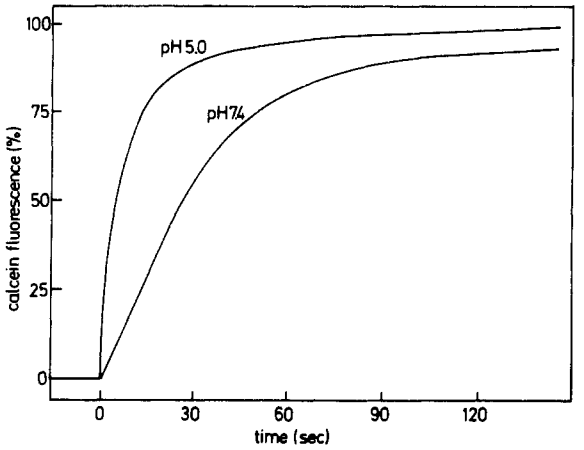

FIGURE 5: Release of calcein from CL LUV upon interaction with influenza virus at pH 7.4 and pH 5.0. Virus was added to calcein-containing liposomes, under conditions as described in the legend to Figure 1, and the increase of the calcein fluorescence, due to relief of self-quenching, was recorded continuously.

Figure 4 show the virus particles and the gold-containing liposomes, respectively, after incubation at pH 5.0 for 1 min. The virus particles can easily be distinguished from the liposomes because of their electron-dense core. In agreement with the results presented in Figures 1 and 2, no transfer of gold from the liposomes to the virus was seen after a 1-min incubation at pH 7.4, even though the liposomes did associate with the viral particles at this pH value (Figure 4c). At pH 5.0, on the other hand, colloidal gold particles appeared in electron-dense structures (Figure 4d), while separate liposomes and virus particles were no longer observed. Free gold could not be detected in this preparation, indicating that the large gold particles are not released into the external medium during the virus–liposome interaction.

Release of Liposomal Contents. Contrary to the large gold particles (Figure 4), low molecular weight substances are released from the liposomal aqueous space into the external medium as a result of virus–liposome interaction. When virus was added to calcein-containing CL LUV, the fluorophore, initially encapsulated in the liposomes at self-quenching concentrations, was rapidly diluted into the medium (Figure 5). At pH 5.0, leakage was fast and complete. Remarkably, also at neutral pH very extensive release occurred, the initial rate of which was considerably faster (Figure 5) than the rate of fusion (Figure 1). Leakage from the liposomes alone, either at pH 7.4 or at pH 5.0, was negligible (not shown).

Cation and Protein Dependence of the Fusion Process. The kinetics and extent of lipid mixing during interaction of the virus with liposomes were affected neither by the presence of EDTA in the medium nor by the addition of Ca^{2+} or Mg^{2+} (1.0 mM) (results not shown). This indicates that the fusion reaction is independent of divalent cations, which is consistent with observations reported by others (White et al., 1982a).

To examine the role of the viral spike proteins in the process, the effect of the presence of an antiserum against the virus and the effect of proteolytic pretreatment of the virus on the fusion reaction were determined. The results are shown in Figure 6. The antiserum completely blocked the fusion activity at pH 5.0 (curve D), consistent with an essential role of the HA protein in the reaction (White et al., 1982b). Treatment of the virus with trypsin for 1 h at pH 7.4 resulted in a slightly reduced fusion activity at pH 5.0 (curve B), relative to that of the control virus (curve A). Treatment with trypsin at pH 7.4, after a preincubation of the virus for 5 min at pH 5.0, caused a major reduction in fusion activity (curve

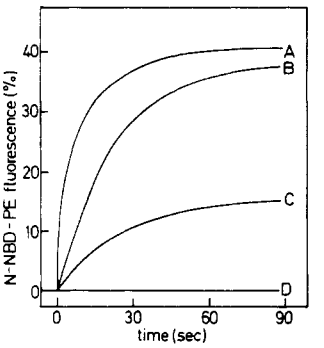

FIGURE 6: Effects of trypsin treatment and antiserum on the fusion activity of influenza virus. Fusion at pH 5.0 was measured as described in the legend to Figure 1. (A) Virus incubated in the absence of trypsin at 37 °C for 1 h at pH 7.4 after a preincubation for 5 min at pH 5.0 (fusion activity was not affected by this treatment); (B) virus treated with trypsin (1 mg/mL) at 37 °C for 1 h at pH 7.4 (virus concentration during the trypsin treatment was 0.15 mg of protein/mL); (C) as in (B), except that trypsin treatment was carried out after preincubation of the virus at 37 °C for 5 min at pH 5.0; (D) virus in the presence of rabbit antiserum against the virus.

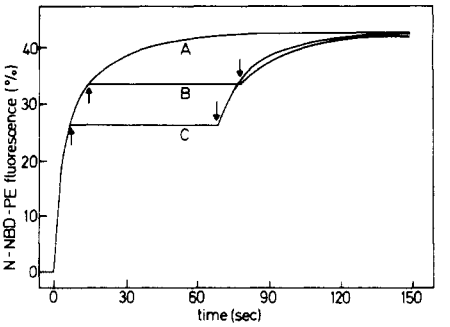

FIGURE 7: Reversibility of the pH-dependent triggering of influenza virus fusion activity. Fusion at pH 5.0 was measured as described in the legend to Figure 1. (A) Control; (B and C) after initiation of the fusion reaction at pH 5.0, the pH was adjusted to 8.0 by addition of a small aliquot of NaOH (↑) and subsequently readjusted to 5.0 by addition of HCl (↓).

C). Incubation of the virus for 1 h at 37 °C in the absence of trypsin, with or without preincubation at pH 5.0, had no effect on the fusion reaction (curve A). These results support previous observations by Skehel et al. (1982) indicating that at low pH an irreversible conformational change in the HA molecule occurs, exposing trypsin cleavage sites.

Reversibility of the Activation of the Fusion Reaction. The pH-dependent conformational change in the HA protein has been proposed to be involved in the activation of the fusion capacity of influenza virus (White et al., 1982a, 1983). The results presented in the preceding section prompted us to investigate whether the conformational change, in itself, is sufficient to initiate the fusion reaction. If so, the conformational change being irreversible (Skehel et al., 1982), one would expect the fusion activity at pH 7.4 of a virus preparation, preincubated at pH 5.0, to be similar to the fusion activity of the virus at pH 5.0. However, the fusion activity at neutral pH was the same, irrespective of whether the virus had been preincubated at pH 5.0 or not (results not shown). Moreover, the results in Figure 7 show that adjustment of the pH to 8.0, after initiation of the fusion at pH 5.0, instantaneously inhibited the reaction, while a subsequent readjustment to low pH immediately reactivated the process. This indicates that the irreversible conformational change in the HA protein, in itself, is not sufficient to trigger fusion activity.

Effect of Liposomal Lipid Composition. Maeda et al. (1981) and White et al. (1982a) have reported that fusion

Table I: Fusion between Influenza Virus and Liposomes of Different Compositions[a]

liposomal lipid composition[b]	initial rate of N-NBD-PE fluorescence increase (%/min)	final level of N-NBD-PE fluorescence (%)
SM	0	0
SM/Chol (1:1)	0	0
DOPC	18	17
DOPC/DOPE (1:1)	40	23
CL	259	44
CL/Chol (1:1)	249	41
CL/DOPC (1:1)	131	37
CL/DOPE (1:1)	148	34
PS	50	39
PS/Chol (1:1)	38	26
PS/DOPC (1:1)	29	30
PS/DOPE (1:1)	39	29

[a] Fusion was measured at pH 5.0, as described in the legend to Figure 1. [b] The ratios in the lipid mixtures, indicated in parentheses, were molar ratios.

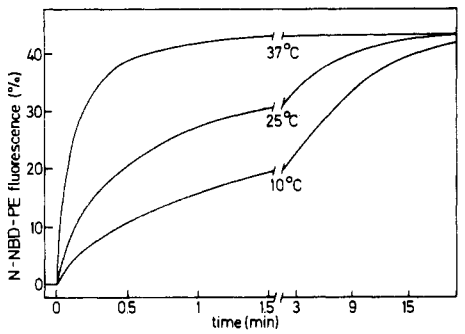

FIGURE 8: Fusion at pH 5.0 between influenza virus and CL LUV at different temperatures. Fusion was measured as described in the legend to Figure 1. The pH was adjusted to 5.0 at the temperatures indicated.

between influenza virus and liposomes is fairly independent of the lipid composition of the liposomes, although Maeda et al. (1981) observed relatively low fusion activities with PC-containing liposomes, while White et al. (1982a) found that PE is required for optimal fusion activity. In our hands, negatively charged liposomes constituted the most efficient target membranes for influenza virus. Therefore, the experiments presented above were carried out with liposomes, consisting of pure bovine heart CL. Results obtained with other lipid compositions are shown in Table I.

Fusion was observed with all lipid compositions studied, except with SM and SM/Chol mixtures. Fast and extensive fusion occurred only with negatively charged liposomes, particularly with those consisting of CL, but also with PS. On the other hand, fusion with PC vesicles was slow and less extensive, in agreement with Maeda et al. (1981). In mixtures with acidic phospholipids, both PC and PE had an inhibitory effect. Liposomes composed of a mixture of PE and PC displayed faster and more extensive fusion than liposomes containing PC alone, confirming the suggestion of White et al. (1982a) that PE has a relative stimulatory effect. The results in Table I clearly indicate that influenza virus, unlike Semliki Forest virus (White et al., 1980), has no requirement for the presence of cholesterol in the target liposomes. This is in agreement with observations by Maeda et al. (1981) and White et al. (1982a).

Quantitation and Kinetic Characterization of the Fusion Reaction. Figure 8 shows the kinetics and final extents of fusion between influenza virus and CL liposomes at 10, 25, and 37 °C. As expected, the initial rate of fusion increased with increasing temperature. Importantly, however, at all three

Table II: Fusion between Influenza Virus and CL Liposomes at Different Ratios[a]

ratio of liposomes:virus	final level of N-NBD-PE fluorescence (%) expected, one round of fusion	expected, total lipid mixing	obsd
16:1	3.13	5.9	4.3
8:1	6.25	11	11
4:1	12.5	20	21
2:1	25	33	33
1:1	50	50	44
1:2	50	66	46
1:4	50	80	47

[a] Fusion was measured at pH 5.0, as described in the legend to Figure 1, at different ratios of liposomes to virus. In all cases the liposomal CL phosphorus concentration was 25 μM. Ratios were based on phospholipid phosphorus.

temperatures studied the same final level of N-NBD-PE fluorescence intensity was attained, indicating that this level corresponds to the maximal possible extent of lipid mixing in the system. As discussed above, in a 1:1 mixture of labeled and unlabeled membrane vesicles complete lipid mixing would result in a 50% increase of the N-NBD-PE fluorescence. The observed increase of 45% is in close agreement with this value.

In a 1:1 mixture of liposomes and virus of equal size one cannot distinguish between one and multiple rounds of fusion, since continued fusion among products of liposome–virus fusion would not result in a further enhancement of the fluorescence. In order to determine whether fusion products continue to fuse with either liposomes or virus, we varied the liposome to virus ratio in the fusion assay. Table II shows (i) the final fluorescence intensities that can be expected when each virus particle fuses with one liposome (assuming equal size of virus and liposomes), (ii) the intensities that can be expected for complete lipid mixing in the system, and (iii) the fluorescence intensities that were observed. The experimental data at high liposome to virus ratios show that complete lipid mixing occurred, indicating that fusion products continued to fuse with liposomes. On the other hand, at high virus to liposome ratios the extent of lipid mixing was essentially the same as that observed at a 1:1 ratio, indicating that fusion products did not fuse with virus particles. Accordingly, when unlabeled liposomes were added to a 1:1 mixture of virus and labeled liposomes *after* the final level of N-NBD-PE fluorescence was reached, the fluorescence further increased to an extent corresponding to the amount of unlabeled liposomes added, whereas addition of virus did not result in any further increase of the fluorescence intensity (results not shown).

Continued fusion between products of virus–liposome fusion and liposomes was also indicated by experiments, in which CL SUV rather than LUV were used. With SUV, at a 1:1 ratio of liposomal to viral phospholipid, the particle ratio of liposomes to virus is much higher than that with LUV. With SUV, the same final level of fluorescence intensity was observed as with LUV (not shown), indicating complete lipid mixing and thus, fusion between virus–SUV fusion products and SUV. The initial rate of fusion with SUV was slightly faster than that with LUV, probably due to the high degree of bilayer curvature, which is likely to render SUV more fusion susceptible compared to LUV.

Discussion

In this paper we have utilized a kinetic fluorescence assay based on RET (Struck et al., 1981) to characterize the fusion of influenza virus with liposomes. Others have studied this fusion process before (White et al., 1982a; Maeda et al., 1981). However, a kinetic interpretation of the results obtained by

these investigators is hampered by the inability of their experimental approaches to accurately reveal the initial rate of the fusion process and also by the fact that multilamellar liposomes were used in a large excess over the virus. The use of LUV of a size roughly equal to that of the virus and in a concentration similar to that of the virus, the high sensitivity of the RET assay, and the ability to monitor the fusion reaction continuously offer obvious advantages over the methods used by White et al. (1982a) and Maeda et al. (1981) to characterize the liposome–virus fusion process. Recently, Eidelman et al. (1984) have used the RET assay to characterize the interaction between liposomes and reconstituted vesicles containing the G protein from vesicular stomatitis virus.

The well-established pH dependence of the fusion activity of influenza virus (Matlin et al., 1981; White et al., 1982a) is clearly expressed by the RET assay. At pH 4.5–5.0, fusion with CL liposomes is extremely fast, occurring on a time scale of seconds (Figures 1 and 2). The final extent of N-NBD-PE fluorescence increase in this pH range (Figure 1) and the observation that this level is independent of temperature (Figure 8) indicate that fusion is essentially complete. This means that in a 1:1 mixture of virus and liposomes on the average each virus particle fuses with a liposome. Furthermore, the data in Table II show that the products of virus–liposome fusion undergo further fusion with liposomes but not with virus. Both the capacity of the fusion products to fuse with liposomes and their refractoriness to fusion with intact virus are likely to decrease with a decreasing surface density of viral spike glycoproteins on the virus–liposome fusion products. Currently, using reconstituted influenza virosomes, we are investigating the relationship between surface density of viral glycoproteins and fusion activity of the virosomes toward liposomes or intact virus.

As shown in Figure 5, fusion of influenza virus with CL LUV is quite leaky to low molecular weight compounds, initially encapsulated in the liposomes. Also at neutral pH, where fusion is relatively slow and incomplete (Figures 1–3), release is fast and extensive (Figure 5). In apparent, partial, discrepancy with these results, Maeda et al. (1981) and Sato et al. (1983) have observed extensive leakage of tempocholine from liposomes upon interaction with influenza virus at low pH, whereas at neutral pH release was very low. This latter observation may have resulted from the use of multilamellar vesicles in a large excess over the virus. Our results clearly show that release of aqueous liposomal contents is an inadequate measure of the fusion activity of viruses toward target liposomes. White et al. (1982a), using fresh influenza virus preparations, have demonstrated partly nonleaky fusion at low pH between the virus and trypsin-loaded liposomes in the presence of trypsin inhibitor in the external medium. However, in this approach the measure for the leakiness of the fusion event is different than that in the experiment shown in Figure 5. In the trypsin assay, considerable amounts of the enzyme may leak into the external medium without being noticed. In addition, the apparent difference between the results of White et al. (1982a) and our results may be due to differences in experimental conditions. When we applied the trypsin assay under our conditions, i.e., using LUV at concentrations similar to that of the virus, no proteolytic degradation products were detected, even when fresh virus was used.

The initial attachment of influenza virus to the cellular plasma membrane presumably occurs via binding to sialic acid containing receptors on the cell surface (Caroll et al., 1981; Rogers et al., 1983). Apparently, an attachment of this kind is not required for membrane fusion, since the virus fuses avidly with liposomes lacking a specific glycoprotein receptor. Yet, also for fusion with liposomes at least two steps have to be distinguished: (i) the initial attachment of the viral particle to the liposome and (ii) the subsequent fusion reaction. The interaction being most efficient with negatively charged liposomes (Table I) suggests that the initial attachment involves, at least in part, an electrostatic interaction. Eidelman et al. (1984) have also observed an enhancement of interaction between liposomes and reconstituted virosomes upon incorporation of PS in the liposomes. It is conceivable that an initial electrostatic interaction is facilitated at low pH due to an increased protonation of the viral proteins. More insight into the nature of the binding of the virus to liposomes can be obtained by determining the *kinetics* of the initial attachment. Recently, by analyzing the data obtained with the RET assay in terms of a mass action kinetic model (Bentz et al., 1983; Nir et al., 1983), we have been able to distinguish the rate of the initial virus–liposome attachment from that of the subsequent fusion reaction (S. Nir, T. Stegmann, and J. Wilschut, unpublished results). Further analyses should reveal to what extent the binding kinetics are dependent on the pH and/or the lipid composition of the liposomes.

It has been unequivocally demonstrated that fusion activity of influenza virus is associated with the HA glycoprotein (White et al., 1982b). The HA_2 subunit of this molecule contains an unusually hydrophobic stretch of amino acid residues at the N-terminus (Min Jou et al., 1980). This segment, which has a striking sequence similarity with a segment in the fusion protein (F) of Sendai virus (Richardson et al., 1980), is thought to be directly involved in the fusion reaction, possibly by penetrating into the target membrane (White et al., 1983). At neutral pH the location of the apolar segment at about 100 Å from the tip of the spike and 30 Å from the viral membrane (Wilson et al., 1981) may seem unfavorable for such a mechanism. However, it has been demonstrated that at low pH a drastic, irreversible, conformational change in the molecule occurs, which exposes the hydrophobic sequence (Skehel et al., 1982) and may induce the distal tip of the spike to fold back to the viral membrane, such that the apolar segment would become accessible to the target membrane. In this case, the positioning of the apolar segment of the spike is not as unfavorable as at first glance it may seem. Indeed, penetration of the segment, located at about 30 Å from the viral membrane, into the target membrane would establish direct lipid bilayer contact, a condition that is obviously required for fusion to occur. The relative inhibitory effect of PC on the liposome–virus fusion reaction and the lack of fusion of SM liposomes (Table I) may well be the result of interference with the establishment of direct lipid bilayer contact, due to the strong hydration of the head group of PC and SM (Rand, 1981). Accordingly, the relative stimulatory effect of PE in mixed PE/PC liposomes (Table I) can be explained by the lower degree of hydration of this phospholipid (Rand, 1981).

An important result of the present study is the observation that the fusion reaction is intantaneously arrested upon readjustment of the pH from 5.0 to neutral (Figure 7). Together with the results in Figure 6, which are fully consistent with the occurrence of a low pH induced, irreversible, conformational change in the HA molecule, the results in Figure 7 indicate that this conformational change, *in itself*, is not sufficient to activate the fusion reaction. At this point, it is interesting to note that there is a remarkable difference between the F protein of Sendai virus, which is known for its fusion activity at neutral pH (White et al., 1983), and the HA

of influenza, in that the hydrophobic sequence of the latter is interrupted by three acidic amino acid residues (Min Jou et al., 1980), whereas that of the former is not (Richardson et al., 1980). It is conceivable that fusion activity of the HA molecule requires, *in addition to* exposure of the apolar segment (Skehel et al., 1982; White et al., 1983), the protonation of these acidic amino acid residues, which would augment the hydrophobicity of the segment and thus facilitate its penetration into the target membrane (Maeda et al., 1981). A similar mechanism may be operative in the case of low pH induced fusion of Semliki Forest virus (Helenius et al., 1980; White & Helenius, 1980) and vesicular stomatitis virus (Matlin et al., 1982). Recently, Eidelman et al. (1984) observed that the low pH induced activation of the fusion capacity of reconstituted vesicles, containing the G protein of vesicular stomatitis virus, is reversed upon readjustment of the pH to neutral.

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