Evaluation of Viral Membrane Fusion Assays. Comparison of the Octadecylrhodamine Dequenching Assay with the Pyrene Excimer Assay†

Toon Stegmann,*,‡ Pieter Schoen,§ Romke Bron,§ Josef Wey,‡ Ingrid Bartoldus,‡ Antonio Ortiz,§,‖ José-Luis Nieva,§,⊥ and Jan Wilschut§

Department of Biophysical Chemistry, Biocenter of the University of Basel, Klingelbergstrasse 70, CH 4056 Basel, Switzerland, and Department of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

Received March 24, 1993; Revised Manuscript Received August 17, 1993®

ABSTRACT: Membrane fusion, in particular the fusion of enveloped viruses, is often measured with an assay based on octadecylrhodamine (R18) fluorescence dequenching. We have studied the association of R18 with membranes and used the R18 assay to measure virus fusion in model systems and in cultured cells. The results were compared with those of an assay based on the decrease in excimer fluorescence of pyrene-labeled phospholipids. For liposomes made from premixed R18 and phosphatidylcholine (PC), R18 fluorescence quenching was proportional to the concentration of the probe up to about 4 mol %. No quenching was found at very low concentrations of R18. However, various artificial and biological membranes labeled by the addition of R18 from an ethanolic solution showed significant quenching at such low R18 concentrations. Thus, some of the R18 was not randomly distributed but likely was associated with the surface of the membranes in the form of highly quenched clusters or micelles. Moreover, in influenza virus membranes, R18 appeared highly quenched at very low concentrations, indicative of the probe interacting with viral proteins. In contrast, pyrene-labeled PC incorporated in either liposomes or reconstituted viral membranes (virosomes) showed an excimer/monomer fluorescence ratio proportional to the concentration of probe. When intracellular membrane fusion was investigated with R18-labeled influenza virus or Semliki Forest virus (SFV), fluorescence dequenching was observed in the absence of fusion, most likely due to spontaneous probe exchange. On the other hand, both SFV, metabolically labeled with pyrene–phospholipids, and influenza virosomes, containing pyrene-labeled PC, showed a decrease in pyrene excimer fluorescence only under conditions permitting fusion with the endosomal membrane. These data indicate that pyrene-labeled lipids, in contrast to R18, are not susceptible to spontaneous exchange. Fusion of influenza virosomes, containing both R18 and pyrene–PC, with erythrocyte ghosts showed that the highly quenched population of R18 can be transferred to the donor membrane; however, the transfer of R18 occurred more slowly than the transfer of pyrene–lipids. The infectivity of metabolically labeled SFV was unaffected. Thus, although the R18 assay gives satisfactory results in many *in vitro* systems, the pyrene assay is better suited for a quantitative assessment of viral membrane fusion.

During the last decade, membrane fusion research has been greatly facilitated by the development of lipid mixing assays based on the use of fluorescent probes. In one type of assay, membranes are labeled with a pair of fluorescent phospholipid analogues. Upon fusion of the labeled with unlabeled membranes, dilution of the probes occurs, resulting in decreased resonance energy transfer between the donor and acceptor fluorophores. The decrease can be measured continuously and is, under certain conditions, proportional to the extent of fusion (Struck et al., 1981; Uster & Deamer, 1985; Silvius et al., 1987). In another type of assay, fusion is monitored as the relief of self-quenching of a fluorescent probe initially incorporated in the donor membrane. By far the most commonly used of these assays is the one based on octadecylrhodamine B (R18)[1] (Hoekstra et al., 1984).

The use of R18 for the measurement of virus-induced cell-cell fusion was first described by Keller et al. (1977). The probe rapidly associates with membranes if added from an ethanolic solution (Hoekstra et al., 1984). Therefore, in contrast to the probes used for the resonance energy transfer assay described above, it can be used to label intact biological membranes. Thus, it allows one to study the fusion between biological membranes both *in vitro* and *in vivo*, such as fusion of enveloped viruses with cells (Stegmann et al., 1987b; Blumenthal et al., 1987; Gilbert et al., 1990; Srinivasakumar et al., 1991; Di Simone & Baldeschwieler, 1992). In spite of its widespread use, several aspects of the R18 assay deserve further attention. Most importantly, there is evidence that the probe can exchange spontaneously between membranes,

† This study was supported by the Swiss National Science Foundation (Grant No. 31-33512.92 to T.S.), the Du Pont-Merck Pharmaceutical Co. (Wilmington, DE; research grant, supporting P.S., to the University of Groningen), the Netherlands Organization for Scientific Research NWO (fellowship to R.B. under the auspices of the Netherlands Chemical Foundation SON), the European Molecular Biology Organization (long-term fellowship to A.O.), the Spanish Government (fellowship to J.L.N.), the U.S. National Institutes of Health (Research Grant AI 22534 to J.W.), and the Concerted Action Programme "Interaction of HIV proteins with the cell membrane" of the European Community.

* Author to whom correspondence should be addressed.

‡ Biocenter of the University of Basel.

§ University of Groningen.

‖ Present address: CEBAS (CSIC), P.O. Box 4195, 30080 Murcia, Spain.

⊥ Present address: Department of Biochemistry, University of the Basque Country, P.O. Box 644, 48080 Bilbao, Spain.

® Abstract published in *Advance ACS Abstracts,* October 1, 1993.

[1] Abbreviations: $C_{12}E_8$, octaethylene glycol *n*-dodecyl monoether; E/M, excimer-to-monomer fluorescence ratio of pyrene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; MDCK, Madin Darby canine kidney; pyrPC, pyrene-labeled phosphatidylcholine; PC, phosphatidylcholine; LUV, large unilamellar vesicles; *Q*, quenching; R18, octadecylrhodamine B; SFV, Semliki Forest virus.

0006-2960/93/0432-11330$04.00/0

which may lead to a false positive fusion signal (Wunderli-Allenspach & Ott, 1990; Comerford & Dawson, 1991; Di Simone & Baldeschwieler, 1992; Puri et al., 1992). In addition, the precise mode of association of R18 with membranes is unknown. Furthermore, the mechanism of self-quenching is not completely understood, although it appears to be mainly due to energy transfer without emission to a nonfluorescent dimer species (MacDonald, 1990). Moreover, in several membranes, especially those containing cholesterol, R18 is much more self-quenched than can be expected on the basis of its concentration (MacDonald, 1990; Loyter et al., 1988; Blumenthal et al., 1987). Finally, it was suggested recently that the biological activities of influenza virus, such as infectivity, may be affected significantly by R18 labeling (Wunderli-Allenspach et al., 1993).

We have investigated the mode of association of R18 with various artificial and biological membranes. It is shown that, depending on the mode of addition of R18 to the membranes and on the type of the membrane used, R18 can be associated with membranes in several different ways. For some membranes, this means that different populations of the probe coexist after labeling, each with different extents of quenching. In some cases, at least part of the R18 was found to be susceptible to transfer to other membranes by nonfusion mechanisms. The usefulness of the R18 assay for measuring fusion induced by viral proteins was compared to that of a fusion assay based on pyrene-labeled phospholipids (Galla & Hartmann, 1980). Enveloped viruses produced from cells that were cultured in the presence of pyrene-labeled fatty acids have pyrene-labeled lipids in their membranes (Pal et al., 1988; Wahlberg et al., 1992; Bron et al., 1993). The pyrene fluorophore forms fluorescent excimers, representing dimers of a molecule in the ground state and a molecule in the excited state (Galla & Hartmann, 1980). The formation of pyrene excimers is dependent on the concentration of the probe, and in membranes, the excimer-to-monomer (E/M) fluorescence ratio is proportional to the surface density of pyrene-labeled lipids. We found that pyrene-labeled phosphatidylcholine (pyrPC), incorporated in various membranes, is present as a homogeneous population. Fusion of pyrene-labeled viruses or reconstituted virosomes with various fusion targets, including cultured cells, could be followed conveniently as a decrease in the excimer fluorescence. In *in vitro* fusion systems, there is no indication of spontaneous transfer of individual pyrene-labeled lipid molecules between donor and acceptor membranes. Moreover, in cells, excimer decrease occurs in the absence, but not in the presence of lysosomotropic weak bases upon the endocytosis of pyrene-labeled Semliki Forest virus (SFV), indicating that *in vivo* pyrene-labeled lipids do not exchange spontaneously either.

MATERIALS AND METHODS

Chemicals. Egg phosphatidylcholine (PC) from Avanti Polar Lipids, Inc. (Alabaster, AL) was used without further purification. Octadecylrhodamine B chloride (R18), 1-palmitoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (pyrPC), and 1-pyrenehexadecanoic acid were from Molecular Probes (Eugene, OR). All other chemicals were of the highest grade available.

Virus. The recombinant strains of influenza virus X-31 [propagated from a single plaque, C-22 (Doms et al., 1986)] and NIB26 were grown in the allantoic cavity of embryonated eggs, isolated, handled, and stored essentially as described previously (Stegmann et al., 1985), except that after sedimentation from the allantoic fluid the virus was further purified by sucrose density gradient centrifugation, as described for SFV. SFV was grown on BHK-21 cells in 900 cm^2 roller bottles (Costar Corp., Cambridge, MA). After the removal of cell debris at 2500*g* for 30 min at 4 °C, the virus was sedimented from the medium at 50000*g* for 4 h at 4 °C in a Ti19 rotor (Beckmann, Palo Alto, CA). Pellets were resuspended in 150 mM NaCl, 5 mM Hepes, and 0.1 mM EDTA (pH 7.4), loaded atop a linear 20–60% sucrose gradient in the same buffer, and centrifuged at 100000*g* for 16 h. The virus-containing band was sampled and stored at −80 °C. Viral phospholipid was determined, after extraction of the lipids (Bligh & Dyer, 1959), by phosphate analysis (Böttcher et al., 1961). Pyrene labeling of SFV was carried out as described (Bron et al., 1993). Briefly, BHK-21 cells were grown for 48 h on medium containing 10 μg/mL pyrene-labeled hexadecanoic acid and subsequently infected with SFV. After an additional 24 h, labeled progeny virus was harvested and purified as above.

Liposomes and Virosomes. Liposomes (large unilamellar vesicles, LUV) were prepared in 150 mM NaCl, 5 mM Hepes, and 0.1 mM EDTA (pH 7.4) by repeated high-pressure extrusion of multilamellar liposomes through polycarbonate filters: three times through 0.4-μm pores and five times through 0.2-μm pores (Mayer et al., 1986). Multilamellar liposomes, prepared by hydration of lipid films exhaustively dried under vacuum, were frozen and thawed 3–5 times before extrusion. After extrusion, residual multilamellar liposomes were removed by centrifugation at 16000*g* for 20 min. Liposomal phospholipid concentrations were determined by phosphate analysis (Böttcher et al., 1961). Reconstituted viral envelopes (virosomes) from NIB26 influenza virus were prepared as described (Stegmann et al., 1987a). SFV virosomes were prepared by an adaptation of this method. Briefly, the virus was sedimented by ultracentrifugation and solubilized with 100 mM C12E8 (Nikko Chemical Co., Tokyo, Japan), the nucleocapsids were removed by ultracentrifugation, and the supernatant (containing the viral envelope proteins and lipids) was shaken with BioBeads SM-2 (Bio-Rad, Richmond, CA) to remove the detergent. Subsequently, the virosomes were purified by centrifugation on a 10–50% discontinuous sucrose gradient at 130000*g* for 90 min at 4 °C (SW 50.1 rotor, Beckmann, Palo Alto, CA). Incorporation of pyrPC or R18 in the reconstituted influenza or SFV virosomes was achieved by adding the detergent-solubilized viral membranes to the dry fluorophore before reconstitution.

Erythrocyte Ghosts. Erythrocyte ghosts (blood type AB, rhesus factor positive) were prepared from outdated red blood cell concentrates by the method of Steck and Kant (1974). Ghost phospholipid content was measured after extraction of the lipids (Bligh & Dyer, 1959) by phosphate analysis (Böttcher et al., 1961). Membrane fusion between influenza virosomes, labeled with R18 or pyrPC, and ghosts was measured as described below.

Exogenous Labeling of Membranes with R18. Membranes were labeled with R18 by injection of an ethanolic solution of R18 into an aqueous buffer containing the membranes under vigorous agitation, followed by incubation for at least 15 min at room temperature. The final concentration of ethanol did not exceed 1%. Unincorporated R18 was then removed on a Sephadex G50 (Pharmacia, Uppsala, Sweden) column (30 × 1 cm), during which procedure the unincorporated fluorophore binds to the column material, or by repeated centrifugation of the membranes (see Results).

Cells. MDCK (Madin Darby canine kidney) cells were grown in Dulbecco's Modified Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum, 100 μg/mL penicillin, 100 units/mL streptomycin, and 2 mM

glutamine (all from Gibco, Paisley, UK). Before labeling with R18, cells were removed from the dishes by trypsinization. Sup T1 cells (a human T cell line) were grown in a suspension in RPMI 1640 supplemented with 10% fetal calf serum, 100 μg/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine (Gibco). BHK-21 cells were grown in Glasgow's Modification of Eagle's Minimal Essential Medium (Flow, Irvine, UK) supplemented with 10% tryptose phosphate broth (Difco Laboratories, Detroit, MI), 5% fetal calf serum, and 2 mM glutamine.

Fluorescence Measurements. Measurements were carried out with continuous stirring in a Jasco FP 777, Schoeffel RRS 1000, or SLM/Aminco Aminco Bowman Series 2 fluorometer, equipped with a thermostated cuvette holder containing 2 mL (final volume) of either 150 mM NaCl and 5 mM Hepes (pH 7.4), or in the case of *in vivo* fusion with intact cells, Hanks/Hepes: 137 mM NaCl, 5.4 mM KCl, 0.40 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.41 mM $MgSO_4$, 0.40 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 10 mM Hepes (pH 7.4). For the fusion of influenza virosomes with erythrocyte ghosts, the medium was acidified to pH 5.1 by the injection of 100 μL of pretitrated 0.4 M Mes and 0.3 M trisodium citrate. R18 fluorescence was measured continuously at excitation and emission wavelengths of 560 and 590 nm, respectively. For calibration of the fluorescence scale in fusion experiments, the initial fluorescence of the labeled membranes was set to zero and the fluorescence at infinite probe dilution at 100%. The latter value was obtained after addition of Triton X-100 (0.5% v/v). Quenching (Q) was calculated according to $Q = 1 - F/F_\infty$, where F is fluorescence and F_∞ is the fluorescence after addition of Triton X-100. Pyrene fluorescence was determined at an excitation wavelength of 345 nm and emission wavelengths of 397 (monomer) and 480 nm (excimer). Fusion extents (f) were calculated as $f = 100(E_0 - E)/(E_0 - E_\infty)$, where E represents the (excimer) fluorescence intensity at 480 nm, and E_0 and E_∞ represent the intensities at 480 nm at time zero and after the addition of C12E8 (final concentration 10 mM), respectively. Fusion curves were traced manually from the original strip chart recordings using a digitizer pad (Sumagraphics, Fairfield, CT) and a drawing program (Canvas, Deneba Software, Miami, FL) or processed directly using the software provided with the Aminco Bowman Series 2 fluorometer.

RESULTS

Self-Quenching of R18 in Liposomes Prepared from Premixed Lipid Films. To investigate the quenching of R18 in pure PC liposomes, various amounts of R18 were mixed with 1 μmol of PC in chloroform/methanol (2:1). The solvent was evaporated under a stream of nitrogen, and the lipid films were dried exhaustively under vacuum. LUV were produced as described in Materials and Methods. It is likely that under these conditions all the R18 is incorporated in the liposomal membranes. Figure 1A shows the quenching of the fluorescence vs the mole fraction of R18 with respect to PC. As reported previously, (Hoekstra et al., 1984; Johansson & Niemi, 1987; MacDonald, 1990), at least up to 4 mol % R18 the quenching was linearly proportional to the concentration of the probe (squared regression coefficient 0.99). Extrapolated to 0% R18, the quenching was zero, indicating that it is caused only by R18 itself ("self-quenching"). These data confirm that, for these membranes, there is a range of concentrations within which the quenching of R18 is linear with the probe concentration and that the quenching disappears at infinitely low probe concentrations (Hoekstra et al., 1984; Johansson & Niemi, 1987; MacDonald, 1990). It should be noted that, in liposomes with compositions other than pure PC, even in the range of 0–4 mol % R18 more pronounced quenching of R18 and, consequently, deviations from a linear relationship between quenching and probe concentration have been observed. These include vesicles containing cholesterol

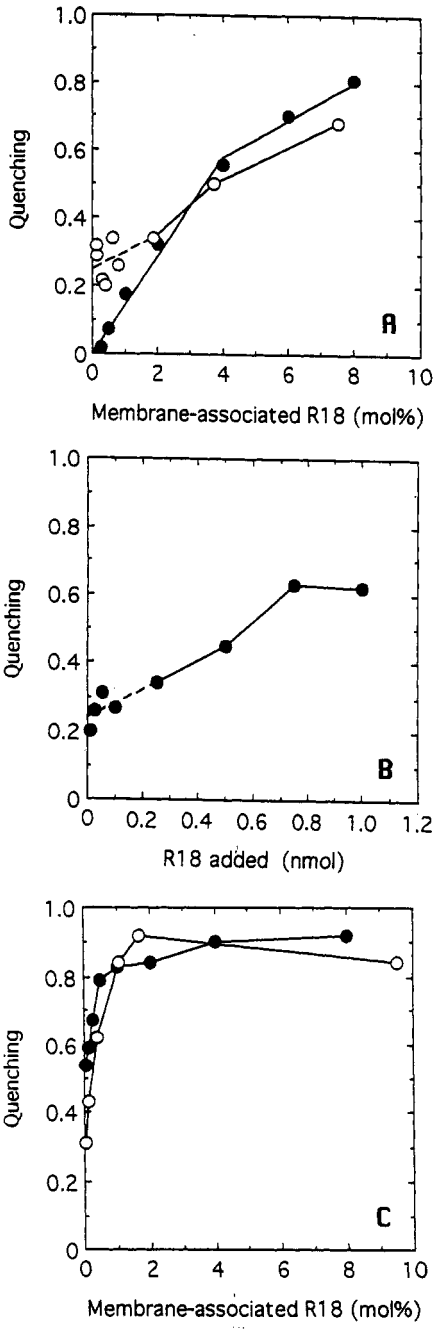

FIGURE 1: Quenching of R18 fluorescence in various different membranes(A) PC LUV, produced from dry lipid films consisting of a mixture of 1 μmol of PC and various amounts of R18 (●) or labeled with R18 by ethanol injection of R18 into a suspension of preformed PC LUV (○). Aliquots contained 50 nmol of phospholipid per data point. Note that the amount of membrane-associated R18 is expressed on the basis of total phospholipid, although it is possible that the probe resided only in the outer leaflet of the membrane after ethanol injection (Hoekstra et al., 1984).(B) Plasma membranes of Sup T1 cells. Cells (2×10^5 per data point) were labeled by ethanol injection of R18 into an agitated cell suspension at 37 °C. Free R18 was removed by repeated centrifugation and washing of the cells with buffer.(C) Membranes of influenza virus or influenza virosomes. Virus (●) was labeled by ethanol injection of R18 at 37 °C. Free R18 was removed by chromatography on a Sephadex G-50 column. Virosomes (○) were made as described in Materials and Methods from a mixture of detergent-solubilized viral membranes and R18. The fluorescence of R18 was measured as described in Materials and Methods, and quenching was calculated from these data.

(MacDonald, 1990) and vesicles containing negatively charged phospholipids, such as phosphatidylserine or cardiolipin (Wilschut et al., unpublished observations), or phosphatidic acid (Wunderli-Allenspach et al., 1993).

Self-Quenching of R18 When Added to Liposomes or Cells by Ethanol Injection. The main advantage of R18 compared to other fluorescent probes for investigating membrane fusion is that membranes can be labeled with the probe simply by injecting an ethanolic solution of R18 into aqueous buffers containing the membranes (Hoekstra et al., 1984). To study the membrane association of the probe under such circumstances, PC LUV were labeled with R18 by injection of aliquots of the probe in ethanol with vigorous agitation. Unincorporated R18 was removed by gel filtration on Sephadex G-50 (Hoekstra et al., 1984). The association of R18 with PC was found to occur by simple partitioning and did not saturate in the concentration range studied (results not shown). Figure 1A shows the quenching of R18 fluorescence as a function of its concentration relative to that of PC. Although in the range of 2–8 mol % R18 the observed quenching was similar to that of PC LUV produced from a dry lipid film of PC and R18, it was found that below 2 mol % R18 quenching no longer decreased with decreasing R18 concentration. Thus, even at very low R18 concentrations, which in the case of a random distribution of probe molecules would not cause appreciable quenching, significant levels of quenching (0.2–0.3) were still observed. This indicates the coexistence of two populations of R18 molecules, one population consisting of molecules randomly distributed in the membrane, and a second highly quenched population possibly consisting of probe molecules bound to the membrane in the form of clusters or micelles.

Similar results were obtained with several biological membranes labeled with R18 by ethanol injection. Figure 1B shows the quenching of R18 incorporated into the plasma membranes of Sup T1 cells. In this case, unincorporated R18 was removed by repeated centrifugation of the cells until the supernatant no longer contained R18. Again, there was a certain range of R18 concentrations within which the quenching of the probe decreased with decreasing concentration. However, at very low concentrations there was still significant quenching; extrapolated to 0 mol % R18 there would be quenching of 0.2–0.3, in good agreement with data reported for Vero cells (Blumenthal et al., 1987). Similar residual quenching was observed with rat pancreatic zymogen granules and bovine chromaffin granules labeled with R18 by ethanol injection (results not shown).

Association of R18 with Viral Membranes. The R18 ethanol injection method for introduction of the probe into biological membranes has been used primarily to label enveloped viruses. Therefore, we investigated the quenching of R18 added to viruses. For influenza virus, labeled as described in Materials and Methods, we found substantial fluorescence quenching at much lower probe concentrations of R18 than for liposomes (Figure 1C vs 1A). Only in the range of 0.078–0.63 mol % did quenching appear linear (squared regression coefficient 0.98). Moreover, extrapolated to infinitely low concentrations of R18, the quenching would still be as high as 0.5, suggesting that the quenching was not caused exclusively by interactions between probe molecules. Note that in this respect the observed relative quenching levels are much higher than those seen in, for example, cholesterol-containing liposomes (MacDonald, 1990). We propose that in the case of influenza virus a fraction of the probe may interact specifically with the viral spike glycoproteins, causing fluorescence quenching independent of probe–probe interactions.

Supporting the latter notion, the high quenching was found not only when R18 was added to influenza virus by ethanol injection but also when it was incorporated into reconstituted influenza virus envelopes (virosomes). In the case of the virosomes, the probe was co-reconstituted by the addition of an ethanolic aliquot of R18 to the detergent-solubilized envelopes (Figure 1C). Like the intact virus, at the lowest R18 concentration measured (0.06%) the residual quenching of virosomes was still quite high (0.3). In summary then, the data in Figure 1C suggest that virus and virosomes have some of their R18 in a form which is highly quenched, most likely through interaction between the probe and viral membrane proteins. Thus, it appears that viral membranes may have three different forms of R18: a freely diffusing population in the membrane, a second population which forms when membranes are labeled by ethanol injection, and a third which interacts specifically with viral proteins. The presence of these different populations may well affect R18-based fusion measurements, not only in the case of influenza virus but perhaps with other enveloped viruses as well (see below).

In Vivo Fusion Activity of Influenza and SFV As Measured with the R18 Assay. Previously, we have shown that the fusion activity of R18-labeled influenza virus with two different cell types could be measured directly inside the living cell (Stegmann et al., 1987b). Since influenza virus enters cells via receptor-mediated endocytosis, and fusion of the viral with the endosomal membrane takes place only after a conformational change induced by low pH, fusion is strictly pH-dependent. One way to elevate the pH inside the endosome and thereby inhibit membrane fusion is by adding a lysosomotropic weak base, such as NH_4Cl, which accumulates in the endosome, preventing the establishment of a low pH. In accordance with these expectations, the increase in fluorescence seen upon the addition of influenza virus, labeled with R18, to MDCK cells was inhibited in the presence of 20 mM NH_4Cl (Figure 2A). In the absence of NH_4Cl, the increase in R18 fluorescence was preceded by a lag phase of about 3 min (curve a), arising from the minimum time it took for the first virions to reach a compartment with a low enough pH for fusion (Stegmann et al., 1987b). Then fusion proceeded for at least 60 min. However, the fluorescence subsequently continued to increase slowly for several hours. In the presence of NH_4Cl (Figure 2A, curve b), a distinct inhibition of the fluorescence increase was observed. However, as in curve a, also in the presence of NH_4Cl, the fluorescence drifted upward for several hours. Most likely, this slow increase was due to exchange of the probe between virus and endosomal and/or lysosomal membranes. In a number of other cell types, such as BHK-21 cells, there was a more pronounced increase in fluorescence in the presence of NH_4Cl, particularly when reconstituted influenza virosomes rather than native virions were used (Table I). Most likely, the more pronounced exchange from virosomes is due to the presence of low levels of residual detergent in the virosome membrane, facilitating the transfer of individual R18 molecules.

This artifact was even more pronounced when the *in vivo* fusion of R18-labeled SFV was investigated. In fact, the lysosomotropic weak base chloroquine, at a concentration of 150 μM, had no effect on the increase in fluorescence found upon addition of the virus to cells (Figure 2B). Since at the concentration tested, infection of cells by SFV is known to be reduced by 2 orders of magnitude (Helenius et al., 1980), most likely through inhibition of the fusion reaction, it would appear that the increase in fluorescence was not due to membrane fusion. Also, other inhibitors of vacuolar acidification such as NH_4Cl(50 mM) or monensin (10 μM) had

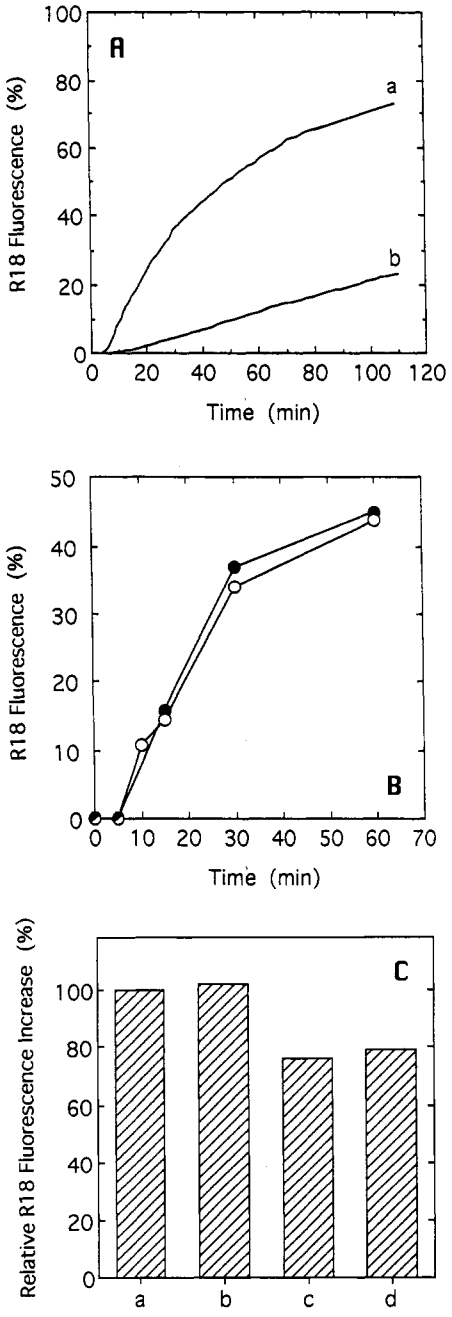

FIGURE 2: Fusion of R18 labeled viruses *in vivo*.(A)Influenza virus, strain NIB26, was labeled with 6.0 mol % R18, as in the legend to Figure 1C. Aliquots of virus were allowed to bind to trypsinized MDCK cells for 1 h at 0 °C, washed with ice-cold Hanks/Hepes, and then added to warm buffer (37 °C) in the cuvette of a fluorometer. Buffers: (a) Hanks/Hepes (pH 7.4); (b) Hanks/Hepes (pH 7.4) with 20 mM NH_4Cl. (B)SFV labeled with R18 was allowed to bind to BHK-21 cells for 1 h at 0 °C in Hanks/Hepes buffer. Unbound virus was removed by two washes with ice-cold buffer, and the cells were further incubated in Hanks/Hepes buffer with (●) or without (○) 150 μM chloroquine at 37 °C. At the times indicated, cells were scraped with a rubber policeman and quickly transferred to ice-cold Hanks/Hepes buffer in the cuvette of a fluorometer, and the quenching of R18 fluorescence was measured at 4 °C. (C) Effect of other inhibitors on SFV fusion. SFV labeled with R18 was bound to cells as described above and kept at 37 °C for 1 h in the absence of inhibitors (a) or in the presence of 150 μM chloroquine (b), 50 mM NH_4Cl (c), or 10 μM monensin (d). Fluorescence changes are expressed relative to the control in the absence of inhibitors (a).

little effect on the increase in R18 fluorescence (Figure 2C). In contrast, in chicken embryo fibroblasts, 75% of the fluorescence dequenching of R18-labeled SFV was inhibited by 50 mM NH_4Cl (Gilbert et al., 1990). Thus, for both influenza virus and SFV, R18 dequenching can occur via a nonfusion mode of lipid transfer, presumably caused by molecular exchange of the probe between viral and endosomal and/or lysosomal membranes or by removal of R18 from the virus by some cellular protein. The amount of this artificial dequenching appears to depend on the cell type. Since the exchange is seen with virus and virosomes, it is likely that the population of R18, which is highly quenched by viral protein as described above, is involved in this reaction.

Table I: Intracellular Fusion of Influenza Virus and Virosomes in BHK-21 Cells[a]

donor membrane	fluorescence change (%)
R18-labeled influenza virus	44 (100)
+ NH_4Cl	17 (39)
R18-labeled influenza virosomes	40 (100)
+ NH_4Cl	26 (65)
pyrPC-labeled influenza virosomes	43 (100)
+ NH_4Cl	2 (5)

[a] Virus (strain NIB26) was labeled with 6.0 mol % R18 (on the basis of total membrane phospholipid); virosomes prepared from the same strain of virus were labeled with 6.0 mol % R18 or 10 mol % pyrPC. The labeled membranes were allowed to bind to trypsinized BHK-21 cells for 1 h at 0 °C and pH 7.4. The cells were washed with ice-cold Hanks/Hepes buffer several times, resuspended, and incubated at 37 °C in the cuvette of a fluorometer for 1 h, after which time the fluorescence increase (R18 assay) or the relative excimer fluorescence decrease (pyrPC assay) was determined as described in Materials and Methods. The numbers in parentheses represent relative changes, where the fluorescence change in the absence of NH_4Cl was set to 100%.

Excimer/Monomer Ratio of Pyrene Phospholipids in Liposomes and Reconstituted Viral Membranes. Since the above data imply that under certain conditions R18 may not reliably reflect viral membrane fusion, we sought an alternative. Recently, it has been demonstrated that pyrene lipids can be incorporated in vesicular stomatitis virus (VSV) (Pal et al., 1988) or SFV (Wahlberg et al., 1992) by producing these viruses on cells cultured in the presence of pyrene-labeled fatty acids. The pyrene fluorophore can form fluorescent excimers (dimers of an excited monomer and a monomer in the ground state) which emit light with a wavelength shifted some 100 nm upward from that of the monomer (Galla & Hartmann, 1980). The extent of pyrene excimer formation is dependent on the concentration of the probe. Therefore, upon the fusion of virus labeled with pyrene lipids with an unlabeled target membrane, the excimer fluorescence intensity decreases and the monomer fluorescence increases concomitantly as a result of dilution of the fluorophore. Thus, fusion of pyrene-labeled viruses with liposomes has been measured by monitoring the decrease in pyrene excimer fluorescence (Wahlberg et al., 1992; Bron et al., 1993). Here we investigated the mode of insertion of a pyrene-labeled phospholipid (pyrPC) in liposomal and virosomal membranes by determination of the fluorescence excimer-to-monomer (E/M) ratio at different probe concentrations (Figure 3). It was found that the ratio decreased proportionally with the probe concentration for both types of membranes and that, extrapolated to very low concentrations of pyrene, the excimer fluorescence disappeared. Moreover, similar concentrations of pyrPC in the different membranes gave comparable E/M ratios. Thus, in contrast to R18, pyrPC appears to be homogeneously distributed in both liposomal and virosomal membranes, and the presence of viral spike glycoproteins does not appear to affect the fluorescence characteristics or intensity of the probe.

Direct Comparison of Influenza Virosome Fusion with Erythrocyte Ghosts, Measured by the R18 and pyrPC Assay. For a direct comparison of the properties of the pyrene excimer and R18 assays, influenza virosomes containing co-reconsti-

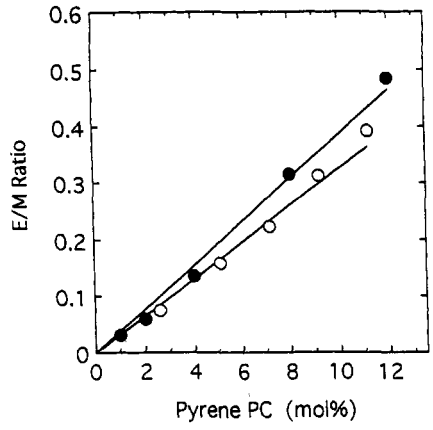

FIGURE 3: Pyrene excimer-to-monomer fluorescence ratios at different concentrations of pyrPC in liposomes or virosomes. Liposomes (●) were made from mixtures of pyrPC and PC. Virosomes (○) were made from solubilized influenza (strain NIB26) envelopes and pyrPC in the detergent C12E8. Excimer and monomer fluorescence intensities were measured at emission wavelengths of 480 and 397 nm, respectively.

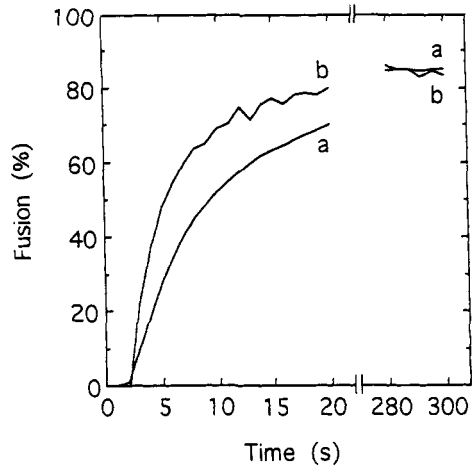

FIGURE 4: Fusion of influenza virosomes, labeled with R18 and pyrPC, with erythrocyte ghosts. Ghosts were incubated with virosomes for 5 min at 37 °C in 150 mM NaCl and 5 mM Hepes (pH 7.4). Then the medium was acidified to pH 5.1 by the injection of an aliquot of a buffered solution containing 0.4 M Mes and 0.3 M trisodium citrate. The final concentrations of ghosts and virosomes were 120 and 2.5 μM membrane phospholipid phosphate, respectively. Fusion was measured as the increase in R18 fluorescence (a) or the decrease in pyrene excimer fluorescence (b), as described in Materials and Methods.

tuted pyrPC were labeled with R18 by ethanol injection. A control preparation received ethanol only. Both preparations were subjected to chromatography on a Sephadex G-50 column. Fusion of these virosomes with erythrocyte ghosts was then measured by monitoring the increase in R18 fluorescence or the decrease in pyrene excimer fluorescence of the mock-treated preparation. Since there is a highly quenched population of R18 in the virosomes, fusion would be underestimated by the R18 assay if the highly quenched probe population were to persist after fusion. Surprisingly, as shown in Figure 4, the final extent of fusion was very high and identical for both assays (82%). However, the initial rate of fusion measured with the pyrene assay (28%/s) was about three times as high as that measured with the R18 assay (8.9%/s). A similar difference in the initial rates of fusion was observed for virosomes labeled separately with co-reconstituted pyrPC (22%/s) or added R18 (8.1%/s), indicating that in the double-labeled virosomes there was no mutual interference between the two probes. These results indicate that, under identical conditions, R18 and pyrPC report similar extents of

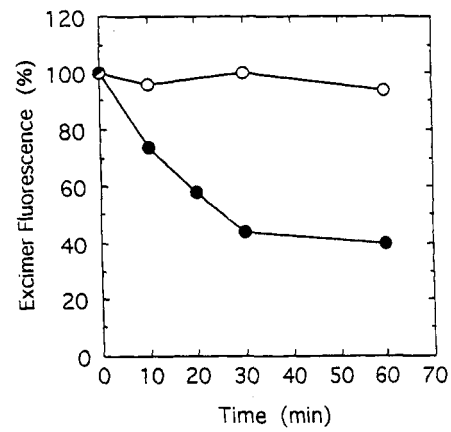

FIGURE 5: *In vivo* fusion of SFV metabolically labeled with pyrene phospholipids. Labeled virus was allowed to bind to BHK-21 cells in the cold. Unbound virus was removed by two washes with ice-cold buffer, and the cells were further incubated in Hanks/Hepes buffer with (○) or without (●) 20 mM NH_4Cl at 37 °C. At the times indicated, the cells were scraped with a rubber policeman and quickly transferred to ice-cold Hanks/Hepes buffer in the cuvette of a fluorometer, and the excimer fluorescence was measured.

fusion of virosomes with erythrocyte ghosts. However, the rate of dilution of the R18 probe appears to be lower than that of pyrPC.

Intracellular Fusion of SFV Metabolically Labeled with Pyrene Phospholipids. Finally, SFV was produced from cells that were previously labeled metabolically with pyrene fatty acids. The presence of pyrene-labeled lipids and the absence of free fluorescent fatty acids in the virus were confirmed by thin-layer chromatography of lipids extracted from the virus (results not shown). The infectivity of the virus was not affected by the pyrene labeling. Plaque titrations on Vero cells yielded 0.9×10^{12} vs 1.0×10^{12} plaque-forming units per milligram of viral protein for labeled and unlabeled virus, respectively. Labeled virus (with an E/M ratio of 0.6 at 37 °C) was allowed to bind to BHK-21 cells for 60 min at 4 °C. The unbound virus was washed away and the cells were incubated for various periods of time at 37 °C in the absence or presence of 20 mM NH_4Cl. Subsequently, the cells were harvested by scraping and quickly transferred to a buffer containing 20 mM NH_4Cl at 4 °C in the cuvette of a fluorometer. Pyrene excimer fluorescence in the samples was determined at 37 °C. In the absence of NH_4Cl, the excimer fluorescence decreased for approximately 30 min, after which time it leveled off (Figure 5). The final extent of fluorescence decrease was about 60%. In the presence of NH_4Cl, the change in excimer fluorescence was marginal, resulting in about a 5% decrease after 60 min. Similarly, influenza virosomes, labeled with pyrPC, showed a decrease in excimer fluorescence in the absence but not in the presence of NH_4Cl during internalization into BHK-21 cells (Table I). These results indicate that the decrease in excimer fluorescence solely reflects membrane fusion and that, in contrast to the observations made with the R18 assay, there is no spurious exchange of pyrene phospholipids between the viral and cellular membranes *in vivo*.

DISCUSSION

Although the fusion assay based on dequenching of R18 in most cases provides a reasonably reliable quantitative method for measuring viral membrane fusion *in vitro*, this article shows that the assay cannot always be relied upon for the measurement of viral membrane fusion *in vivo*. For example, during *in vivo* fusion of the influenza virus, or particularly SFV, part of the R18 initially associated with the viral membranes is subject to dequenching via a nonfusion mech-

anism. In contrast, as demonstrated here for the first time, an assay based on the decrease in excimer fluorescence of pyrene phospholipids does reliably report membrane fusion of enveloped viruses *in vivo*.

R18 is widely used because the probe permits facile labeling of biological membranes. It spontaneously associates with membranes when added from an ethanolic solution. Because of its hydrophobic nature, it is assumed to enter the hydrophobic interior of the membrane (Hoekstra et al., 1984). Although the latter does appear to be the case for some of the probe molecules, we have found that, when model or biological membranes are labeled with R18 by ethanol injection, there is also a population of R18 which is more highly quenched than can be expected on the basis of a random probe distribution. Since in PC liposomes produced from a mixture of R18 and PC such a population was not present (Figure 1A), the highly quenched R18 population probably is not located *within* the membrane interior, but rather *on* the membrane surface. We suggest that this R18 is present in the form of membrane-associated clusters or micelles. Apparently, these micelles cannot be removed by the gel filtration step described for the removal of unincorporated R18 (Hoekstra et al., 1984).

Upon R18 labeling of viral membranes, yet another population of highly quenched probe molecules was detected. Since this population was found not only when R18 was added by ethanol injection but also when viral membranes were coreconstituted with R18, it appeared to be specific for membranes containing viral proteins. Cell membranes (with similar lipids but different proteins) and model membranes without proteins did not exhibit the extremely high degree of R18 quenching observed with influenza virus or reconstituted viral envelopes. It is possible that R18 binds to viral proteins and that its fluorescence is quenched as a result of that binding. Neither this highly quenched population of R18 nor the population that is generated by the addition of probe to preexisting membranes, as discussed above, appears to be identical to the one described by MacDonald (1990) for membranes containing cholesterol.

Concerning the very high quenching of R18 observed in viral membranes, we note that Wunderli-Allenspach et al. (1993) have recently reported on labeling of the PR8/34 strain of influenza virus with different amounts of R18. In contrast to our observations, these authors conclude that, in order to obtain the same quenching in the virus, much higher overall surface densities of the probe are required than in liposomes. However, close scrutiny of the data (Wunderli-Allenspach et al., 1993) reveals that in their experiments, like in ours, quench values below about 0.6 were not observed and that extrapolated to infinitely low R18 levels, quenching would still be quite significant (around 0.4–0.5). At higher levels of R18 the data cannot be compared directly to our present results, since the authors present the R18 membrane concentration in number of molecules per nm^2. At the highest probe concentrations tested, this would amount to more than 50 mol % of R18 relative to viral phospholipid. If indeed such very high R18 concentrations are used, we are concerned that the probe may not have been quantitatively associated with the viral membrane. This concern is strengthened by the fact that the authors did not separate unincorporated from virus-associated dye. We also note that only partially purified virus was used under dilute conditions, which may have resulted in probe binding to contaminating proteins. In our hands, pure influenza virus invariably exhibits very high R18 quenching at low surface concentrations of the probe. The latter observation has now also been made for Sendai virus (K. J. M. Klappe and D. Hoekstra, personal communication). The high concentrations used by Wunderli-Allenspach et al. are probably the cause of the 17-fold reduction in viral infectivity resulting from labeling with a concentration of R18 that caused 79% quenching (Wunderli-Allenspach et al., 1993). We have not observed such a reduction in infectivity. Use of virus labeled with 0.8 mol % R18, which gave about 80% quenching (Figure 1C), produced $(2.27 \pm 0.1) \times 10^8$ plaques/mg on MDCK cells vs $(2.1 \pm 0.2) \times 10^8$ plaques/mg for unlabeled virus.

One of the potential consequences of the presence of highly quenched populations of R18 is an underestimation of fusion by the assay, if these R18 molecules are to persist after fusion. We consider the possibility of probe-induced inactivation of fusion activity, as suggested by Wunderli-Allenspach et al. (1993), less likely, at least at probe concentrations well below 10 mol %, since influenza labeled with either 0.3 or 6.0 mol % R18 exhibited the same extent of fusion with erythrocyte ghosts (results not shown). With respect to underestimation of the extent of fusion due to the presence of highly quenched R18, it should be possible, in principle, to correct for this effect by the introduction of a certain correction factor (Blumenthal et al., 1987). However, first, it is difficult to determine the relative amount of highly quenched R18, and second, the quenched population does not necessarily persist after fusion. Indeed, we found that upon the fusion of influenza virosomes with erythrocyte ghosts (Figure 4) the final level of fusion was high and the same, irrespective of whether the R18 or pyrPC assay was used, indicating that the R18 probe dilutes to the same extent as the pyrPC probe. Previously we have found different extents of fusion of R18-labeled influenza virus and pyrPC-labeled virosomes in an erythrocyte ghost system (Wilschut et al., 1991; Bron et al., 1992). This difference was attributed to incomplete dilution of R18. However, in our present study, in which the two probes were compared directly under identical conditions, almost all R18 and pyrPC molecules appeared to dilute from virosomal into erythrocyte membranes. Importantly, however, fusion as measured with the R18 assay was slower. Studying single fusion events between influenza virus and erythrocytes by video-enhanced fluorescence microscopy, Lowy et al. found that R18 redistribution after fusion was slower than would be expected on the basis of a free-diffusion model (Lowy et al., 1990). These and our observations are consistent with the notion that highly quenched R18 is transferred from the donor membrane to the hydrophobic interior of the acceptor membrane, but that this occurs more slowly than for the population that is free to diffuse in the membrane.

In some cases, particularly during the fusion of R18-labeled viruses in endosomes of cultured cells, we found significant R18 fluorescence dequenching in the absence of membrane fusion. Such nonfusion dequenching events have also been observed by others in *in vitro* systems as well as in cells (Blumenthal et al., 1987; Wunderli-Allenspach & Ott, 1990; Comerford & Dawson, 1991; Di Simone & Baldeschwieler, 1992; Puri et al., 1992; Wunderli-Allenspach et al., 1993). Whatever the cause of this artifactual dequenching (direct molecular transfer of R18 from donor to acceptor membranes without fusion, redistribution of donor membrane-bound R18, or removal of R18 from the membrane by cellular components), it indicates that R18 is not always completely or tightly sequestered in the donor membrane. Since in our experiments the nonfusion dequenching was much more pronounced for SFV than for influenza virus (Figure 2), and SFV virions are considerably smaller than influenza virions, the effect may be related to the degree of curvature of the donor membrane.

This would be consistent with the relatively high degree of spontaneous dequenching observed for R18-labeled small liposomes, as compared to large liposomes, interacting with influenza virus at neutral pH (Wunderli-Allenspach et al., 1993). Since the magnitude of the effect varies for different membranes and for the same donor membrane in different cell lines, its extent is very difficult to predict. In order to assess the extent of nonfusion dequenching of R18, one should devise a control experiment in which the donor membrane is tightly bound to the acceptor membrane but unable to fuse with it. For influenza virus, which fuses at low pH but binds to sialic acid containing receptors at neutral pH, such a control is provided by virus associated with erythrocyte ghosts at neutral pH. Under these conditions, we have observed rates of spontaneous R18 dequenching of around 0.2%/min at 37 °C (results not shown), which is at least 3 orders of magnitude lower than the rate observed during fusion at pH 5.1 and 37 °C (8.9%/s). For low pH induced fusion of viruses in the endosomes of cells, the control can consist of endocytosis in the presence of lysosomotropic weak bases, as was done in this study. In these cases, the control is more critical than in the case of *in vitro* fusion, since the time course of *in vivo* virus internalization experiments is usually much longer than that of *in vitro* fusion. Another important control could involve viruses having incorporated nonfusogenic precursors of membrane fusion proteins. Finally, we note that for viruses such as Sendai virus and the human immunodeficiency virus (Potash et al., 1992), which fuse with the plasma membrane of cultured cells at neutral pH, it is extremely difficult to design a proper control experiment for assessing the nonfusion dequenching of fluorescent probes such as R18.

As an alternative for the R18 assay, we have used a fusion assay based on pyrene-labeled lipids recently introduced for the measurement of vesicular stomatitis virus (Pal et al., 1988) and SFV (Wahlberg et al., 1992; Bron et al., 1993) fusion with liposomes *in vitro*. The main advantage of this assay compared with the R18 assay is that the pyrene fluorophore is metabolically incorporated into membrane lipids. These are known to be refractory to spontaneous transfer. Thus with the pyrPC assay, nonfusion dilution of the probe is much less likely to occur than with the R18 assay. Here, we have used the assay to measure SFV fusion *in vivo*. We found changes in fluorescence only under conditions permitting fusion, which means that there was indeed no spurious lipid exchange. The E/M fluorescence ratio of pyrPC was proportional to the concentration of the probe in both liposomal and virosomal membranes, and there was no excimer fluorescence at infinitely low probe concentrations. This indicates that in either membrane the probe is distributed randomly and that it does not interact specifically with viral spike glycoproteins. While previous observations have demonstrated that the pyrene excimer assay reliably reports the fusion of enveloped viruses with liposomes (Wahlberg et al., 1992; Bron et al., 1993), the results of the measurements presented in this article clearly demonstrate the usefulness of the assay for *in vivo* fusion measurements.

REFERENCES

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37,* 911–917.

Blumenthal, R., Bali-Puri, A., Walter, A., Covell, D., & Eidelman, O. (1987) *J. Biol. Chem. 262,* 13614–19.

Böttcher, C. J. F., Van Gent, C. M., & Fries, C. (1961) *Anal. Chim. Acta 24,* 203–204.

Bron, R., Ortiz, A., Dijkstra, J., Stegmann, T., & Wilschut, J. (1992) *Methods Enzymol. 220,* 313–331.

Bron, R., Wahlberg, J. M., Garoff, H., & Wilschut, J. (1993) *EMBO J. 12,* 693–701.

Comerford, J. G., & Dawson, A. P. (1991) *Biochem. J. 280,* 335–340.

Di Simone, C., & Baldeschwieler, J. D. (1992) *Virology 191,* 338–345.

Doms, R. W., Gething, M. J., Henneberry, J., White, J., & Helenius, A. (1986) *J. Virol. 57,* 603–13.

Galla, H. J., & Hartmann, W. (1980) *Chem. Phys. Lipids 27,* 199–219.

Gilbert, J. M., Mason, D., & White, J. M. (1990) *J. Virol. 64,* 5106–5113.

Helenius, A., Kartenbeck, J., Simons, K., & Fries, E. (1980) *J. Cell Biol. 84,* 404–420.

Hoekstra, D., de Boer, T., Klappe, K., & Wilschut, J. (1984) *Biochemistry 23,* 5675–5681.

Johansson, L. B. Å., & Niemi, A. (1987) *J. Phys. Chem. 91,* 3020–3023.

Keller, P. M., Person, S., & Snipes, W. (1977) *J. Cell Sci. 28,* 167–177.

Lowy, R. J., Sarkar, D. P., Chen, Y., & Blumenthal, R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 1850–1854.

Loyter, A., Citovsky, V., & Blumenthal, R. (1988) in *Methods of Biochemical Analysis* (Glick, D., Ed.) Vol. 33, pp 129–164, John Wiley & Sons, New York.

MacDonald, R. I. (1990) *J. Biol. Chem. 265,* 13533–13539.

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochem. Biophys. Acta 858,* 161–168.

Pal, R., Barenholz, Y., & Wagner, R. R. (1988) *Biochemistry 27,* 30–36.

Potash, M. J., Zeira, M., Huang, Z. B., Pearce, T. E., Eden, E., Gendelman, H. E., & Volsky, D. J. (1992) *Virology 188,* 864–868.

Puri, A., Grimaldi, S., & Blumenthal, R. (1992) *Biochemistry 31,* 10108–10113.

Silvius, J. R., Leventis, R., Brown, P. M., & Zuckermann, M. (1987) *Biochemistry 26,* 4679–4287.

Srinivasakumar, N., Ogra, P. L., & Flanagan, T. D. (1991) *J. Virol. 65,* 4063–4069.

Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31,* 172–180.

Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1985) *Biochemistry 24,* 3107–3113.

Stegmann, T., Morselt, H. W., Booy, F. P., van Breemen, J. F., Scherphof, G., & Wilschut, J. (1987a) *EMBO J. 6,* 2651–2659.

Stegmann, T., Morselt, H. W., Scholma, J., & Wilschut, J. (1987b) *Biochim. Biophys. Acta 904,* 165–70.

Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20,* 4093–4099.

Uster, P. S., & Deamer, D. W. (1985) *Biochemistry 24,* 1–8.

Wahlberg, J. M., Bron, R., Wilschut, J., & Garoff, H. (1992) *J. Virol. 66,* 7309–7318.

Wilschut, J., Bron, R., Dijkstra, J., Ortiz, A., van Ginkel, L. C., DeGrado, W. F., Rafalski, M., & Lear, J. D. (1991) in *Progress in Membrane Biotechnology* (Gómez-Fernández, J. C., Chapman, D., & Packer, L., Eds.) pp 317–334, Birkhäuser Verlag, Basel, Switzerland.

Wunderli-Allenspach, H., & Ott, S. (1990) *Biochemistry 29,* 1990–1997.

Wunderli-Allenspach, H., Günthert, M., & Ott, S. (1993) *Biochemistry 32,* 900–907.