Articles

Kinetics of Fusion and Lipid Transfer between Virus Receptor Containing Liposomes and Influenza Viruses As Measured with the Octadecylrhodamine B Chloride Assay†

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ABSTRACT: Octadecylrhodamine B chloride (R18) and ganglioside G_{D1a} (virus receptor) were incorporated into small unilamellar liposomes [Hoekstra et al. (1984) *Biochemistry 23*, 5675-5681]. Upon interaction of these liposomes with PR8 influenza viruses without prebinding, two types of dequenching were observed at 37 °C, both second-order processes: a fast reaction at pH 5.3, $^2k = 17.53 \times 10^{-3}$ $(Q \cdot s)^{-1}$, and a slow reaction at pH 7.4, $^2k = 0.335 \times 10^{-3}$ $(Q \cdot s)^{-1}$. The maximal level of dequenching was the same for both. Upon prebinding of liposomes to PR8 viruses (30 min, 0 °C, pH 7.4) at high concentrations, a very fast dequenching occurred when the prebinding mixture was diluted into prewarmed (37 °C) 10 mM PBS, pH 5.3. For the initial phase, a first-order rate constant of 0.5 s^{-1} could be extrapolated. After a quick drop in velocity during the first 30 s, the reaction was kinetically indistinguishable from the one found without prebinding. A second-order process with $^2k = 16.52 \times 10^{-3}$ $(Q \cdot s)^{-1}$ became rate-limiting. The fast reactions at pH 5.3 can be abolished by inactivation or removal of the virus hemagglutinin. We conclude that the reaction at pH 5.3 reflects the hemagglutinin-dependent fusion process known to occur between influenza viruses and partner membranes at low pH; however, second-order kinetics indicate that specific binding rather than fusion is the rate-limiting step. For the slow dequenching, which is not affected by prebinding, the rate constant is 20 times lower than for the fast reaction, and the process is independent of viral hemagglutinin. We postulate that it arises through collision-mediated lipid transfer. This is the first evidence for a nonspecific transfer of the R18 marker. In view of the therapeutical application of small unilamellar vesicles, the phenomenon of nonspecific lipid transfer has to be carefully controlled.

Strong evidence has accumulated over the last few years that influenza viruses infect cells by fusion with cellular membranes. This fusion process is mediated by the viral hemagglutinin molecule, which at acidic pH undergoes a conformational change, as has been demonstrated by several groups (Maeda et al., 1981; Skehel et al., 1982; White et al., 1983). The same authors could show that fusion of viral envelopes with target membranes occurred at acidic but not at neutral pH. The prelysosomal compartment with a pH value between 5 and 6 has thus been identified as the site of virus entry into cells. The pH dependence of the specific fusion reaction correlates very well with the pH dependence of hemolysis (Yewdell et al., 1983). The significance of the low-pH step for the viral fusion has been challenged by Haywood and Boyer (1985), who claimed that fusion also occurs at pH 7.4 and that the preferential fusion observed at pH 5.3 is only a consequence of more efficient binding. Binding of viruses to target membranes is obviously a prerequisite for a possible fusion event. It has been shown that adsorption of viruses to artificial membranes of various lipid compositions strongly depends on the surface charge (Haywood & Boyer, 1985) and thus is very likely to be affected by pH. Comparative kinetic studies on the membrane interactions of influenza viruses with various target membranes have recently been performed by using quantitative fluorescence assays (Struck et al., 1981; Stegmann et al., 1989). Rate constants were, however, only calculated for the reaction at pH 5.3 and not for the reaction at neutral pH, which was treated as a background phenomenon. Haywood and Boyer (1985) themselves based their evidence for fusion at pH 7.4 on quantitation by gradient centrifugation and on electron microscopic studies.

In this paper, we present quantitative measurements on membrane interactions at neutral (pH 7.4) and low pH (5.3) between ganglioside G_{D1a}[1] (virus receptor)-containing liposomes and influenza viruses. Specific binding between influenza viruses and the sialic acid residues of G_{D1a} has been shown to be independent of pH (Matlin et al., 1981; Yoshimura et al., 1982). Thus, potential differences measured between the kinetics of membrane interactions at acidic and neutral pH will not simply reflect differences in the binding step but will be due to the subsequent lipid bilayer interactions. For kinetic studies, we used the octadecylrhodamine B chloride (R18) assay which had been introduced by Hoekstra et al. (1984). This assay is based on the incorporation of the R18 marker into liposomes or biological membranes at quenched concentrations. Upon interaction with an unlabeled target membrane, the marker is diluted, and the accompanying dequenching can be measured quantitatively in a fluorescence spectrophotometer. A very fast dequenching reaction was found at pH 5.3,

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; G_{D1a}, disialoganglioside; HA, hemagglutinin; ^1k and ^2k, first- and second-order rate constants, respectively; PA, phosphatidic acid; PC, phosphatidylcholine; R18, octadecylrhodamine B chloride; SUV and LUV, small and large unilamellar vesicle(s), respectively.

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which reached a plateau of maximal dequenching within a few minutes. At pH 7.4, a slow dequenching reaction could be measured, which after 3–4 h reached the same maximal dequenching value as determined for the pH 5.3 reaction. Using bromelain-treated influenza viruses as fusion partners, we could demonstrate that only the fast dequenching reaction represents hemagglutinin-specific fusion whereas the slow reaction occurred independently of the presence of the hemagglutinin fusion molecule.

Materials and Methods

Chemicals. Egg yolk phosphatidylcholine (PC) and phosphatidic acid, both grade 1, were purchased from Lipid Products (Nutfield, U.K.). Ganglioside G_{D1a} was from Bachem (Bubendorf, Switzerland), and octadecylrhodamine B chloride (R18) was from Molecular Probes (Junction City, OR). The radioactively labeled substances were 1,2-dipalmitoyl-L-3-phosphatidyl[*N-methyl*-^{3}H]choline (76 Ci/mmol; Amersham) and [*carboxyl*-^{14}C]cholic acid, sodium salt (55.7 mCi/mmol; Amersham). Cholesterol and EDTA were from Sigma; the other chemicals were from Merck (all analytical grade). Buffers used: 10 mM PBS (Na_2HPO_4 and KH_2PO_4) containing 130 mM NaCl at pH values between 5.0 and 7.7 as indicated, and NTE (10 mM Tris-HCl, 50 mM NaCl, and 1 mM EDTA), pH 7.4.

Virus. (A) Growth and Purification. Influenza PR8 virus [A/PR/8/34 (H1N1)] from chicken egg allantoic fluid (gift from Dr. Th. Bächi, Institute for Immunology and Virology, University of Zürich, Switzerland) was adapted to growth in MDCK cells (Madin Darby canine kidney cells, a gift from Dr. M. Paccaud, Institut d'Hygiêne, Geneva, Switzerland). Routinely, cells were infected with about 10^{-5} hemagglutinating unit of the adapted virus per cell and incubated at 35 °C in Eagle's minimal essential medium (MEM) to which trypsin was added at a final concentration of 2 μg/mL 1 h after infection. After 48–72 h, cells were disrupted by mild sonication (TUC 300, Telsonic sonifier, 3 min, room temperature), and cell debris were removed by low-speed centrifugation (2000*g*, 10 min, 4 °C). Virus was sedimented from the supernatant (200000*g*, 30 min, 4 °C) and resuspended in NTE buffer, pH 7.4, at a final concentration of approximately 700 μg of protein/mL corresponding to about 3×10^5 hemagglutinating units/mL. Virus titers were determined with the hemagglutination assay using chicken erythrocytes (Fazekas de St. Groth & Webster, 1966). For protein measurements, virus was heated to 37 °C for 30 min in the presence of 0.5% Triton X-100 to solubilize membrane proteins. The protein content was determined with the method of Bradford (1976) using the Bio-Rad microassay (Bio-Rad Laboratories, Munich, FRG). The final concentration of detergent in the assay was 0.05% Triton. Bovine serum albumin was used as standard protein. Small aliquots of concentrated virus were frozen in liquid nitrogen after addition of bovine serum albumin to a final concentration of 0.2%. For dequenching assays, aliquots were freshly thawed. They were kept on ice and used within the next 6–8 h without refreezing and thawing.

(B) Removal of HA. To remove the hemagglutinin coat from the virus surface, PR8 virus particles were treated with bromelain (Brand & Skehel, 1972). A virus suspension (65–80 μg of virus protein) was incubated in a final volume of 200 μL of Tris/HCl/EDTA buffer (100 mM Tris-HCl and 1 mM EDTA, pH 7.4) supplemented with 50 mM β-mercaptoethanol and 400 mg (4 units) of bromelain (EC 3.4.22.4; Sigma). The sample was incubated for about 16 h at 35 °C, thereafter cooled to 4 °C, and diluted 50-fold with ice-cold Tris/HCl/EDTA buffer (pH 7.4). The smooth-surfaced virus particles were separated from the solubilized hemagglutinin and neuraminidase molecules by centrifugation (200000*g*, 30 min, 4 °C). The residual hemolytic activity of the bromelain-treated virus was determined by using human erythrocytes (Yewdell et al., 1983). It was below 1% as compared to a control preparation of virus which had been incubated under identical conditions without bromelain. The diameter of bromelain-treated influenza viruses was determined by dynamic laser light scattering (Schurtenberger et al., 1983). A mean diameter of about 50 nm was measured. As compared to SUV prepared by detergent dialysis (see below), the virus population is much less homogeneous.

(C) Acid Inactivation. Acid inactivation of PR8 influenza virus (Yewdell et al., 1983) was performed by incubation in 10 mM PBS, pH 5.3, at 37 °C for increasing periods of time as indicated. The degree of inactivation was determined by measuring the hemolytic activity as described above. Already after 1-min acid incubation the hemolytic activity was reduced to below 1% of the control virus.

Liposomes. Small unilamellar liposomes (SUV) were prepared by the detergent dialysis method (Zumbühl & Weder, 1981) with sodium cholate as detergent, using a Liposomat (Diachema, Munich, FRG). The lipid to detergent ratio was always 0.6 (M/M). Stock solutions of the lipids in methanol/chloroform (1/1) were mixed in the indicated proportions (R18 included), and the solvent was evaporated, yielding a clear lipid film. Micelles were formed by shaking with 10 mM PBS, pH 7.4, and subsequently, cholate was removed by dialysis at room temperature for 18 h against the same buffer. Residual cholate was determined by use of [^{14}C]cholate (0.5 μCi/mL of micelles). It was less than 0.2%, corresponding to less than 1 detergent molecule per 280 lipid molecules (total lipid) in the liposomes. The recovery of lipid after dialysis as determined with ^{3}H-labeled PC (0.5 μCi/mL of micelles) was 90 ± 5%. The total final lipid concentration of all liposome preparations was 0.7–0.8 mg/mL. The lipid compositions were as follows: for "standard" liposomes, PC/PA/cholesterol/G_{D1a}/R18 = 0.57/0.07/0.21/0.06/0.09; for G_{D1a}-free liposomes, PC/PA/cholesterol/R18 = 0.57/0.07/0.21/0.09. Liposomes were filtered through 0.2-μm pore size filters and stored at 4 °C in the dark. They were used within 2 weeks. During this time and up to 4 weeks, preparations were stable as judged by macroscopic appearance and behavior in the dequenching assay. The diameter of the standard liposomes was measured by dynamic laser light scattering and found to be 26.7 ± 4.9 nm (≥99% of the vesicles). For G_{D1a}-free liposomes, the size was not significantly different. Calculated according to Huang and Mason (1978), the number of liposomes was about 1.2×10^{14}/mL.

For certain experiments, G_{D1a} was removed from standard liposomes by digestion with neuraminidase as follows: Thirty microliters of liposome stock solution was added to 240 μL of 10 mM PBS, pH 5.3, and the pH was adjusted to 5.3 with 0.1 N HCl (2 μL). The sample was brought to 37 °C and the reaction started by addition of 30 μL (0.03 unit) of neuraminidase (*Vibrio cholerae*; SERVA 30294). After 15-min incubation, 100 μL of the mixture was added to 400 μL of 10 mM PBS, pH 7.4, and the pH was brought up to 7.4 with 0.2 N NaOH (4 μL). For R18 dequenching assays, 15 μL of the neuraminidase-treated liposomes was incubated with 15 μL of PR8 viruses as usual (see below).

Fluorescence Measurements. For fluorescence measurements, a LS-5B spectrophotofluorometer (Perkin-Elmer) was used equipped with a thermostated circulating water bath. To avoid bleaching of the samples, the shutter was kept closed

between measurements. The excitation and emission wavelengths were 545 and 585 nm, respectively; the slit width was 10 nm. Quenching of the R18-liposome preparations was measured and calculated as described by Hoekstra et al. (1984). Measurements were related to a calibration standard which was obtained as follows: Liposomes were diluted into a 1% Triton X-100 solution in PBS, pH 7.4, which causes complete solubilization of the membranes and therefore maximal increase in fluorescence. Standard liposomes [R18/total lipid (PC, PA, cholesterol, G_{D1a}) = 0.10 (M/M)] showed 94.2 ± 3.5% quenching. G_{D1a}-free liposomes [R18/total lipid (PC, PA, cholesterol) = 0.11 (M/M)] showed 95.5 ± 0.7% quenching.

(*A*) *R18 Assay*. Interaction of R18-labeled liposomes with PR8 influenza virus was monitored by measuring the dequenching kinetics of R18 at pH 7.4 and 5.3, respectively. Temperature and pH were controlled during the incubation. A standard dequenching assay was run as follows: 15 μL of concentrated PR8 virus (corresponding to 10–12 μg of virus protein) and 15 μL of a 50-fold dilution of standard liposomes in PBS, pH 7.4 (corresponding to 210–240 ng of total lipid), were mixed and incubated on ice for 30 min. No dequenching occurred during this prebinding step (data not shown). Unless otherwise stated, prebinding of the fusion partners was routinely performed. The reaction was then started by addition of 25 μL of the incubation mixture to 975 μL of PBS, pH 7.4 or 5.3 (as indicated), which had been equilibrated in a water-jacketed cuvette to the desired temperature. The increase in fluorescence due to R18 dequenching was monitored as a function of time. The fluorescence intensity measured at 0 min was subtracted from all subsequent measurements as background. The measured intensities, which are directly proportional to the concentration of dequenched R18, were expressed as fraction of the fluorescence at infinite dilution. The latter value was obtained by dilution of R18-liposomes (same concentration as used in the assay) in 1% Triton X-100 after subtraction of Triton X-100 background fluorescence. This calibration standard was incubated in a parallel cuvette and periodically checked throughout the incubation time. The corrected dequenching, DQ(t), can take values between 0 and 1.

For dequenching assays with acid-inactivated PR8 virus, the standard procedure was modified as follows. No prebinding of liposomes and viruses was performed. Instead, 12.5 μL of virus suspension (about 8–10 μg of virus protein) was added to 975 μL of PBS, pH 5.3, prewarmed to 37 °C directly in the cuvette. After the indicated time intervals, the dequenching reaction was started by addition of 12.5 μL of a 50-fold dilution of standard liposomes (final liposomal lipid concentration 175–200 ng/mL). For dequenching assays at pH 7.4, the pH was adjusted with 7 μL of 1 N NaOH prior to addition of the liposomes, i.e., start of the reaction. Monitoring of the dequenching and calculations were performed as described above. Unless stated otherwise, the virus/liposome particle ratio was about 3/1 in all assays, corresponding to a lipid ratio (virus/liposome) of 12/1. Viral to liposomal lipid ratios were calculated on the basis that viral proteins and nucleic acids account for 75–80% and viral lipids for about 20% of the total weight (Barrett & Inglis, 1985).

(*B*) *Data Analysis*. Membrane interactions can be analyzed by using a mass action kinetic model (Nir et al., 1986). Fusion and lipid transfer are described by a sequence of kinetic processes. Among them, collision and aggregation are second order whereas fusion and transfer follow first-order kinetics. At least two additional first-order reactions have to be accounted for under certain conditions: virus inactivation at pH 5.3 (37 °C) and action of the endogenous viral neuraminidase at pH 7.4 (37 °C), the latter leading to the destruction of the virus receptor and thus to the abolishment of specific binding.

To determine the order of the dequenching reactions, we used the fractional-life period method (Frost & Pearson, 1961). This method was applied to single runs and also to two or more runs with different initial virus or liposome concentrations. In addition, by use of polynomial regression analysis (StatView^{512+TM} program; Brain Power, Inc., Calabasas, CA), dequenching curves were sequentially fitted to the following first- or second-order model-derived formulas:

first-order fit:

$$v = \mathrm{d(DQ)}/\mathrm{d}t = {}^1k(\mathrm{DQ_{max}} - \mathrm{DQ}) \quad (1)$$

$$v = C_1 - {}^1k\mathrm{DQ} \quad (2)$$

$$C_1 = {}^1k\mathrm{DQ_{max}}$$

second-order fit:

$$v = \mathrm{d(DQ)}/\mathrm{d}t = {}^2k(\mathrm{DQ_{max}} - \mathrm{DQ})^2 \quad (3)$$

$$v = C_2 - C_3\mathrm{DQ} + {}^2k\mathrm{DQ}^2 \quad (4)$$

$$C_2 = {}^2k\mathrm{DQ_{max}}^2$$

$$C_3 = 2{}^2k\mathrm{DQ_{max}}$$

The reaction velocity (v) was determined from the dequenching increments between successive time points. Rate constants ${}^1k$ or ${}^2k$, respectively, as well as $\mathrm{DQ_{max}}$, the asymptotically reached maximal dequenching, were calculated from the best fit. The choice between first- and second-order fits was based on residual analysis and on a higher correlation coefficient. The program also performs t tests to indicate the "goodness of the fit", and the respective p values are given in parentheses under Results.

The dimensions of the rate constants are $(\mathrm{time})^{-1}$ for ${}^1k$ and $(\mathrm{concentration})^{-1}(\mathrm{time})^{-1}$ for ${}^2k$. DQ and $\mathrm{DQ_{max}}$ are expressed as fractions of a standard; therefore, although they correspond to a certain quenched concentration of R18, these values are dimensionless. To recall this fact and to prevent mixing up of first- and second-order rate constants, the dimensions of ${}^2k$ are given as $(Q{\cdot}\mathrm{s})^{-1}$ in which Q stands for the quenched R18 concentration.

Results

R18 Dequenching Kinetics: Calculation of Rate Constants. Membrane interactions between virus receptor (ganglioside)-containing liposomes and influenza viruses were studied by measuring the dequenching kinetics of the R18 marker. As a standard procedure, G_{D1a}-containing R18-liposomes were preadsorbed to PR8 viruses for 30 min at 0 °C. The reaction was then started by diluting 25 μL of the preincubation mixture to 975 μL of prewarmed (37 °C) PBS, and dequenching was measured as described. Dequenching curves from incubations at pH 5.3 and 7.4, respectively, are shown in Figure 1A. At pH 5.3, a plateau of about 0.75 dequenching is reached within 30 min. At pH 7.4, dequenching is much slower; however, within 3–4 h, about the same maximal dequenching results as at low pH. Curves were analyzed with the fractional-life period method (see Materials and Methods) to determine n, the order of the reaction. The following values were obtained: $n = 1.95$ for pH 5.3 incubations and $n = 2.1$ for pH 7.4 incubations. This means that in both cases we deal with second-order processes. The same result ($n \approx 2$) was obtained when runs were analyzed in which the virus con-

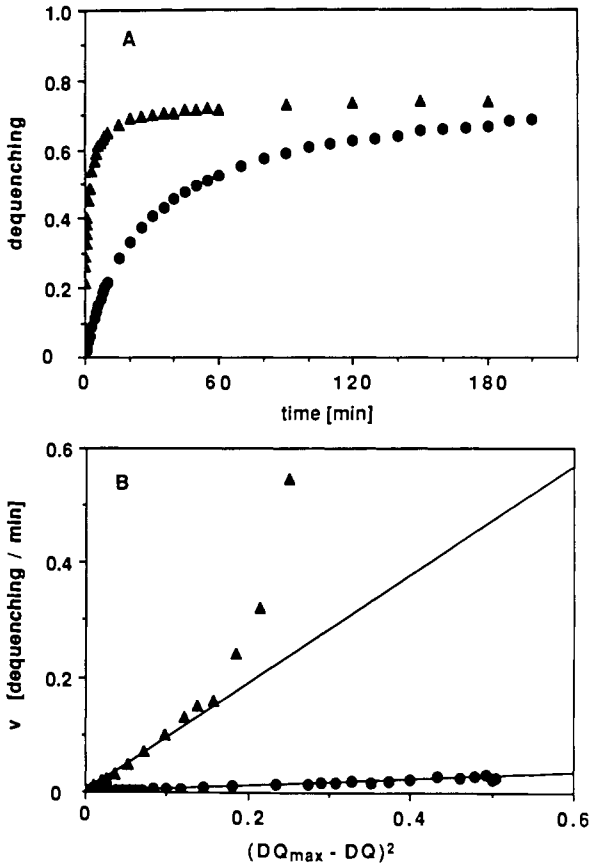

FIGURE 1: R18 dequenching upon interaction of PR8 influenza viruses with receptor (G_{D1a})-containing R18-liposomes. (Panel A) Standard liposomes were prebound to PR8 viruses at 0 °C for 30 min. The reaction was started by addition of 25 μL of the preincubation mixture to 975 μL of prewarmed (37 °C) PBS, pH 5.3 (▲) or pH 7.4 (●). R18 dequenching was measured as described under Materials and Methods. Final concentrations in the assay were 175–200 ng of liposomal lipids/mL and 8–10 μg of viral proteins/mL corresponding to about 2–2.5 μg of viral lipids. Data points are mean values from three (▲) or two (●) independent experiments. (Panel B) Data are calculated as described under Materials and Methods. The slopes correspond to the rate constant ^2k (see eq 3). Symbols as in panel A.

centration had been varied down to one-eighth of the standard conditions, i.e., to a lipid ratio of about 1.5/1 (virus/liposome), and also when the liposome concentration was varied between a lipid ratio of 3/1 and 45/1 (virus/liposome) (data not shown). Dequenching curves at both pH 5.3 and pH 7.4 were then fitted with polynomial regression analysis (see Materials and Methods), and the rate constants were calculated. For pH 7.4 incubations, the second-order fit (eq 4) showed a correlation of $r^2 = 0.9941$ ($p \leq 0.0001$) as compared to a first-order fit (eq 2) with $r^2 = 0.9571$. The second-order rate constant ^2k of the pH 7.4 reaction equals 0.942×10^{-3} $(Q \cdot s)^{-1}$, and DQ_{max} is 0.6828. These constants were obtained for the time interval between 5 and 200 min. If earlier times (0–5 min) are included, r^2 gets worse due to the low readings, but constants do not significantly change. For the dequenching curve at pH 5.3, a second-order fit yielded a very good correlation of $r^2 = 0.9951$ ($p \leq 0.0001$) for the time interval between 1 and 200 min (first-order fit for the same interval: $r^2 = 0.8956$). ^2k equals 16.52×10^{-3} $(Q \cdot s)^{-1}$, and DQ_{max} is 0.7157. Contrary to the pH 7.4 situation, the curve for pH 5.3 incubations shows a significant deviation from second-order kinetics during the first minute. The apparent ^2k value increases very steeply for $t \rightarrow 0$. This can best be illustrated by plotting the velocity v versus $(DQ_{max} - DQ)^2$ according to formula 3 (see Figure 1B). Due to the limited time resolution of our measuring device, statistical analysis of this initial phase is difficult. Approximation with a first-order fit (eq 2) yields an apparent rate constant of $^1k = 0.072$ s^{-1}. Extrapolation via the initial velocity provides us with an initial rate constant which is much higher ($^1k \approx 0.5$ s^{-1}). The implications of these findings will be discussed. To test the stability of R18 fluorescence in liposomes, they were incubated without PR8 viruses at 37 °C both at pH 5.3 and at pH 7.4. For both pH's, less than 0.5% dequenching were found up to 6 h (data not shown), indicating that the liposomes are very stable under the assay conditions. Two types of dequenching can thus be distinguished under prebinding conditions: a fast reaction at pH 5.3 and a slow reaction at pH 7.4, both reaching the same final level of dequenching. Both reactions can adequately be described as second-order reactions except for the initial phase at pH 5.3 where (a) first-order process(es) is (are) superposed.

If prebinding is omitted when standard liposomes are incubated with PR8 viruses, the dequenching kinetics at pH 5.3 can be described with a second-order reaction over the whole time range ($r^2 = 0.9980$). ^2k equals 17.53×10^{-3} $(Q \cdot s)^{-1}$, and DQ_{max} is 0.8114. These values are very similar to the ones found at times later than 2 min [$(DQ_{max} - DQ)^2 \leq 0.125$] for incubations with prebinding (see above). For incubations at pH 7.4, without prebinding, a ^2k value of 0.335×10^{-3} $(Q \cdot s)^{-1}$ is found ($r^2 = 0.9684$). This is about 3 times lower than the one determined with prebinding. In the following sections, we will analyze the nature of the fast and the slow dequenching reactions more closely.

Influence of pH on R18 Dequenching. To study the pH dependence of the dequenching reaction in more detail, incubations were performed at various pH values between 5.0 and 7.7. Standard liposomes were preadsorbed to PR8 virus in the cold as described above, and the reaction was started by diluting the preincubation mixture into PBS of the appropriate pH value. Incubations were performed at 30 °C. Dequenching was measured and ^2k calculated as described. Between pH 5.0 and 6.0, a 10-fold drop in the rate constant ^2k occurred (Figure 2A). No significant decrease was found for DQ_{max} over the whole pH range; therefore, the pH dependence also shows up in a rapidly decreasing initial velocity (v_i) as the pH value increases from 5 to 6 (Figure 2B). These results indicate that there is a pH-dependent transition from a fast dequenching at low pH values to a slow dequenching at higher pH values. It occurs in a very narrow range.

Influence of PR8 Virus Inactivation on R18 Dequenching. Fusion of influenza viruses with membranes only occurs in the presence of active hemagglutinin (HA) molecules on the virus particles (Maeda et al., 1981; White et al., 1982; Doms et al., 1985). Yewdell et al. (1983) demonstrated an irreversible loss of infectivity as well as hemolytic activity upon low pH treatment in the absence of a partner membrane, e.g., erythrocytes. Therefore, the influence of acid inactivation of PR8 virus on the dequenching kinetics of the liposome/virus interaction at pH 5.3 and 7.4 was studied. PR8 virus was treated with pH 5.3 buffer for 0, 1, 5, or 10 min as described under Materials and Methods, and the dequenching was measured at pH 5.3 and 7.4. For technical reasons, no preincubation of virus and liposomes was performed in this series of experiments (see Materials and Methods). The fast dequenching reaction occurring at pH 5.3 is slowed down dramatically if acid-treated virus is used for the assay (Figure 3). Already with 1-min acid inactivation a 5-fold reduction of the rate constant is observed (^2k values, see Table I), and after 10 min of acid pretreatment, the reaction is slowed down to the same

Table I: Rate Constants (k) for Various R18-Liposome/PR8 Virus Interactions

incubation	prebinding	$^2k \times 10^3$ [$(Q \cdot s)^{-1}$] pH 5.3	pH 7.4
standard liposomes × PR8 virus	+	16.52[a]	0.942
standard liposomes × PR8 virus	−	17.53	0.335
standard liposomes × PR8, 1 min acid-treated	−	2.62	ND[b]
standard liposomes × PR8, 5 min acid-treated	−	1.70	ND
standard liposomes × PR8, 10 min acid-treated	−	0.350	0.333
standard liposomes × bromelain-treated PR8 virus	+	0.477	0.430
neuraminidase-treated liposomes × PR8 virus	+	0.890[a]	0.552
G_{Dla}-free liposomes × PR8 virus	+	0.622[a]	0.630

[a] At $t > 2$ min (for $t < 30$ s; see text). [b] ND = not determined.

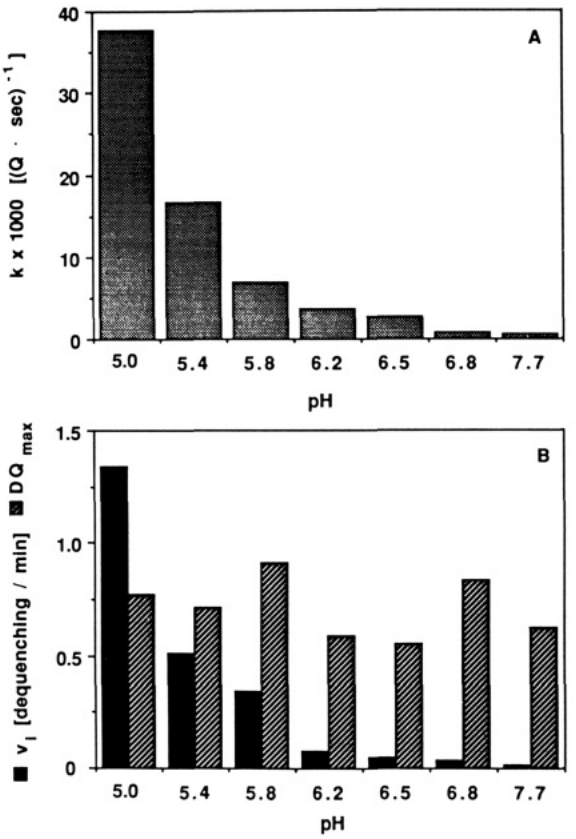

FIGURE 2: pH dependence of liposome/virus-mediated R18 dequenching. Standard liposomes and PR8 viruses were incubated at the indicated pH values. Experimental conditions as described in Figure 1 except that the incubation temperature was 30 °C. (Panel A) Rate constants ^2k as a function of pH. (Panel B) DQ_{max} and v_i as a function of pH. Rate constants ^2k and DQ_{max} were calculated as described under Materials and Methods. Initial velocities v_i were calculated by using formula 3 with $t \rightarrow 0$. Data are from one set of experiments. They were confirmed in a second, independent set.

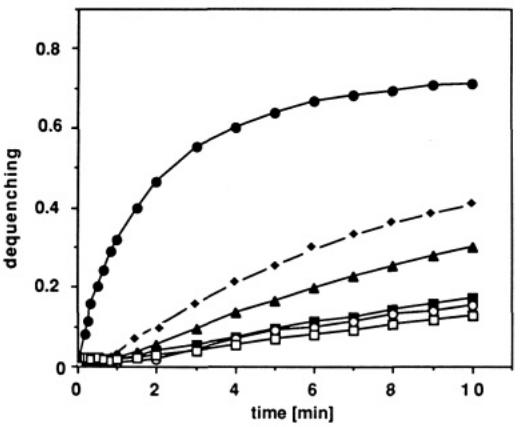

FIGURE 3: Influence of acid inactivation of PR8 virus on R18 dequenching. PR8 virus was incubated in low-pH buffer (PBS, pH 5.3) at 37 °C for 0 (●, ○), 1 (♦), 5 (▲), and 10 min (■, □), and immediately afterward used for dequenching assays (37 °C) with standard liposomes at pH 5.3 (closed symbols) or pH 7.4 (open symbols). For the latter, treated virus was neutralized with NaOH (see Materials and Methods) before the dequenching reaction was started. Data are from one set of experiments. They were confirmed in two independent sets. Rate constants ^2k, see Table I.

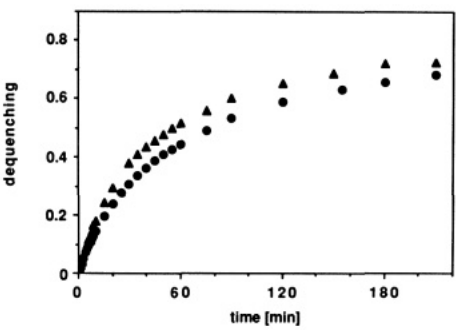

FIGURE 4: Influence of bromelain treatment of PR8 virus on R18 dequenching. Bromelain-treated PR8 viruses (see Materials and Methods) were used for dequenching assays (37 °C) with standard liposomes at pH 5.3 (▲) and pH 7.4 (●). Experimental conditions as described in Figure 1. Data are means of two independent experiments.

^2k range as found with pH 7.4 incubations of standard liposomes with untreated virus. No significant difference was found between the dequenching at pH 7.4 with acid-treated virus as compared to untreated control virus. The DQ_{max} values were not significantly reduced by the acid inactivation (range 0.769–0.944). The fast dequenching reaction occurring at pH 5.3 can thus be abolished by inactivation of the hemagglutinin molecules, whereas no effect is found on the slow dequenching process occurring at pH 7.4.

Influence of Bromelain Treatment of Virus on the Fast and Slow Dequenching Reactions. Treatment of PR8 virus with the protease bromelain has been reported to remove hemagglutinin as well as neuraminidase from the virus surface (Compans et al., 1970). The residual virus particles are completely devoid of the characteristic spike proteins. To study the role of hemagglutinin in the fast dequenching reaction, bromelain-treated PR8 viruses were used for incubations with standard liposomes both at pH 5.3 and at pH 7.4 (Figure 4). The fast reaction usually observed at pH 5.3 was abolished, and the dequenching kinetics were very similar for both pH values. When eq 4 was used, the following rate constants were determined: $^2k = 0.663 \times 10^{-3}$ $(Q \cdot s)^{-1}$ ($r^2 = 0.9665$) at pH 5.3 and $^2k = 0.437 \times 10^{-3}$ $(Q \cdot s)^{-1}$ ($r^2 = 0.9714$) at pH 7.4. The DQ_{max} values were 0.7826 and 0.7961, respectively. At pH 5.3 as well as pH 7.4, only the slow type of dequenching thus occurs with bromelain-treated viruses. Together with the data from the acid inactivation of the virus hemagglutinin, this

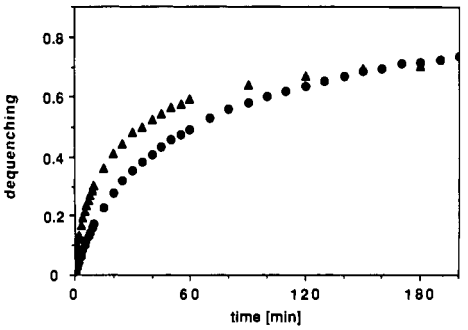

FIGURE 5: Influence of neuraminidase treatment of liposomes on R18 dequenching. Liposomes were treated with neuraminidase (see Materials and Methods) and incubated at 37 °C under standard conditions (see Figure 1) with PR8 virus at pH 5.3 (▲) and pH 7.4 (●). Data are means of two independent experiments.

result strongly suggests that a slow and HA-independent dequenching reaction takes place under conditions in which active fusion is abolished.

Influence of G_{D1a} Virus Receptor on R18 Dequenching. To study the influence of the G_{D1a} ganglioside virus receptor on the fast and slow dequenching reaction, two types of liposomes devoid of G_{D1a} virus receptor were prepared: On one hand, standard liposomes were digested with neuraminidase to remove the sialic acid residues (see Materials and Methods), and on the other hand, liposomes were prepared which had no G_{D1a} incorporated from the beginning (see Materials and Methods). Both types were used in standard R18 assays with prebinding for 30 min at 0 °C. Figure 5 shows the dequenching curves obtained with neuraminidase-treated liposomes. Rate constants are ${}^2k = 0.532 \times 10^{-3}$ $(Q \cdot s)^{-1}$ ($r^2 = 0.9908$) for incubation at pH 7.4 and ${}^2k = 1.058 \times 10^{-3}$ $(Q \cdot s)^{-1}$ ($r^2 = 0.9870$) for incubations at pH 5.3. Results from incubations with G_{D1a}-free liposomes are not significantly different (rate constants ${}^2k$ are listed in Table I). The curves obtained are very similar to those obtained with bromelain-treated viruses (Figure 4) except that during the initial phase of the pH 5.3 reaction dequenching in the case of neuraminidase-treated viruses seems to be faster. Analysis of the dequenching curves by formula 3 shows a tendency for an increase in the apparent ${}^2k$ value for G_{D1a}-free and neuraminidase-treated liposomes, but not for the bromelain experiment (data not shown). The deviation from second-order kinetics is not as pronounced as in the case of pH 5.3 incubations of standard liposomes and viruses after prebinding. In an effort to quantitate this effect, initial velocities were extrapolated and compared. For G_{D1a}-free and neuraminidase-treated liposomes, initial velocities were between 0.04 and 0.07 per minute, whereas for incubations with bromelain-treated viruses it was between 0.01 and 0.02 per minute. The implications of these findings will be discussed.

Discussion

Kinetics of membrane interactions between virus receptor (G_{D1a})-containing liposomes and influenza viruses have previously been determined by using a fluorescence resonance energy-transfer (RET) method (Stegmann et al., 1985, 1989) or the R18 fluorescence assay (Hoekstra et al., 1984). Efforts concentrated on the process at low pH, under which condition specific fusion of influenza viruses with target membranes is well documented (Matlin et al., 1981; White et al., 1983). As the issue of a potential fusion process at neutral pH is still controversial (Haywood & Boyer, 1985; Stegmann et al., 1986), we undertook to analyze the kinetics of the dequenching reaction at pH 7.4 in parallel to pH 5.3. In order to obtain reproducible measurements also at neutral pH, where the reaction runs much more slowly, the octadecylrhodamine B chloride (R18) membrane marker was used as follows. R18 was incorporated into liposomes, which were prepared by a controlled detergent dialysis procedure (Zumbühl & Weder, 1981). With this method, a relatively homogeneous population of small unilamellar vesicles is produced, in which all lipid components, marker included, are evenly and reproducibly distributed. This approach avoids the problems of apparently nonuniform incorporation of the R18 marker into virus particles which had been encountered with external labeling (Hoekstra et al., 1984). In addition, incubation conditions were chosen such that between two and five virus particles per liposome were present. As Nir et al. (1986) have demonstrated, several liposomes can fuse with one virus particle but only one virus particle with one liposome. Therefore, under our assay conditions, only one round of fusion can occur. Taking into account the respective diameters of liposomes and viral lipid vesicles, i.e., bromelain-treated viruses, a one to one fusion leads to a 5–6-fold dilution of R18. This results in a residual quenching of about 0.2 (quench curve not shown). Indeed, between 0.7 and 0.9 of maximal dequenching was found in all our assays. Stegmann et al. (1989) have demonstrated that there is a subpopulation of inactive viruses unable to fuse. The fact that we find the same amount of maximal dequenching at pH 5.3, with fusion taking place, and at pH 7.4, where no fusion is possible (see below), makes it unlikely that our residual quenching is due to blockage of liposomes with inactive virus.

According to these standard conditions, the dequenching reactions at pH 5.3 and 7.4 were quantitatively followed. In both cases, the same amount of maximal dequenching was reached, although at pH 5.3 this was achieved within minutes, whereas at pH 7.4 it took 3–4 h. The important question is now whether the dequenching reactions at acidic and neutral pH have the same molecular basis. Theoretically, R18 dequenching can result from either fusion of labeled membranes with unlabeled membranes or transfer of individual R18 molecules from a labeled to an unlabeled membrane. To distinguish between the two possibilities, the role of the hemagglutinin molecules in the respective reactions was first studied. It is well established that the specific fusion activity of the influenza virus is mediated by the hemagglutinin glycoprotein (Maeda et al., 1981; White et al., 1982; Doms et al., 1985; Stegmann et al., 1987). As we demonstrate, the fast dequenching reaction at pH 5.3 described in this paper depends on the presence of intact, active hemagglutinin molecules. The first evidence for this was obtained by using acid-inactivated influenza viruses. The fusion activity of influenza viruses is known to be irreversibly abolished by acid inactivation of the HA molecules in the absence of a fusion partner (White et al., 1982; Sato et al., 1983; Stegmann et al., 1986). The second evidence stems from experiments performed with bromelain-treated influenza viruses, which are devoid of the HA molecules. Inactivation as well as removal of the HA molecules was found to suppress the fast dequenching. The third evidence for a correlation of the fast reaction at low pH with the HA-mediated fusion activity was obtained from studies on the pH dependence of the R18 dequenching process. The 10-fold drop in ${}^2k$ observed in the present study corresponds well to the pH dependence of the influenza virus fusion activity described by other groups (Matlin et al., 1981; White et al., 1982). In addition, measuring the hemolytic activity of the PR8 influenza virus strain, Yewdell et al. (1983) found a drastic decrease in the same pH range. All evidence thus

indicates that the fast dequenching reaction observed at acidic pH indeed reflects the HA-mediated, specific influenza virus fusion process. These findings correspond well to the data on specific fusion obtained by Stegmann et al. (1986) with either erythrocyte ghosts or ganglioside-containing liposomes. There was no indication for an additional, "non physiological" fusion process as found with cardiolipin liposomes by Stegmann et al. (1989). The reaction occurring at neutral pH could clearly be shown to be independent of viral hemagglutinin.

To get more information about the fast and slow dequenching process, the respective curves were kinetically analyzed. Both showed second-order characteristics which implicates that both are collision-mediated. For the fast dequenching reaction, this means that not the actual fusion process but rather specific receptor binding is rate-limiting. Theoretically, the rate-limiting collision step could be overcome by increasing the virus and liposome concentrations (Nir et al., 1986). With a 6-fold increase of both incubation partners, we still found second-order kinetics (data not shown). Only by use of prebinding of viruses and liposomes at high concentrations (see Materials and Methods), first-order kinetics were detectable at least during the initial phase at pH 5.3. By extrapolation from data in Figure 1B, an initial rate constant ^1k of 0.5 s^{-1} is obtained which corresponds very well to the fusion constant reported by Stegmann et al. (1989) for G_{D1a}- and cholesterol-containing liposomes. The apparent rate constant then drops off very quickly until at about 2 min after the start of the reaction specific binding is rate-limiting also in this case. Due to the limited time resolution of our measuring device, very fast reactions like the fusion process cannot directly be studied. From curve simulations (not shown), we got indirect evidence that the experimental values obtained must result from the superposition of the fusion reaction (first order) with specific binding (second order) and at least one additional first-order process with a rate constant ^1k in the range of 0.05 s^{-1}. A possible candidate is virus inactivation which is known to occur at low pH and 37 °C with any unbound virus ($^1k \approx 0.03$ s^{-1} estimated from Figure 3). This can be demonstrated under conditions in which specific binding is abolished, i.e., with G_{D1a}-free or neuraminidase-treated liposomes. Fusion is drastically reduced in these cases and only shows up in elevated initial velocities as compared to the HA-independent R18 dequenching. As mentioned above, analysis of the slow reaction reproducibly revealed second-order kinetics. Rate constants were about 20 times lower than for the fast dequenching, indicating that not the same type of collision is rate-limiting for both. As van Meer and Simons (1983) have demonstrated, virus receptors are very quickly destroyed by viral neuraminidase at pH 7.4 (37 °C) and the virus particles released ($^1k \approx 0.2$ s^{-1}). Under our incubation conditions, digestion of G_{D1a} will readily occur. As a result, the rate constants found for the slow dequenching processes reflect the nonspecific collision between viruses and liposomes devoid of receptor molecules. The fact that rate constants for the slow process vary within a factor of 2.5 (see Table I), depending on pH and incubation partners, indicates that electrostatic interactions govern the nonspecific collisions.

Having demonstrated that the slow R18 dequenching process is not only independent of the presence of the viral HA but also of the virus receptor, we think we can exclude the occurrence of a specific fusion at neutral pH between G_{D1a}-containing liposomes and influenza viruses which had been postulated by Haywood and Boyer (1985). The slow dequenching reaction at neutral pH can best be explained with nonspecific lipid transfer of R18. Lipid transfer or lipid exchange between bilayers has been described for various membrane components like phosphatidylcholine (Gardam et al., 1989; Nichols & Pagano, 1982), cholesterol (Fugler et al., 1985), and others. For R18, such a process has not been described up to now. Hoekstra et al. (1984), when introducing R18 as a marker for membrane interactions, checked for spontaneous transfer between R18-labeled and unlabeled large unilamellar vesicles or erythrocyte ghosts. No evidence was found for a fusion-independent R18 dequenching under these circumstances. The most likely explanation for the apparent discrepancy between their results and ours lies in the size of the incubation partners. As has been shown with cholesterol, transfer from SUV is much faster than transfer from LUV (McLean & Phillips, 1984; Thomas & Poznansky, 1988). In comparison to the LUV and ghosts used by Hoekstra et al. (1984), the diameters of our incubation partners are small, about 25-nm diameter for the SUV and about 50-nm diameter for the viruses (only the lipid envelopes, measured with bromelain-treated influenza viruses). In our case, R18 transfer was also demonstrated to occur from R18-labeled SUV to unlabeled ones (data not shown). All these data can be interpreted to indicate that the curvature of the interacting partners plays an important role in lipid transfer or exchange processes. To elucidate the underlying mechanisms, the conditions which might influence R18 transfer, like the size of the interacting particles and also the lipid composition of the partner membranes, are now under study. In view of the use of liposomes as specific drug carriers, nonspecific lipid transfer is an important parameter to be controlled. Lipid carriers cannot a priori be considered as inert vectors unless the necessary controls have been performed.

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Effect of Partial Delipidation of Purple Membrane on the Photodynamics of Bacteriorhodopsin[†,‡]

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ABSTRACT: The effect of lipid-protein interaction on the photodynamics of bacteriorhodopsin (bR) was investigated by using partially delipidated purple membrane (pm). When pm was incubated with a mild detergent, Tween 20, the two major lipid components of pm, phospholipids and glycolipids, were released in different ways: the amount of phospholipids released was proportional to the logarithm of the incubation time; the release of glycolipids became noticeable after the release of ~2 phospholipids/bR, but soon leveled off at ~50% of the initial content. It was found that the thermal decay of the photocycle intermediate N_{560} was inhibited by the removal of less than 2 phospholipids per bR. This inhibition was partly explained by an increase in the local pH near the membrane surface. More significant changes in the bR photoreactions were observed when >2 phospholipids/bR were removed: (1) the extent of light adaptation became much smaller, and this reduction correlated with the release of glycolipids; (2) N_{560} became difficult to detect; (3) the M_{412} intermediate, which is characterized by a pH-insensitive lifetime, was replaced by a long-lived M-like photoproduct with a pH-sensitive lifetime. The heavy delipidation apparently altered the mechanism by which the deprotonated Schiff base receives a proton. An important conformational change in the protein moiety is suggested to take place during the M_{412} state, this conformational change being inhibited in the rigid lipid environment.

Bacteriorhodopsin (bR),[1] the sole protein in the purple membrane (pm) of *Halobacterium halobium*, functions as a light-driven proton pump. [for reviews, see Stoeckenius et al. (1979), Khorana (1988), Kouyama et al. (1988a), and Rothschild (1988)]. In the living cells and isolated pm fragments, bR exists in two interconvertible isomers, one containing *all-trans*- and the other 13-*cis*-retinal. The equilibrium between them is slowly established in the dark (dark-adapted state). The trans isomer (bR_{570}) accumulates rapidly in the light (light-adapted state), and its cyclic photoreaction drives the translocation of proton from the cytoplasmic side to the outside. At low light intensity, the trans photocycle is approximated by the scheme (Kouyama et al., 1988b; Lozier et

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[1] Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tween 20, poly(oxyethylene) (20) sorbitan monolaurate; DOC, deoxycholic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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