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Parameters Affecting Fusion between Sendai Virus and Liposomes. Role of Viral Proteins, Liposome Composition, and pH

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ABSTRACT: A kinetic and quantitative characterization of the fusion process between Sendai virus and phospholipid vesicles is presented. Membrane fusion was monitored in a direct and continuous manner by employing an assay which relies on the relief of fluorescence self-quenching of the probe octadecylrhodamine B chloride which was located in the viral membrane. Viral fusion activity was strongly dependent on the vesicle lipid composition and was most efficient with vesicles solely consisting of acidic phospholipids, particularly cardiolipin (CL). This result implies that the fusion of viruses with liposomes does not display an absolute requirement for specific membrane receptors. Incorporation of phosphatidylcholine (PC), rather than phosphatidylethanolamine (PE), into CL bilayers strongly inhibited fusion, suggesting that repulsive hydration forces interfere with the close approach of viral and target membrane. Virus-liposome fusion products were capable of fusing with liposomes, but not with virus. In contrast to fusion with erythrocyte membranes, fusion between virus and acidic phospholipid vesicles was triggered immediately, did not strictly depend on viral protein conformation, and did not display a pH optimum around pH 7.5. On the other hand, with vesicles consisting of PC, PE, cholesterol, and the ganglioside GD_{1a}, the virus resembled more closely the fusogenic properties that were seen with erythrocyte target membranes. Upon decreasing the pH below 5.0, the viral fusion activity increased dramatically. With acidic phospholipid vesicles, maximal activity was observed around pH 4.0, while with GD_{1a}-containing zwitterionic vesicles the fusion activity continued to increase with decreasing pH down to values as low as 3.0. This indicates that the fusion susceptibility of the virus at low pH also depends on the molecular properties of the target membrane. Presumably, these properties determine the ability of the viral proteins to interact hydrophobically with the membranes, thus triggering fusion. Fusion between Sendai virus and CL liposomes at low pH was not affected when the virus had been pretreated with trypsin, suggesting that low pH induced fusion was largely mediated by the viral binding protein HN rather than the fusion protein F. It is concluded that, depending on the pH, different mechanisms are operational in the fusion process between Sendai virus and *liposomes*; i.e., fusion with liposomes can, at least partly, be accomplished in a manner which may not bear physiological significance.

Because of their simplicity and the ease of manipulating their composition, liposomes or phospholipid vesicles are widely used as model systems for studying membrane interaction processes as they occur, e.g., during membrane fusion (Papahadjopoulos et al., 1979; Nir et al., 1983; Wilschut & Hoekstra, 1984, 1986). These studies have yielded valuable information on the physicochemical properties of the lipid molecules per se and their role in artificial membrane fusion. Recently, these model membranes have also been introduced as appropriate target membranes for enveloped viruses (Landsberger et al., 1981; Maeda et al., 1981; Haywood & Boyer, 1982, 1984; Hsu et al., 1983; White et al., 1983; Stegmann et al., 1985). Such viruses display membrane fusion activity as an imperative mechanism to deliver their nucleocapsids into host cells for viral replication (Bächi et al., 1977; Choppin & Scheid, 1980; Lenard & Miller, 1983). It has been demonstrated that those enveloped viruses which enter cells by a process of receptor-mediated endocytosis fuse with liposomes at acidic pH (White & Helenius, 1980; White et al., 1983; Maeda et al., 1981; Stegmann et al., 1985). This is consistent with the concept that these viruses fuse in vivo "from within" with the endosomal membrane (Marsh & Helenius, 1984) triggered by the low pH generated in this organelle (Tycko & Maxfield, 1982). Their relatively high fusogenic capacity has greatly facilitated the ability to investigate the fusion between such viruses and liposomes in a kinetic and quantitative manner (Eidelman et al., 1984; Stegmann et al., 1985; Nir et al., 1986a) using a fluorescence assay based upon resonance energy transfer (Struck et al., 1981; Hoekstra, 1982). Efforts to adapt this procedure in order to study the fusion properties of Sendai virus were less successful due to the much less potent fusion capacity of the virus.

Sendai virus, a negative-strand RNA virus, belongs to the family of paramyxoviruses and fuses with biological target

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membranes at neutral pH. Previous work has revealed that fusion between Sendai virus and liposomes is a fairly slow process, while the number of virus particles displaying fusion activity is rather low (Landsberger et al., 1981; Haywood & Boyer, 1982, 1984; Hsu et al., 1983; Hoekstra et al., 1985a; Hoekstra & Klappe, 1986). Recently, we have developed a new procedure which enables one to monitor continuously fusion of biological membranes, including viruses, in a sensitive and quantitative manner (Hoekstra et al., 1984, 1985a). In this report, we have applied this assay, which relies on the relief of fluorescence self-quenching, to investigate the fusogenic properties of Sendai virus with liposomes as the target membranes. The biological relevance of virus–liposome fusion as a model for the fusion step in the infectious entry of viruses into cells is discussed in conjunction with previous work (Hoekstra et al., 1985a; Hoekstra & Klappe, 1986) on virus–erythrocyte membrane interaction.

Experimental Procedures

Chemicals. Octadecylrhodamine B chloride (R_{18})[1] was obtained from Molecular Probes Inc., Junction City, OR. Cardiolipin (CL, from bovine heart), phosphatidylserine (PS, from bovine brain), dioleoylphosphatidylethanolamine (DOPE), and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. Birmingham, AL. The ganglioside GD_{1a} and cholesterol were obtained from Supelco and Sigma, respectively. Trypsin and trypsin inhibitor (soybean) were from Boehringer (Mannheim, FRG); dithiothreitol (DTT) was a product from Calbiochem.

Virus. The Z strain of Sendai virus was grown in the allantoic cavity of 10-day-old embryonated eggs. The virus was harvested 72 h after infection, purified as described (Hoekstra et al., 1984; Hoekstra & Klappe, 1986), suspended in phosphate-buffered saline, and stored at −70 °C. The amount of virus as indicated in the text refers to the amount of viral protein, determined by protein measurement. Fluorescently tagged viruses were prepared as described in detail elsewhere (Hoekstra et al., 1984, 1985a). Briefly, 10 μL of a 2.2 mM ethanolic solution of R_{18} was injected, under vigorous vortexing, into 1 mL of a suspension of Sendai virus (1.1 mg of protein) in 120 mM KCl/30 mM NaCl buffered with 10 mM sodium phosphate, pH 7.4 (KNP buffer). The mixture was incubated in the dark at room temperature for 1 h. Noninserted R_{18} was separated from the fluorescently labeled virus by chromatography on a Sephadex G-75 column (1 × 20 cm) with KNP buffer as the elution buffer. The amount of R_{18}-labeled virus was determined by protein measurement, and the preparation was stored on ice.

Vesicle Preparation. Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation (Wilschut et al., 1983) in 150 mM NaCl/5 mM Hepes, pH 7.4 (NaCl/Hepes buffer). The vesicles (for composition, see figure legends) were sized to an average diameter of 0.1 μm by extrusion through Unipore polycarbonate filters (Bio-Rad). The lipid concentration of the vesicles was determined by phosphorus determination.

Fusion between Sendai Virus and Lipid Vesicles. The details of the R_{18} fusion assay have been described elsewhere (Hoekstra et al., 1984, 1985a). At the conditions of labeling, a surface density of the probe in the viral membrane is obtained that causes self-quenching of fluorescence. When the labeled viral membrane fuses with a nonlabeled target membrane, relief of self-quenching will occur, which results in a proportional increase in the fluorescence intensity due to randomization of viral and target membrane components. The increase in fluorescence, as a measure of fusion, is monitored continuously in a fluorometer.

Fusion was initiated by injecting the phospholipid vesicles into a cuvette containing the appropriate amount of R_{18}-labeled virus in NaCl/Hepes buffer. The final incubation volume was 2 mL. Fluorescence was monitored with a Perkin-Elmer MPF 43 spectrophotofluorometer (λ_{ex} = 560 nm; λ_{em} = 590 nm). The sample chamber of the fluorometer was equipped with a magnetic stirring device, and the temperature was controlled with a thermostated circulating water bath. Unless indicated otherwise, the experiments were carried out at 37 °C.

When the effect of pH on fusion between the virus and the vesicles was examined, R_{18}-labeled virus was preincubated for 10 min at 37 °C in 130 mM NaCl/10 mM sodium acetate/10 mM sodium phosphate/10 mM Tris buffer adjusted to the desired pH. The mixture was then transferred to a cuvette, and fusion was initiated by injecting the liposomes with a Hamilton syringe. Alterations in the pH of the incubation medium *during* the course of fusion were induced by injecting small volumes of either 1 N HCl or 1 N NaOH.

Calibration of the fluorescence scale was done by taking the residual fluorescence of R_{18}-labeled virus as the zero level ("0% fusion") and the fluorescence obtained after addition of Triton X-100 (1% v/v), corrected for sample dilution, as 100% (infinite dilution). The initial fusion rates were determined from the slopes of the fluorescence readings. To calculate the maximal extent of fluorescence that would be reached upon complete fusion of all viruses and liposomes present in the system, we have explicitly taken into account the difference in size between the liposomes (0.1 μm) and the virus particles (0.15 μm; Harmsen et al., 1985). For example, when mixed at a 1:1 ratio, complete merging of labeled (virus) and nonlabeled membranes (liposomes) should lead to a net increase of fluorescence of ca. 40%. Note that the dilution would be 50% when the particles are of equal size [see following paper for a more detailed treatment (Nir et al., 1986b)].

Sucrose Gradient Analysis of Fusion Products. Linear sucrose density gradients of 10–60% sucrose (w/w) were prepared in NaCl/Hepes buffer. The samples (ca. 800 μL) were layered on top of the gradient (5 mL). After centrifugation at 40000 rpm for 22 h at 4 °C in an SW50.1 Beckman rotor, fractions of ca. 250 μL were collected from the bottom of the tube. The density of each fraction was determined by measuring the refractive index. In addition, each fraction was analyzed for protein and R_{18} fluorescence. The latter was measured after solubilizing small aliquots in 2 mL of 1% (v/v) Triton X-100. Because of interference of the viral nucleocapsid with the phosphorus assay, only the top fractions were screened for liposomal phospholipid.

Other Procedures. Trypsinization of Sendai virus was carried out essentially as described by Shimizo and Ishida (1975). The virus was incubated with the enzyme in 100 μL of sodium phosphate buffer, pH 7.2, for 20 min at 37 °C. The reaction was terminated by the addition of a 2-fold excess of soybean trypsin inhibitor and placing the mixture on ice.

Dithiothreitol (DTT) treatment was done by incubation of virus in NaCl/Hepes buffer, pH 7.4, with various concentrations of DTT in a final volume of 400 μL at 37 °C. After 10 min, an appropriate volume of NaCl/Hepes buffer was

[1] Abbreviations: CL, cardiolipin; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol; R_{18}, octadecylrhodamine B chloride; LUV, large unilamellar vesicles; KNP, 120 mM KCl/30 mM NaCl buffered with 10 mM sodium phosphate, pH 7.4; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaCl/Hepes, 150 mM NaCl buffered with 5 mM Hepes, pH 7.4; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

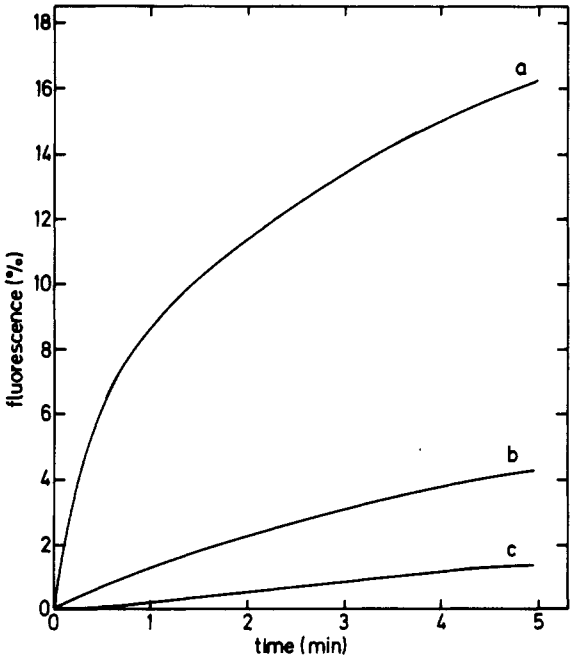

FIGURE 1: Kinetics of fusion between Sendai virus and liposomes of various lipid compositions. R_{18}-labeled virus (19.8 μg of protein) was suspended in NaCl/Hepes buffer, pH 7.4, at 37 °C. Fusion was initiated by rapid injection of the liposomes, and the increase of R_{18} fluorescence as a measure of fusion was monitored continuously. (a) CL, 30 nmol of lipid; (b) PS, 60 nmol of lipid; (c) DOPC/DOPE/cholesterol/GD_{1a}, molar ratio 7:3:6.6:1.1, 60 nmol of lipid.

added, the mixture was transferred to a cuvette, and fusion was initiated by injection of the lipid vesicles.

RESULTS

Preferential Fusion of Sendai Virus with Negatively Charged LUV. In previous work (Hoekstra et al., 1985a), we have shown that liposomes composed of neutral lipids can constitute a proper target membrane for Sendai virus, provided that they contain ganglioside GD_{1a} as a receptor for the virus. The kinetics of fusion with such liposomes are very slow, while the extent of fusion is quite moderate. As shown in Figure 1, PS LUV imparted a much higher fusion capacity to the virus. However, pure cardiolipin (CL) vesicles proved to be the most efficient target membrane. Note in this respect that, for illustrative purposes, the concentration of CL liposomes (curve a) was only half of that of the PS (curve b) and GD_{1a}-containing vesicles (curve c). The final extents of viral fusion reached at these conditions, as determined after 24 h, were 45% and 25% with CL and PS LUV, respectively. With GD_{1a}-containing vesicles, the final level of fluorescence corresponded to ca. 15% fusion. This result is consistent with data reported by Haywood and Boyer (1982) using isotopically labeled virions. Because of the remarkably high susceptibility of Sendai virus to fuse with CL vesicles, the significance of this fusion event was further examined.

Fusion between Sendai Virus and CL Vesicles. Both the initial rate (○) and the extent (●) of fusion increased when the amount of target membrane, available for viral interaction, increased (Figure 2). When comparing the extent of fusion observed experimentally (●) to that calculated (×), i.e., the extent that would be reached upon complete merging of all particles present in the system, it becomes apparent that on average maximally 64% (range 60–67%) of the viral dose added fused. Note that this maximal number will only be reached when it is assumed that fusion is limited to the merging of one virus particle with only one liposome.

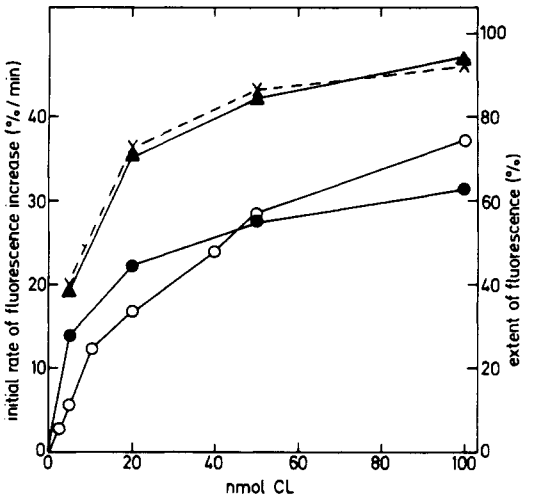

FIGURE 2: Interaction of Sendai virus with CL vesicles as a function of the vesicle concentration. Fusion between Sendai virus (18.6 μg of protein) and various amounts of CL vesicles was monitored. At pH 7.4, the final extent of fusion (●) was determined, and the initial fusion rate (○ was calculated. The theoretical extent of fusion (×), which would be reached upon complete randomization of viral and liposomal membranes, was calculated as described under Experimental Procedures. A similar experiment was performed at pH 5.0; the final extent of fusion (▲) was estimated and plotted as a function of the amount of CL vesicles.

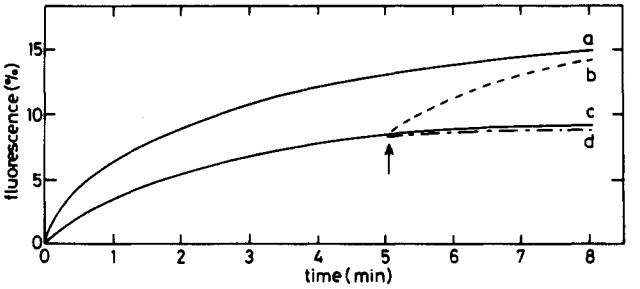

FIGURE 3: Ability of liposomes and virus to fuse with virus–liposome fusion products. R_{18}-labeled Sendai virus (15 μg) was incubated with 5 nmol of CL vesicles (c), and fusion was monitored as described in the legend to Figure 1. In parallel experiments, either 5 nmol of CL vesicles (b) or 15 μg of nonlabeled virus (d) was added 5 min after initiation of the fusion reaction with 5 nmol of CL per se. Curve a shows the course of fluorescence obtained for fusion between the virus and 10 nmol of CL vesicles.

Therefore, at least 36% of the total virus fraction did not participate in fusion, in spite of the large excess of liposomes which was eventually added (at 100 nmol of CL vesicles, the ratio of virus to liposomes is 1:20, based on phospholipid). Taking into account, however, that (i) only a fraction of the virus is "fusion-active" whereas (ii) the extent of fluorescence continues to increase with increasing vesicle concentration, the results would suggest that fusion of the "active" virus fraction is not limited to one round of fusion only. Rather, it would appear that the fusion products, formed upon merging of one virus particle and one LUV, can subsequently fuse with other liposomes. Indeed, experimental support was provided by the experiment shown in Figure 3. After an initial round of fusion between virus and CL vesicles was allowed to equilibrate (curve c), a subsequent increase in fluorescence was only triggered upon another addition of liposomes (arrow, curve b), rather than R_{18}-labeled or nonlabeled virus (curve d). The results further indicated that essentially all liposomes participated in the initial fusion event as another addition of R_{18}-labeled virus also did not result in an increase in fluorescence.

Effect of PC and PE. Sendai virus does not fuse with pure PC bilayers (Haywood & Boyer, 1984; Hoekstra et al., 1985a).

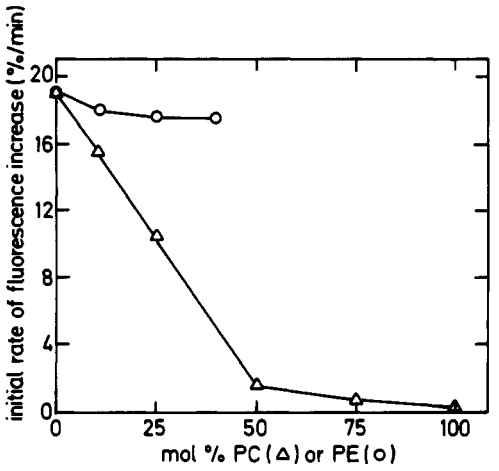

FIGURE 4: Effect of the incorporation of PC and PE into CL liposomes on the viral fusion activity. Increasing amounts of PC or PE were incorporated into CL bilayers. Fusion of the mixed vesicles (lipid concentration was 60 nmol of phosphate in a final volume of 2 mL) with Sendai virus (19.3 μg of protein) was monitored, and the initial fusion rates were calculated.

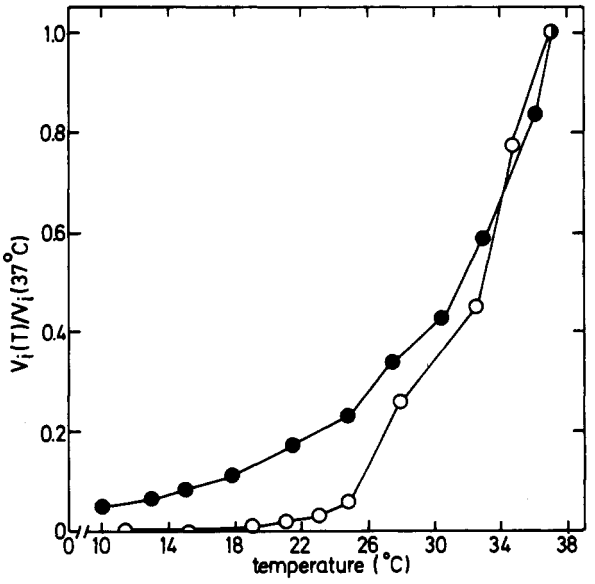

FIGURE 5: Effect of temperature on the fusion of Sendai virus with artificial and biological target membranes. Fusion between virus (16.4 μg of protein) and CL vesicles (30 nmol of lipid) was monitored as described in the legend to Figure 1 after equilibrating the virus, suspended in NaCl/Hepes, pH 7.4, at the desired temperature. The initial fusion rates [$V_i(T)$] were calculated and normalized to the initial rate obtained at 37 °C [V_i(37 °C)]. This ratio was plotted as a function of temperature (●). Similar data [taken from Hoekstra et al. (1985a)] were calculated and plotted for the fusion between Sendai virus and erythrocyte membranes (○).

As shown in Figure 4, the presence of increasing amounts of this lipid in the CL bilayers also had a pronounced effect on the ability of the virus to fuse with such mixed vesicles. A virtually complete inhibition occurred when the amount of PC exceeded 50 mol % of the total lipid. At comparable conditions, the insertion of PE, instead of PC, caused only minor inhibition, and the fusion reaction was sustained rather than inhibited in the concentration range tested (up to 40 mol %).

Temperature Dependence of Sendai Virus–CL Vesicle Fusion. In contrast to fusion with erythrocyte ghosts, fusion of the virus with CL vesicles showed a much less defined threshold temperature, although the overall shape for the temperature-dependent fusion activity with either target membrane was similar (Figure 5). Upon addition of CL

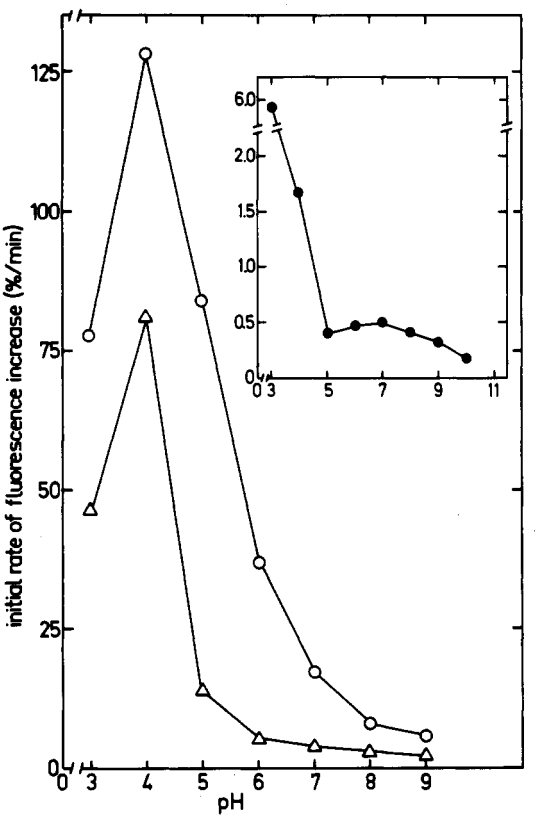

FIGURE 6: pH dependence of fusion of Sendai virus with liposomes of various compositions. R_{18}-labeled Sendai virus (19.3 μg of protein) was incubated in 120 mM NaCl/10 mM sodium acetate/10 mM sodium phosphate/10 mM Tris, adjusted to the appropriate pH at 37 °C for 10 min. Subsequently, 30 nmol of CL (○), 60 nmol of PS (Δ), or 60 nmol of DOPC/DOPE/Chol/GD_{1a} (molar ratio 7:3:6.6:1.1) vesicles (●, insert) was injected into the medium, and fusion was monitored. The initial fusion rates were calculated and plotted as a function of the pH.

vesicles to R_{18}-labeled virus, the fluorescence intensity increased immediately, albeit rather slow at temperatures below 18 °C (●). Upon further raising of the incubation temperature (22–37 °C), the rate rapidly increased, similarly as seen for ghosts (○). This increase commensurates with and is probably related to the rapid increase in the mobility of the viral proteins [Lee et al., 1983; cf. Hoekstra et al. (1985a)] occurring within the same temperature range. In case of ghosts (○), fusion becomes apparent only at temperatures above 20 °C, and, depending on the incubation temperature, a lag time (cf. Figure 1, curve c) of ca. 1 min (at 37 °C) up to 16 min (at 21 °C) is observed (Hoekstra et al., 1985a), before the fluorescence increases significantly. A maximum in the fusion rate between Sendai virus and CL liposomes was seen around 38 °C; above 40 °C, the rate decreased again (not shown), similar to the fusion reaction with ghosts (Hoekstra et al., 1985a). Presumably, the occurrence of fusion with CL vesicles observed at temperatures where fusion of ghosts does not take place reflects the higher susceptibility of the vesicles as a fusogenic target membrane for Sendai virus. In conjunction with the instantaneous triggering of the fusion reaction upon mixing of the virus and the vesicles, the results entail that viral fusion with CL LUV is less strictly controlled than fusion with erythrocyte membranes, thus suggesting a difference in the mechanism of fusion.

pH Dependence of Virus–Liposome Fusion. Figure 6 shows that with LUV consisting of CL (○) or PS (Δ) both the initial rate and the extent (not shown) of fusion increased dramatically with decreasing pH, displaying an optimum around pH 4.0 rather than around pH 7.5, i.e., the optimal pH for vi-

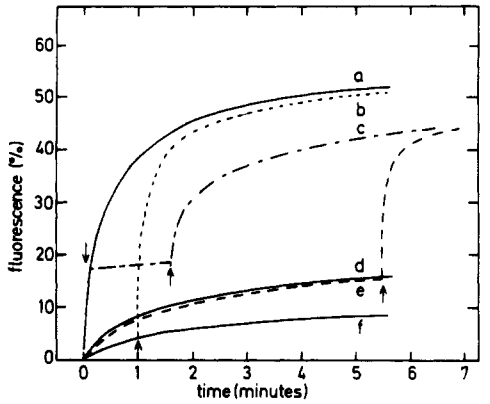

FIGURE 7: Reversibility of low pH induced fusion of Sendai virus with CL vesicles. Fusion between virus (18.9 μg of protein) and CL vesicles (30 nmol of lipid) was monitored at various pH values as described in the legend to Figure 6; Solid curves; (a) pH 5.0; (d) pH 7.4; (f) pH 9.0. After initiation of the fusion reaction at pH 5.0 (curve a), the pH was adjusted to 7.4 (↓) by addition of a small aliquot of NaOH and subsequently readjusted to 5.0 (↑) by addition of HCl (curve c). Curve b was obtained when, after initiation of fusion at pH 9.0, the pH was adjusted to 5.0 (↑, curve f). Curve e shows the fluorescence development obtained when the virus, prior to fusion with vesicles at pH 7.4, had been preincubated for 10 min at pH 5.0. After 5.5 min, the pH was adjusted to 5.0 (curve e, ↑).

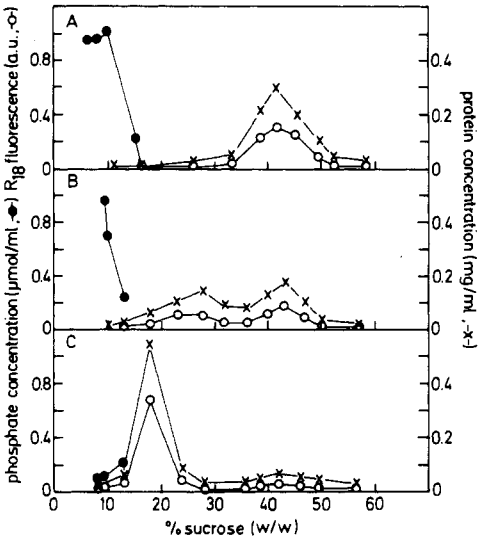

FIGURE 8: Density gradient analysis of virus–liposome fusion products. R_{18}-labeled Sendai virus (375 μg of protein) was incubated with CL vesicles (562 nmol of lipid) at 37 °C, either at pH 7.4 or at pH 5.0. After 15 min, the pH of the latter incubation mixture was adjusted to 7.4, and subsequently, both samples were centrifuged on a continuous sucrose gradient (10–60%). Fractions were collected and analyzed for (liposomal) phospholipid by phosphate determination (●), protein (×), and R_{18} fluorescence (○) as described under Experimental Procedures and plotted as a function of the sucrose density. (B) Incubation at pH 7.4; (C) incubation at pH 5.0. As a control, liposomes and virus were run on separate gradients and analyzed similarly. The combined results are shown in panel A.

rus–erythrocyte membrane fusion (Hoekstra et al., 1985a). This remarkable fusion behavior of Sendai virus with pure CL and PS membranes might indicate that the mode of this virus–target membrane interaction differs from that observed under physiological conditions. At the latter, the virus initially attaches to sialic acid residues contained in glycoproteins and/or glycolipids (Wu et al., 1980), rather than interacting directly with the lipid bilayer. Therefore, the above experiment was repeated with vesicles consisting of PC, PE, cholesterol, and the viral receptor ganglioside GD_{1a}. As shown in Figure 6 (insert) in the range of pH 5–10, the fusion pattern differs from that observed for the pure negatively charged bilayers in that an apparent optimum for viral fusion activity was seen around pH 7.0, which is slightly lower than the pH optimum observed with erythrocyte ghosts as the target membranes. However, with the artificial bilayers, the optimum is far less pronounced than with the ghosts [cf. Hoekstra et al. (1985a)]. Yet, when the pH was further lowered (below pH 5), the initial rate of fluorescence increased steeply as in the case of CL and PS LUV. Interestingly, with GD_{1a}-containing vesicles, no apparent maximum was seen around pH 4.0. Rather, the rate further increased with decreasing pH to a value as low as 3.0.

As shown in Figure 7, the low pH induced fusion activity is a reversible process. After fusion was initiated at pH 5.0 (curve a), the reaction was suppressed upon readjustment of the pH to 7.4 (curve a, ↓). A subsequent lowering of the pH to 5.0 reactivated the fusion reaction (curve c, ↑). Furthermore, the fusion kinetics as monitored at pH 7.4 were the same, irrespective of whether the virus had been preincubated at pH 5.0 (curve d vs. curve e). Hence, these results further indicate that a mild acidic pretreatment of the virus does not irreversibly activate the fusion activity (curves a vs. c and d vs. e). Clearly, these results are in striking contrast with previous results on the Sendai virus–erythrocyte membrane interaction (Hoekstra et al., 1985a) which revealed that mild acidic treatment of the virus results in an irreversible diminishment of its fusion capacity. Finally, the fusion activity observed at low pH (5.0) was not completely restored when at some stage during the fusion reaction the medium pH was readjusted to 7.4 and subsequently lowered again to pH 5.0 (curves c and e). Only upon immediate lowering of the pH of the neutral (not shown) or alkaline medium (curve b) to 5.0, a fusion level was attained which was virtually identical with that seen when fusion had been initiated at pH 5.0 per se.

Analysis of Virus–Liposome Fusion Products by Sucrose Density Gradient Centrifugation. Relative to fusion at pH 5.0, fusion between Sendai virus and CL LUV at pH 7.4 is a rather slow process while the extent of fusion reached at either pH reflects similar differences. Indeed, when liposomes were injected into the virus-containing medium at a ratio of 5:1 (based on phospholipid), the final level of fluorescence obtained at pH 7.4 was ca. 55% and ca. 85% at pH 5.0. Moreover, as may be inferred from Figure 2, fusion at pH 5.0 is essentially complete (▲). Since, as demonstrated in Figure 3, the fusion products of one virus particle and one liposome subsequently fuse with other liposomes, but not among themselves or with viruses, maximal fusion at pH 5.0 would thus imply that at a liposome:virus ratio of 5:1, on average a virus particle must have fused with five liposomes. Similarly, one would estimate that at pH 7.4 one virus particle can maximally fuse with only two liposomes (cf. Figure 2). Accordingly, the result of the pH-dependent fusion events would be that the *density* of the fusion products obtained at pH 7.4 and 5.0 should differ as the virus will be more "diluted" with exogenous phospholipid at pH 5.0 than at pH 7.4. To obtain experimental support for these arguments, R_{18}-labeled Sendai virus was fused with a 5-fold excess of CL liposomes, either at pH 7.4 or at pH 5.0. After 15 min at 37 °C, when ca. 40% of the virus fraction will be fused at pH 7.4 and ca. 90% at pH 5.0 (see above), the mixtures were analyzed by sucrose density gradient centrifugation. As shown in Figure 8, the gradient obtained at pH 7.4 (panel B) revealed two bands, one of high density representing the nonfused virions (cf. panel A) and another fraction with a density which was intermediate between the densities of the pure vesicles (panel A) and the virus. If the fusion products would mainly consist of virus particles

Table I: Fusion between Trypsinized or DTT-Treated Sendai Virus and Liposomes. Effect of Vesicle Composition and pH[a]

		CL vesicles		PS vesicles		PC/PE/Chol/GD_{1a} vesicles	
treatment	pH[b]	V_i (%/min)	inhibn (%)	V_i (%/min)	inhibn (%)	V_i (%/min)	inhibn (%)
none	7.4	13.7	0	3.6	0	0.45	0
trypsin[c]	7.4	2.5	82	0.7	81	0.03	92
DTT[d]	7.4	9.5	30	nd[e]	nd	0.12	74
none	5.0	74.1	0	13.0	0	0.41	0
trypsin	5.0	39.3	47	1.9	85	0.04	90
DTT	5.0	75.2	0	12.1	7	0.17	59

[a] Virus pretreated with trypsin or DTT as described under Experimental Procedures was suspended (19.2 μg of protein) in the medium and adjusted to the appropriate pH. Fusion was initiated by injection of the liposomes: CL (30 nmol of lipid), PS (60 nmol), or PC/PE/Chol/GD_{1a} vesicles (60 nmol). The initial fusion rates were determined as described. [b] Values given represent the pH of the fusion medium. [c] Trypsin concentration was 20 μg/mL. [d] DTT concentration was 1 mM. [e] Not determined.

fused with one liposome, and taking into account that ca. 40% of the total virus fraction had fused, the majority of the liposomes should still be present as nonfused particles after 15 min. This appears to be the case as is shown in panel B (closed symbols). On the other hand, at pH 5.0 only a fraction of nonfused virus was traced on the gradient while the remaining liposomal fraction was also virtually negligible. Instead, a major band, intermediate between the nonfused liposomes and virions, was apparent, at a lower density than that observed for the fusion product at pH 7.4. In passing, these experiments also provide another means to show that probe dilution reflects membrane fusion rather than transfer of free monomers. Previously, we have shown (Hoekstra et al., 1984; Harmsen et al., 1985) that the kinetics of R_{18} dilution are the same as those determined with a fusion assay based on resonance energy transfer (Struck et al., 1981).

Multiple Rounds of Fusion at Low pH. To provide further support for the occurrence of multiple fusion events at pH 5.0 between one virus particle and several liposomes rather than nonspecific merging of the fusion products among themselves, virions and CL LUV were mixed at a 1:1 ratio at pH 5.0, and fusion was monitored continuously (Figure 9). After an apparent maximal fluorescence level was reached, an amount of CL vesicles equal to the total amount of phospholipid in the cuvette was added such that the lipid ratio of fusion product and newly added LUV became 1:1. Again, the fluorescence development was monitored. This procedure was repeated twice. As shown in Figure 9, the enhancement in the final fluorescence level after each addition as observed experimentally correlates fairly well with the level expected theoretically (bars), i.e., upon complete randomization of viruses and liposomes. Moreover, upon addition of labeled or nonlabeled virus rather than liposomes to the fusion products, no increase in fluorescence was observed (cf. also figure 3). This indicates that essentially all (nonlabeled) liposomes participated in the fusion process and that the virus per se did not fuse with the fusion products.

Dependence of Virus–Liposome Fusion on Viral Proteins. The envelope of Sendai virus contains only two proteins (Choppin & Scheid, 1980; Hsu et al., 1982; Blumberg et al., 1985a,b). One protein, HN, is required for viral attachment to the target membrane receptors, while the other, F, exclusively mediates the actual fusion process. The role of these glycoproteins in virus–*liposome* fusion was examined after pretreatment of the virus with trypsin and the reducing agent dithiothreitol (DTT). Trypsin specifically cleaves the F protein, thereby inactivating viral fusion activity at physiological conditions. DTT perturbs the conformation of the proteins by reducing disulfide bonds which are present in both F and HN. At neutral pH (Table I), the fusion between the trypsinized virions and all three types of vesicles used in this work was inhibited by more than 80%. Furthermore, at pH 5.0,

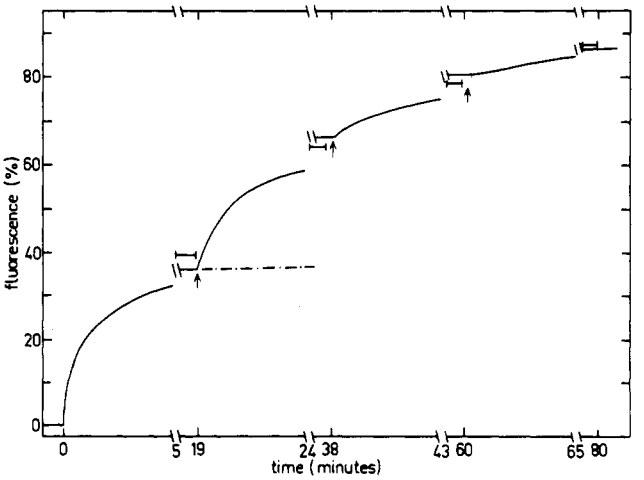

FIGURE 9: Multiple fusion events between Sendai virus and CL vesicles at pH 5.0. Twenty micrograms R_{18}-labeled virus was preincubated at pH 5.0 for 10 min at 37 °C. Subsequently, fusion was initiated by adding an equal amount (based on phospholipid) of CL vesicles (5.5 nmol of lipid). After an apparent fusion maximum was reached, additional CL vesicles were injected into the medium (arrow) corresponding to an amount equal to the total amount of viral and CL phospholipid after the first addition (i.e., 11 nmol). These additions were repeated twice (by adding 22 and 44 nmol of CL, respectively) such that a ratio of phospholipid (present in the incubation medium) to newly added vesicle lipid of 1:1 was obtained. The bars indicate the level of fluorescence that would be reached upon complete randomization of the lipids in the system, calculated as described under Experimental Procedures. When virus was added instead of liposomes after one round of fusion, a curve designated by (–·–) was obtained.

trypsinized virions barely fused with either PS or GD_{1a}-containing vesicles, the extent of inhibition (85% and 90%, respectively) being similar to that observed at pH 7.4. However, in the case of CL LUV, the degree of inhibition at acidic pH was substantially less (47% vs. 82% at pH 7.4), indicating that a considerable part of the fusion reaction at low pH between Sendai virus and CL liposomes was accomplished by an F protein independent mechanism. The inhibition of the extent of fusion between the trypsin-treated virus and liposomal membranes was similar to that seen for the initial fusion rates.

The effect of DTT treatment is also shown in Table I. For GD_{1a}-containing vesicles as target membranes, the viral fusion activity decreased to ca. 34% at 0.1 mM DTT up to ca. 62% at concentrations ≥1 mM. At neutral as well as acidic pH, both the rate and maximal extent of fusion were inhibited to the same degree. A different picture emerged, however, with the pure negatively charged vesicles as target membranes. At neutral pH, the initial rate of fusion between the DTT-treated virus and CL vesicles decreased, displaying an inhibition relative to nontreated virus of ca. 60%; however, the level of fluorescence reached after 5–10 min was the same as that obtained for the nontreated virus. Hence, in this case, the

fusion reaction was delayed rather than inhibited. Furthermore, at low pH (5.0), both the rate and the extent of fusion between DTT-treated virus and CL or PS LUV were virtually indistinguishable from that observed for the control virus.

Discussion

A number of observations reported in this work raise doubts on the *general* applicability of phospholipid vesicles as reliable target membranes for investigating the *biological* fusion properties of viruses. It would appear that at least part of the fusion event between Sendai virus and the liposomes used in this work can be accounted for by a mechanism which does not seem to be operational at biological conditions.

The ability to readily fuse with pure acidic phospholipid vesicles implies that the virus does not display an absolute requirement for binding to specific, sialic acid containing receptors (Bächi et al., 1977; Wu et al., 1980), which is consistent with recent work of others (Haywood & Boyer, 1984; Landsberger et al., 1981). To trigger the subsequent fusion reaction, a sufficiently close interbilayer contact has to be established. It seems conceivable that this requires a spatial and/or lateral reorganization of the viral proteins, as they protrude ca. 120 Å beyond the bilayer/water interface (Bächi et al., 1977; Knutton, 1977; Harmsen et al., 1985) and might therefore interfere sterically with close approach of apposed bilayers (Hoekstra et al., 1985b; Hoekstra & Düzgünes, 1986). This process could be reflected by the brief delay in the fusion reaction between the virus and GD_{1a}-containing vesicles (Figure 1) or erythrocyte membranes (Hoekstra et al., 1985a, 1986). By contrast, both CL and PS vesicles fused instantaneously with the virus.

In addition to an interference of viral proteins with the close approach and subsequent fusion of the viral and liposomal membranes (Hoekstra et al., 1985a), repulsive hydration forces (Rand, 1981; Marra & Israelachvili, 1985), due to the lipid head-group bound water, have to be taken into account as well. In contrast to PE, PC strongly inhibits viral fusion activity (Figure 4), analogous to its inhibiting effect noted in studies on vesicle–vesicle fusion (Wilschut & Hoekstra, 1984, 1986; Düzgünes, 1985). These distinct effects of PE and PC are best explained in terms of differences in the state of hydration, PC being substantially more hydrated than PE (Israelachvili et al., 1980). It is possible that the relatively low fusion susceptibility of the GD_{1a}-containing vesicles is (partly) related to the high amount of PC contained in these bilayers.

At *neutral* pH, the fusion of trypsinized viruses was almost completely abolished, irrespective of the composition of the liposomal target membrane (Table I). This observation would be consistent with an F_1-mediated fusion mechanism, as trypsin specifically removes this polypeptide (Shimizu & Ishida, 1975; Lyles, 1979; Abidi & Yeagle, 1984). An absolute dependence of the fusion reaction on the conformation of the viral proteins was less apparent. DTT treatment revealed an inhibition of 74% with GD_{1a}-containing liposomes and only 30% with CL liposomes (Table I), whereas almost complete inhibition is observed with erythrocyte membranes (Hoekstra et al., 1985a; Hoekstra & Klappe, 1986). Hence, at neutral pH, fusion with liposomes does not display an absolute dependence on viral protein conformation. The occurrence of nonspecific fusion between Sendai virus and liposomes was further indicated by the pH-dependence experiments. In a pH range between 5.0 and 9.0, the pH exclusively affects the F protein and induces an irreversible conformational change (Hsu et al., 1982) which results in a strongly reduced fusion activity of the virus with erythrocyte membranes (Hoekstra et al., 1985a) at mild acidic (pH 5.0) and alkaline conditions (pH 9.0), while optimal fusion activity is seen around pH 7.5. With liposomes, these properties were reflected to some extent only with GD_{1a}-containing vesicles (Figure 7). The apparent fusion optimum at neutral pH is far less pronounced, however, than that observed for virus–ghost fusion (Hoekstra et al., 1985a). Presumably, this difference can be attributed to an only partial dependence of the fusion process on viral protein conformation (see above).

With CL and PS vesicles, a different picture emerged as fusion steadily increased with decreasing pH. Quite unexpectedly, at pH <5, extensive fusion also occurred with GD_{1a}-containing vesicles. Yet, as far as the fusion mechanism is concerned, distinct lipid-dependent differences became apparent. With GD_{1a}-containing vesicles, viral fusion activity relied predominantly on an F_1-mediated mechanism. A similar but not identical (see below) mechanism appeared to account for fusion with PS vesicles. By contrast, more than 50% of the fusion reaction with CL LUV was accomplished by an F_1-independent mechanism. Furthermore, fusion with CL and PS vesicles was essentially unaffected upon altering the protein conformation by treatment of the virus with DTT. Remarkably, fusion with GD_{1a}-containing vesicles was still substantially governed by the viral protein conformation. Recently, Citovsky et al. (1985) reported that the fusion of DTT-treated Sendai viruses with PS vesicles was only slightly inhibited at neutral pH.

In spite of the nonspecific fusion process between Sendai virus and CL liposomes, which was also reflected by the reversible pH effect on fusion [Figure 7; cf. Hoekstra et al. (1985a)], the interaction appeared to be protein dependent. Thus, fusion was not observed between CL LUV and vesicles prepared from total viral lipids (not shown; incidently, this result also excludes spontaneous transfer of the probe between membranes at the experimental conditions, thereby confirming the reliability of the assay in this system). Furthermore, as fusion proceeded upon removal of F_1 by trypsinization (Table I), the evidence would thus point toward a direct involvement of HN in the fusion process. The absence of a lag phase in the initiation of the fusion reaction (Figure 1) as well as the occurrence of fusion below 21 °C (cf. Figure 5) would support this suggestion. Whether such a mechanism involves a hydrophobic interaction with the target membrane, similarly as proposed for F_1-triggered fusion, remains to be established. In this regard, it is interesting to note, however, that the ability of a variety of proteins and polypeptides (Schenkman et al., 1981; Banerjee et al., 1981; Blumenthal et al., 1983; Eytan & Almary, 1983; Gad & Eytan, 1983) to induce membrane fusion has been related to the exposure of hydrophobic surfaces, provided that cationic charges were available for initial interaction with the membranes. For the viral proteins, the latter condition is clearly met upon decreasing the pH (as the p*I*'s for HN and F are 6.5 and 4.9, respectively; Shimizu et al., 1974). Furthermore, at low pH, the HN molecule contains at least 10 peptide segments consisting of 13–30 apolar amino acids (Blumberg et al., 1985b). Similar to the penetrating capacity of the N-terminal segment of F_1, it seems likely that these segments may also be capable of penetrating into the hydrocarbon region of the liposomal target membrane.

The ability of the virus to fuse with the various liposomal systems may thus be governed by differences in the lipid head-group region, presumably involving differences in lateral packing. As demonstrated by Jähnig et al. (1980), the bilayer packing density will decrease with increasing surface charge due to electrostatic inter-head-group repulsion, which causes the hydrocarbon chains to tilt. It is quite conceivable that this property facilitates the penetration and subsequent hydro-

phobic interaction of viral proteins with a target membrane and the observed order in which fusion increases (PC/PE/Chol/GD_{1a} < PS << CL) would be consistent with this notion. Interestingly, myelin basic protein, which also displays membrane fusion properties (Lampe et al., 1983), has been demonstrated to interact hydrophobically with artificial bilayers according to a very similar lipid-species dependence as described here for the viral proteins (London & Vossenberg, 1973; Boggs, 1980). Furthermore, in contrast to CL, PS can interact intermolecularly via hydrogen bonds with adjacent polar head groups (MacDonald et al., 1976). Such interactions may in addition contribute to a relatively closer packing in PS than in CL vesicles (Boggs et al., 1980) and hence further restrict the ability of the viral proteins to interact hydrophobically with the former vesicles. Presumably, these differences in packing constraints between CL and PS are reflected by the poor ability of PS to sustain the fusion reaction according to an HN-mediated mechanism at low pH. It seems likely that to accommodate the HN protein, which contains only *internal* hydrophobic stretches of amino acids at low pH, a larger inter-head-group spacing will be required than will be necessary to accommodate the *N-terminal* hydrophobic segment of F. Upon lowering the pH, the relative net increase in cationic charge and the concomitant conference of a higher hydrophobicity would suffice to explain the increased capacity of the viral proteins to mediate the fusion with negatively charged bilayers. However, besides protonation of acidic amino acid residues, the acidic lipid head groups will also become increasingly protonated. As a result, electrostatic inter-head-group repulsions will decrease which leads to an increased packing density (Israelachvili et al., 1980) particularly in the case of CL and PS vesicles. This propensity is also reflected by the increase in the main transition temperature of acidic phospholipids as the pH decreases (Watts et al., 1978; Sacrē et al., 1979). Hence, one would anticipate that if viral protein penetration constitutes the trigger for fusion, eventually the ability of the virus to fuse will diminish wiht a decrease in pH in spite of an increase in the net cationic charge and hydrophobicity of the viral proteins. Apparently, an optimal balance between the various parameters which determine virus–liposome fusion was achieved for CL and PS around pH 4.0, which is close to the p*K*'s of the ionizable groups of these lipids (Boggs et al., 1980). The steady increase of fusion between the virus and GD_{1a}-containing liposomes below pH 4 is consistent with this notion as the sialic acid residues of GD_{1a} only become titrated around pH 2 (Maggio et al., 1978). Thus, around pH 4.0, alterations in the bilayer constraints of the negatively charged vesicles may become effective, presumably related to changes in molecular geometry (Cullis et al., 1984) and possibly further modulated by interactions between the lipid molecules and viral proteins. It would appear, therefore, that below pH 4.0 the viral proteins fit less easily into the pure negatively charged target membranes and, hence, the virus displays less fusion susceptibility. Similarly as observed for the interaction of myelin basic protein with acidic phospholipid vesicles (Boggs et al., 1980), it would thus appear that the closer the lipid packing the less capable the proteins will be to interact hydrophobically with the target membrane.

In summary, the present work indicates that with zwitterionic liposomes containing GD_{1a}, the virus displays the closest approximation of the fusion reaction seen with the biological membranes. The observations support a relationship between the ability of the viral proteins to penetrate into the target membrane and their capacity to trigger fusion. Depending on the pH and on the target membrane composition, the fusion reaction can be partly governed, however, by nonspecific hydrophobic interactions. When assuming that a hydrophobically mediated perturbation of the bilayer triggers fusion, it would appear that this type of interaction is far less subtle in liposomal than in biological systems.

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