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Functional reconstitution of influenza virus envelopes

Toon Stegmann, Henriëtte W.M.Morselt, Frank P.Booy[1], Jan F.L.van Breemen[1], Gerrit Scherphof and Jan Wilschut

Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, and [1]Biochemical Laboratory, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

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We have examined several procedures for the reconstitution of influenza virus envelopes, based on detergent removal from solubilized viral membranes. With octylglucoside, no functionally active virosomes are formed, irrespective of the rate of detergent removal: in the final preparation the viral spike proteins appear predominantly as rosettes. Protein incorporation in reconstituted vesicles is improved when a method based on reverse-phase evaporation of octylglucoside-solubilized viral membranes in an ether/water system is employed. However, the resulting vesicles do not fuse with biological membranes, but exhibit only a non-physiological fusion reaction with negatively charged liposomes. Functional reconstitution of viral envelopes is achieved after solubilization with octaethyleneglycol mono(*n*-dodecyl)ether ($C_{12}E_8$), and subsequent detergent removal with Bio-Beads SM-2. The spike protein molecules are quantitatively incorporated in a single population of virosomes of uniform buoyant density and appear on both sides of the membrane. The virosomes display hemagglutination activity and a strictly pH-dependent hemolytic activity. The virosomes fuse with erythrocyte ghosts, as revealed by a fluorescence resonance energy transfer assay. The rate and the pH dependence of fusion are essentially the same as those of the intact virus. The virosomes also fuse with cultured cells, either at the level of the endosomal membrane or directly with the cellular plasma membrane upon a brief exposure to low pH.

Key words: influenza virus/reconstitution/$C_{12}E_8$/octylglucoside/membrane fusion

Introduction

Influenza virus infects a cell by fusion of the viral membrane with the endosomal membrane after entry of the virus particles through receptor-mediated endocytosis (Matlin *et al.*, 1981; Yoshimura and Ohnisni, 1984). As a result of this fusion process, the viral genome is released into the cytoplasm. The fusion reaction is induced by the low pH inside the endosomes (Tycko and Maxfield, 1982). It is mediated by a viral membrane glycoprotein, the hemagglutinin (HA) (White *et al.*, 1982; Skehel *et al.*, 1982). In addition to HA, the viral envelope also contains another glycoprotein, the neuraminidase (NA), and lipids. The lipids are derived from the host cell during the release of virus particles in a process involving membrane budding. Thus, the viral membrane closely resembles the host cell plasma membrane in terms of composition and transverse distribution of the lipids (Lenard and Compans, 1974; Tsai and Lenard, 1975).

We have previously studied the fusion activity of influenza virus using liposomes and erythrocyte ghosts as target membranes (Stegmann *et al.*, 1985,1986). In these studies we examined the dependence of the fusion characteristics on the nature and composition of the *target* membrane (Stegmann *et al.*, 1986). To study the role of the *viral* membrane components in the fusion reaction in detail, it is necessary to be able to manipulate these components. For this purpose a method is required for the isolation and reconstitution of the viral spike proteins, producing reconstituted virosomes with full biological fusion activity. Despite various efforts (Almeida *et al.*, 1975; Huang *et al.*, 1979, 1980; Kawasaki *et al.*, 1983; Hosaka *et al.*, 1983), reconstitution of influenza virus envelopes, displaying biological fusion activity, has not been reported.

Most of the methods that have been employed to reconstitute viral envelopes are based on solubilization of the viral membrane with a detergent and, after sedimentation of the internal viral proteins and genetic material, removal of the detergent from the supernatant. Detergents with a high critical micelle concentration (c.m.c.), such as octylglucoside, can be removed effectively by dialysis. Reconstitution employing octylglucoside has been reported for Semliki Forest virus (SFV) (Helenius *et al.*, 1977,1981), vesicular stomatitis virus (VSV) (Petri and Wagner, 1979; Miller *et al.*, 1980; Eidelman *et al.*, 1984), influenza virus (Huang *et al.*, 1979,1980) and Sendai virus (Harmsen *et al.*, 1985). However, properly reconstituted viral envelopes were not produced in all cases. For example, virosomes formed from SFV had a protein to lipid ratio deviating from that of the viral membrane (Helenius *et al.*, 1977,1981) and virosomes produced from VSV did not exhibit biological fusion activity (Eidelman *et al.*, 1984; Metsikkö *et al.*, 1986).

Detergents with a low c.m.c., like Triton X-100, cannot readily be dialysed. Detergent removal in this case requires the addition of a hydrophobic resin, such as Bio-Beads SM-2, a styrene–divinyl–benzene co-polymer (Holloway, 1973). Triton X-100 has been used successfully for the reconstitution of Sendai virus (Volsky and Loyter, 1978; Vainstein *et al.*, 1984), even though the reconstituted vesicles may have contained significant amounts of residual detergent. Influenza virus envelopes have also been reconstituted with Triton X-100 (Kawasaki *et al.*, 1983) or another non-ionic detergent with a low c.m.c., Nonidet P-40 (Hosaka *et al.*, 1983). However, in these cases the reconstituted virosomes were not extensively characterized in terms of biological fusion activity.

In this paper, we describe various methods for the reconstitution of influenza virus envelopes. One approach was based on octylglucoside removal, while another combined octylglucoside dilution with the reverse-phase evaporation method for the production of liposomes (Szoka and Papahadjopoulos, 1978). However, only a recently published method employing the detergent octaethyleneglycol mono(*n*-dodecyl)ether ($C_{12}E_8$) as a solubilizing agent and its subsequent two-step removal with Bio-Beads SM-2 (Metsikkö *et al.*, 1986) yielded virosomes with full biological fusion activity.

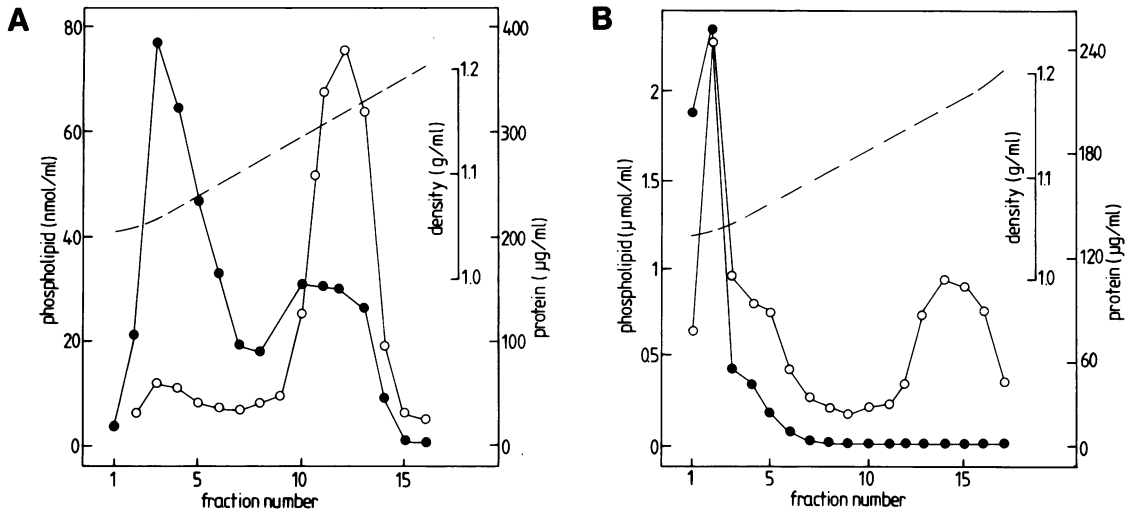

Fig. 1. Equilibrium density centrifugation of reconstitution products on sucrose gradients **A**, octylglucoside dialysis reconstitution products **B**, REV reconstitution products. (●), phospholipid phosphate; (○), protein; dashed line, buoyant density.

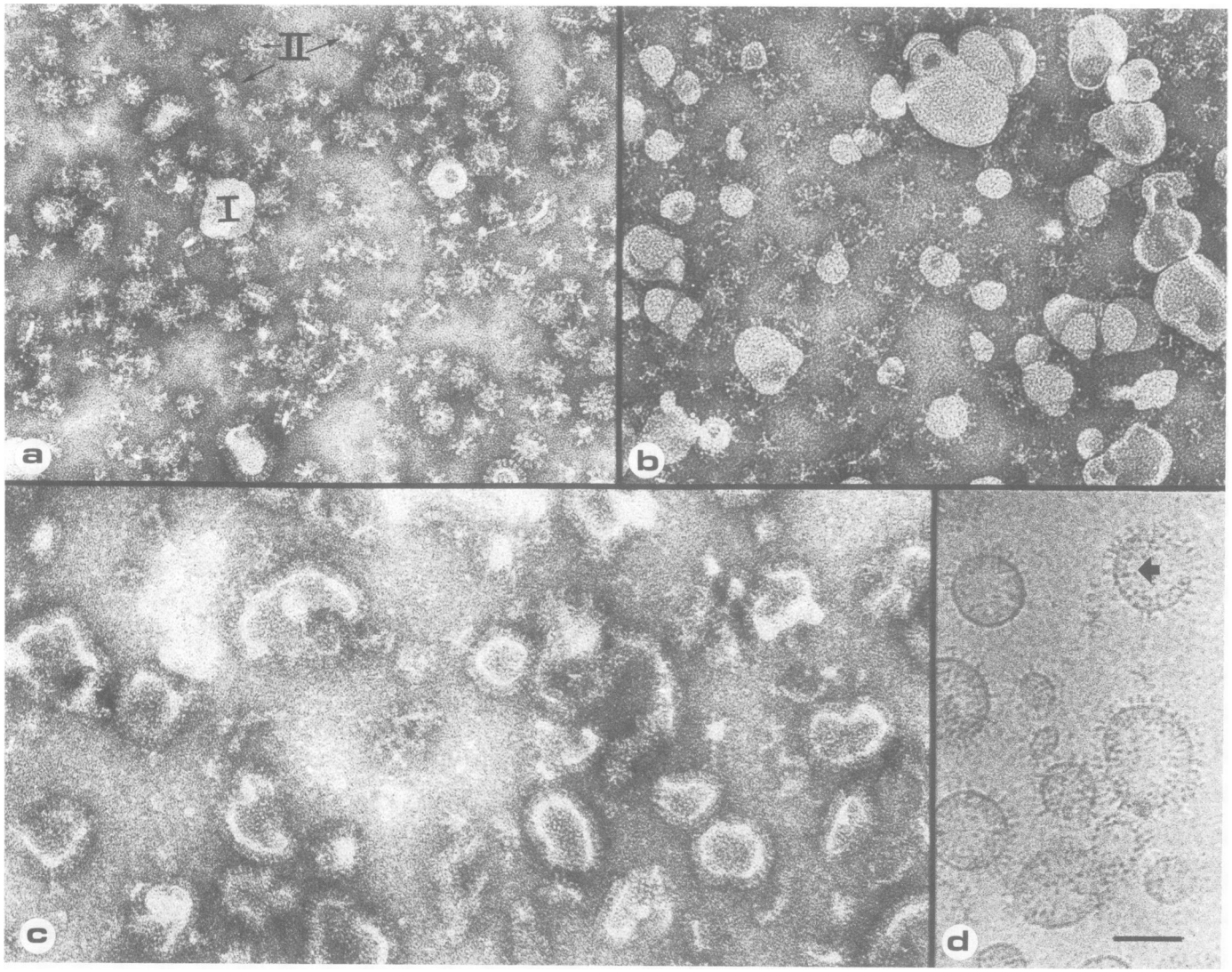

Fig. 2. Electron micrographs of reconstitution products, negatively stained with phosphotungstate (**A, B** and **C**) or unstained frozen (**D**). **A**, octylglucoside-dialysis reconstitution products; **B**, REV reconstitution products; **C,D,** $C_{12}E_8$ reconstitution products. Bar represents 100 nm.

Results

Octylglucoside reconstitution of viral envelopes

A first series of attempts to reconstitute the influenza virus envelope was based on solubilization of the viral membrane with octylglucoside and subsequent removal of the detergent by dialysis. The detergent was used at a concentration of 45 mM to solubilize the viral envelope at a membrane phospholipid concentration of 1.25 mM. After removal of the viral matrix protein and nucleocapsid by ultracentrifugation, 82% of the viral phospholipid and 27% of the total viral protein were recovered in the supernatant. As judged by gel electrophoresis of the supernatant, the solubilized protein represented viral envelope protein only. For reconstitution the supernatant was dialysed against four changes of 1500 volumes of buffer over a period of 48 h at 4°C. The products of the reconstitution were analysed by centrifugation on a sucrose gradient and visualized by electron microscopy. Two main density classes were recovered from the gradient, one lipid-rich and one protein-rich (Figure 1A). As the reconstitution products tended to aggregate, separation of the two density classes on the gradient required the presence of 0.5 M NaCl. Electron-microscopic examination of the reconstituted preparation after negative staining demonstrated the presence of large vesicles, containing few spikes, if any (Figure 2A:I). Protein was concentrated in seemingly lipid-free rosettes or in extremely small vesicles, densely covered with spikes (Figure 2A:II). All protein-containing fractions from the sucrose gradient showed hemagglutination activity, equal to or slightly higher than that of the virus (on the basis of membrane protein). However, because of the heterogeneity of the reconstituate and the poor protein incorporation in vesicles, the reconstitution products did not suit our purpose of studying fusion activity. Similar results were obtained with another detergent with a high c.m.c., CHAPS (Hjelmeland *et al.*, 1980). With cholate and deoxycholate the association of viral protein with lipid was improved, but the reconstitution products had neither fusion nor hemagglutination activity.

In an attempt to improve protein incorporation in virosomes we adapted the procedure by which octylglucoside was removed from the supernatant. Eidelman *et al.* (1984) have argued that a slow removal of octylglucoside is essential to achieve an efficient and functional reconstitution of viral spike proteins. However, using their protocol, we did not observe any improvement of protein incorporation in vesicles. Extremely fast removal by dilution, under continuous vortex mixing, of 1 vol. of viral supernatant into 25 vols of buffer (Jackson and Litman, 1985) did not significantly alter the final results either . Dialysis at 37°C, rather than first at room temperature and subsequently in the cold (see Materials and methods), or addition of exogenous lipids to the solubilized viral membrane [dioleoylphosphatidylcholine/dioleoylphosphatidylethanolamine (DOPC/DOPE, molar ratio 2:1, in a 3-fold excess over the viral lipid] did not improve the results either. Increasing the NaCl concentration to 1 M during dialysis enhanced the concentration of protein in the lipid-rich density class, but the resulting vesicles produced large aggregates after removal of the salt.

A protein and lipid distribution very similar to that shown in Figures 1A and 2A has been reported previously for the octylglucoside reconstitution of SFV (Helenius *et al.*, 1977,1981). The authors rationalized that bilayers form and seal from the solubilized viral lipids below a threshold concentration of octylglucoside during dialysis. At and below this concentration, the proteins form stable complexes that do not dissociate to free monomeric proteins, thus precluding their insertion into the lipid bilayers (Helenius *et al.*, 1977,1981).

Reconstitution during reverse-phase evaporation

If the mechanism responsible for the formation of the different classes of reconstitution products formed from influenza virus envelopes were similar to that proposed for SFV, successful formation of influenza virosomes would require protein insertion simultaneous with the formation of lipid bilayers during the removal of the detergent. We tried to achieve this by adapting the reverse-phase evaporation (REV) method for the formation of liposomes (Szoka and Papahadjopoulos, 1978) to include viral membrane components. In this method lipids dissolved in ether are sonicated with an aqueous buffer to form a stable emulsion of water in ether stabilized by monolayers of phospholipids ('inverted micelles'). Upon subsequent partial removal of the ether under reduced pressure a gel is formed which collapses upon further removal of the ether in combination with mechanical agitation. During this collapse liposomes are formed. We added the supernatant of octylglucoside-solubilized virus to the gel of DOPC/DOPE (molar ratio, 1:1) and followed the REV procedure (Szoka and Papahadjopoulos), 1978). At the collapse of the gel the viral supernatant is instantly diluted by the buffer released from the gel to an octylglucoside concentration of 10−15 mM, simultaneous with the formation of liposomes from the inverted micelles. In control experiments, in which a solution of 30 mM octylglucoside rather than viral supernatant was added to the gel, it could be demonstrated by absorbance and light scattering measurements that at this stage there are indeed liposomes present and not just detergent micelles (results not shown). After the collapse, residual ether is evaporated under reduced pressure and residual octylglucoside is removed by dialysis (as described in Materials and methods). Following this procedure we found ~55% of the viral protein to be incorporated into a single population of virosomes of uniform buoyant density, while 45% of the viral protein was not incorporated in vesicles (Figure 1B), but formed rosettes (Figure 2B). A high concentration of NaCl (0.5−1.0 M) was necessary both during reconstitution and separation of the products on the gradient. A limitation of the method is that an initial stable emulsion is only formed at a defined ratio of lipid, buffer and ether (Szoka and Papahadjopoulos, 1978). Thus, dilution of the viral lipid with exogenous lipids is unavoidable, causing the virosomes to have a relatively low protein to lipid ratio (Figure 1B) and to be not as densely covered with spikes as the virus (Figure 2B).

To determine the fusion activity of the virosomes we incorporated the fluorescent phospholipids *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (*N*-Rh-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) in the virosomal membrane. The probes were added to the exogenous lipid mixture before formation of the ether/buffer emulsion. The final probe concentration in the virosomes was 0.6 mol % each (based on membrane phospholipid). Fusion was monitored continuously as an increase of *N*-NBD-PE fluorescence intensity resulting from a decreased resonance energy transfer (RET) between the probes upon their dilution into the target membrane (Struck *et al.*, 1981; Stegmann *et al.*, 1985,1986). The REV virosomes fused efficiently with pure cardiolipin liposomes at pH 5.0 (Figure 3). However, with erythrocyte ghosts as target membranes, fusion activity was negligible over the entire pH range (4.0−7.4) investigated (Figure 3). We have shown previously that the characteristics of the fusion activity of influenza virus toward cardiolipin liposomes deviate in several respects

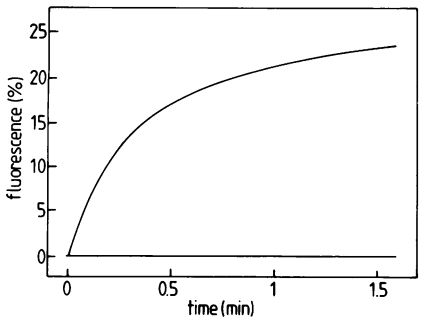

Fig. 3. Fusion of REV virosomes at pH 5.0, 37°C, with cardiolipin liposomes (upper curve) or erythrocyte ghosts (lower curve). Concentration of virosomes or liposomes was 5 μM of phospholipid; erythrocyte ghost concentration was 50 μg/ml of membrane protein.

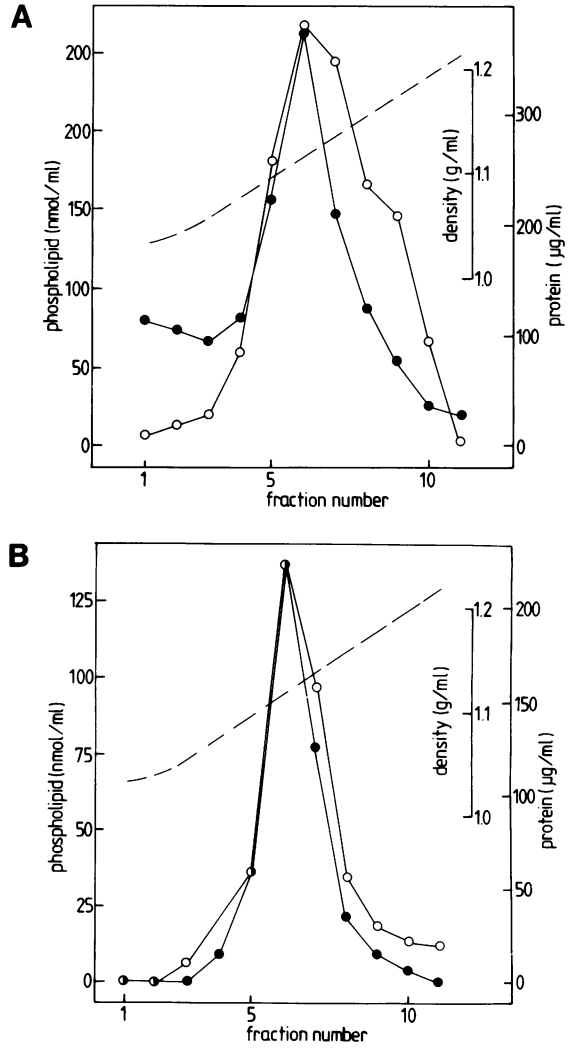

Fig. 4. Equilibrium density gradient centrifugation of $C_{12}E_8$ virosomes on sucrose gradients. **A**, $C_{12}E_8$ virosomes applied directly; **B**, $C_{12}E_8$ virosomes, after a prior purification on a discontinuous sucrose gradient (see text). (●), phospholipid phosphate; (○), protein; dashed line, buoyant density.

from those of the biological fusion activity of the virus (Stegmann *et al.*, 1986). Therefore, virosomes produced by the REV method, although capable of fusing with the negatively charged cardiolipin liposomes, do not possess biological fusion activity.

Recently, comparable results have been obtained with virosomes produced from VSV by dialysis of octylglucoside. The virosomes did fuse with liposomes carrying a negative surface charge (Eidelman *et al.*, 1984), but failed to induce cell–cell fusion, indicating a lack of biological fusion activity (Metsikkö *et al.*, 1986). As an alternative, Metsikkö *et al.* (1986) produced virosomes from VSV using the non-ionic detergent $C_{12}E_8$ and demonstrated full biological fusion activity at low pH. We applied this method to the reconstitution of influenza virus.

$C_{12}E_8$ reconstitution: physical characteristics of the virosomes

Approximately 90% of the viral membrane lipid and 36% of the viral protein were solubilized by 100 mM $C_{12}E_8$. After centrifugation only the subunits of the viral HA protein could be identified in the supernatant by gel electrophoresis. However, there is no reason to assume that only the HA, and not the NA, is solubilized from the viral envelope. It should be noted that with the virus strain used the NA almost co-migrates with the HA_1 subunit on the gel. Internal virus proteins were not present in the supernatant. For reconstitution the detergent was removed by shaking the supernatant with Bio-Beads SM-2 (Metsikkö *et al.*, 1986; Vainstein *et al.*, 1984). The resulting turbid suspension was applied to a linear sucrose gradient for analysis either directly (Figure 4A) or after a prior purification on a discontinuous sucrose gradient (Figure 4B). The purification step on the discontinuous gradient removed some unincorporated lipid (cf. Figure 4A, fractions 1–3) and protein (cf. Figure 4A, fractions 8–10) from the preparation. In addition, it is likely to result in a further removal of residual detergent from the virosomes (Ueno *et al.*, 1984; Metsikkö *et al.*, 1986). Routinely, virosomes isolated from this discontinuous gradient were used for further experiments. The reconstitution yielded a single population of virosomes with a uniform density of 1.12 g/ml (Figure 4B). The virosomes had a protein to phospholipid ratio of 1.42 mg/μmol, identical to the corresponding ratio in the solubilized viral supernatant. The recovery of protein and lipid relative to the viral supernatant was ~20%. The virosomes were densely covered with spikes (Figure 2C). Figure 2D shows an unstained, frozen sample of the virosome preparation, which reveals (arrow) that the spikes are present on both sides of the virosomal membrane. The virosomes are unilamellar and relatively homogeneous in size. Their mean diameter is ~100 nm.

To determine the amount of residual detergent left in the virosomes, 10 μCi [^{14}C]$C_{12}E_8$ was included in 0.6 ml of the initial solubilization mixture, containing 100 mM $C_{12}E_8$ and 900 nmol viral phospholipid. In the virosome preparation, after purification on the sucrose gradient, 0.06% of the radioactivity and 15% of the phospholipid was recovered, corresponding to a molar ratio of detergent to phospholipid of 0.25. However, in agreement with observations of Metsikkö *et al.* (1986), using two-dimensional thin-layer chromatography we found that ~40% of the radioactivity in the virosomes was not $C_{12}E_8$ but a contaminant, which was enriched in the virosomes. At present we do not know what the contaminant is nor do we know its specific radioactivity. The presence of the contaminant implies that the ratio of residual $C_{12}E_8$ to phospholipid in the virosomes was 0.15 rather than 0.25. Since the membrane of the virus contains approximately equal molar fractions of cholesterol and phospholipid the amount of residual $C_{12}E_8$ was 7.5 mol% relative to the total lipid.

$C_{12}E_8$ reconstitution: functional activity of the virosomes

Based on membrane phospholipid, the hemagglutination activity of the virosomes was 4–8 times higher than that of the intact

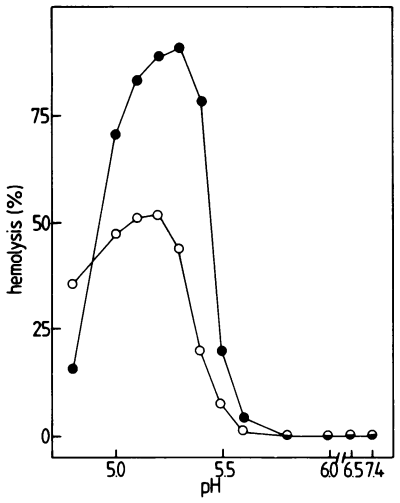

Fig. 5. Hemolysis of human A^+ erythrocytes at a range of pH values, 37°C, for 30 min. (○), intact virus; (●), $C_{12}E_8$ virosomes. Concentration of virus and virosomes was 1 μM of membrane phospholipid; concentration of erythocytes 4 × 10^7 ml.

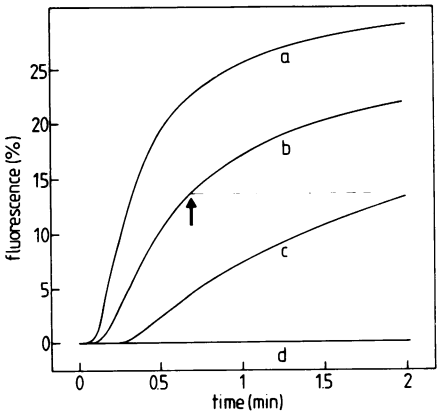

Fig. 6. Fusion of virosomes, labelled with *N*-NBD-PE and *N*-Rh-PE, and R_{18}-labelled intact virus with erythrocytes ghosts at 37°C. **Curve a**, fusion of the virus at pH 5.1; **curve b**, fusion of the virosomes at pH 5.1; **curve c**, fusion of the virosomes at pH 5.3; **curve d**, fusion of the virosomes at pH 5.1 in the presence of a rabbit anti-HA antiserum, added to the cuvette before injection of the virosomes. The arrow indicates pH neutralization by addition of NaOH and the dashed line the fluorescence intensity after this neutralization. Fusion was measured as described in Materials and methods.

virus. We have no explanation for this relative increase in activity.

The hemolytic activity of the virosomes was determined within a range of pH values at an HA concentration that would result in 50% hemolysis by intact virus at the optimal pH (Figure 5). Hemolysis by the virosomes and the virus both increased sharply below pH 5.5 reaching an optimum at pH 5.0–5.2. Particularly in the case of the virosomes, hemolytic activity dropped sharply below the optimal pH. At the optimal pH, virosomes induced nearly twice as much hemolysis as the intact virus. Importantly, no hemolysis was observed at neutral pH, even when the virosome concentration was increased 10-fold. It is possible that the residual detergent in the membranes of the virosomes contributed to destabilize the erythrocyte membrane after fusion at low pH.

To examine the fusion activity of the virosomes the RET probes, *N*-NBD-PE and *N*-Rh-PE, were added to the viral supernatant before removal of the $C_{12}E_8$. Subsequently, fusion was

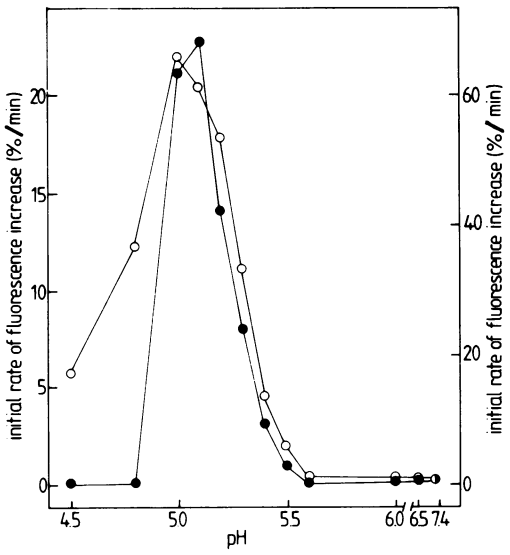

Fig. 7. pH dependence of the initial rate of fusion of R_{18}-labelled intact virus (○, right-hand axis) or virosomes, labelled with *N*-NBD-PE and *N*-Rh-PE, (●, left-hand axis) with erythrocyte ghosts at 37°C. Concentration of virus or virosomes was 2.5 μM membrane phospholipid, concentration of ghosts 50 μg/ml membrane protein.

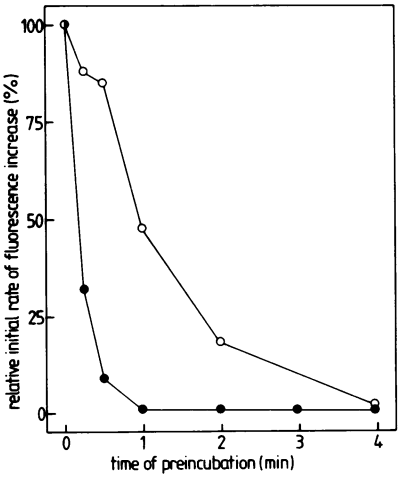

Fig. 8. Initial rate of fusion of R_{18}-labelled intact virus (○) or virosomes labelled with *N*-NBD-PE and *N*-Rh-PE (●) with erythrocyte ghosts at pH 5.1, 37°C, as a function of the time of pre-incubation of virus or virosomes at pH 5.1, 37°C. Fusion is expressed relative to the fusion activity of samples that had not been pre-incubated.

monitored continuously as an increase of *N*-NBD-PE fluorescence intensity upon dilution of the probes into the target membrane. As shown in Figure 6, fusion activity of the virosomes toward erythrocyte ghosts was quite similar to that of the intact virus, monitored by the dilution of octadecyl rhodamine B from the viral into the ghost membrane (Hoekstra *et al.*, 1984; Stegmann *et al.*, 1986). Fusion of the virosomes with erythrocyte ghosts was totally abolished by an antiserum against the virus. Neutralization during fusion instantaneously arrested the fusion reaction. Moreover, fusion of the virosomes with ghosts showed the same pH-dependent lag phase (Figure 6) previously observed with the intact virus (Stegmann *et al.*, 1986).

Figure 7 shows the pH dependence of the fusion of $C_{12}E_8$-reconstituted virosomes with erythrocyte ghosts. Clearly, the pH dependence is essentially identical to that of the fu-

Table I. Fusion of influenza virosomes with BHK-21 cells

Condition		Fluorescence increase (%)
Virosomes	pH 5.0[a]	26.7 ± 1.5
Pre-incubated	pH 5.0[b]	0
Virosomes	pH 7.4[c]	25 ± 1.8
	pH 7.4 + NH_4Cl[d]	8.8 ± 5.2

Virosomes, labelled with *N*-NBD-PE and *N*-Rh-PE, were allowed to bind to cells at 4°C for 1 h. Subsequently fusion with the plasma membrane was induced by replacing the medium with fusion buffer at pH 5.0, 37°C, for 5 min, or the cells were incubated for 1 h at pH 7.4 in Hanks/Hepes buffer, with or without NH_4Cl, at 37°C.
[a]Fusion with the plasma membrane, untreated virosomes.
[b]As [a], but now the virosomes were pre-treated at pH 5.0, 37°C, for 5 min.
[c]Uptake at pH 7.4.
[d]As [c], but in the presence of 20 mM NH_4Cl.

sion of intact virus with the same target membrane. Previously we have shown this pH dependence of fusion to be an important criterion for the biological fusion activity of influenza virus (Stegmann *et al.*, 1986).

In addition to the pH dependence of fusion, a second criterion for biological fusion activity of the virus is presented by the rapid irreversible loss of fusion capacity upon pre-incubation of the virus alone at low pH, before the addition of target membranes (Stegmann *et al.*, 1986). Virosomes also met this criterion: the loss of fusion activity occurred even faster than with the intact virus (Figure 8). Hemolytic activity was also lost after a low-pH pre-incubation of the virosomes alone (not shown).

Finally we examined the interaction of the virosomes with cultured cells. Virosomes labelled with *N*-NBD-PE and *N*-Rh-PE were allowed to bind to BHK-21 cells at 4°C. Then either fusion at pH 5.0, 37°C, with the plasma membrane of cells, or after uptake of the virosomes at neutral pH was measured. Low-pH-induced fusion with the plasma membrane readily occurred (Table I) and was fully inhibited after pre-treatment of the virosomes alone at pH 5.0, 37°C. After 1 h of incubation at 37°C in a medium of neutral pH, a fluorescence increase of 25% was seen, which was inhibited at 20 mM NH_4Cl, an inhibitor of vacuolar acidification (Mellman *et al.*, 1986). The latter observation indicates that virosomes, after internalization through endocytosis, fuse within an intracellular acidic compartment, presumably the endosome (Yoshimura and Ohnishi, 1984).

Discussion

Octylglucoside reconstitution of influenza virus envelopes

Reconstitution of influenza virus envelopes by octylglucoside dialysis produces two density classes of reconstitution products in which viral lipids and spike proteins are largely separated (Figures 1A and 2A). Similar reconstitution products have been observed by Helenius *et al.* (1977,1981) after dialysis of octylglucoside from solubilized envelopes of SFV. The authors suggest (Helenius *et al.*, 1981) that the crucial factor precluding proper protein incorporation in lipid bilayers during removal of the detergent is the aggregation state of the protein at the stage of the process in which vesicles are formed.

In principle, in the course of detergent removal, proteins can become membrane-incorporated by either one of two mechanisms: first, incorporation simultaneous with the formation of lipid bilayers (i.e. at a detergent concentration close to the c.m.c.) or, second, insertion into pre-formed membranes (i.e., below the c.m.c.). The former mechanism would, in general, result in a symmetric orientation of proteins across the membrane, whereas the latter would produce vesicles with an asymmetric (facing outward) protein orientation. Helenius *et al.* (1981) argued that protein incorporation is effectively precluded when, at the stage of lipid bilayer formation, the proteins are stabilized in oligomeric protein/lipid/detergent complexes, unless equilibration between such complexes and monomeric protein would proceed sufficiently fast to permit secondary insertion of proteins into pre-formed vesicles at detergent concentrations below the c.m.c. The rationale behind extremely slow removal of detergent (Eidelman *et al.*, 1984; Harmsen *et al.*, 1985) is to allow for the conversion of the oligomeric complexes to monomeric proteins. However, we did not observe improved protein incorporation in vesicles when the octylglucoside was removed very slowly. Irrespective of the rate of dialysis, protein was recovered predominantly in rosettes and very small protein-rich vesicles, presumably resulting from detergent depletion of oligomeric protein/lipid/detergent complexes. The somewhat improved incorporation of protein into the lipid-rich class of vesicles at high ionic strength points to a stabilization of the oligomeric protein complexes by electrostatic interactions.

In agreement with our observations, the studies of Jackson and Litman (1982) on the reconstitution of rhodopsin by octylglucoside dialysis also indicate the autonomous formation of protein-rich vesicles irrespective of the amount of lipid present. In a more recent study the authors achieved a more homogeneous reconstituted preparation by increasing rather than decreasing the rate of octylglucoside removal (Jackson and Litman, 1985). It was argued that rapid dilution of the detergent would not allow for the separate formation of protein-free and protein-rich vesicles. In our hands rapid dilution of octylglucoside did not result in an improved incorporation of viral spike proteins in vesicles. We did achieve, however, a reasonably effective, although not complete, protein incorporation by dilution of octylglucoside simultaneous with the formation of lipid bilayers in the REV procedure (Figure 1B). This supports the idea that if destabilization of protein/lipid/detergent micelles occurs at the stage of bilayer formation, it does result in an enhanced incorporation of proteins in the membranes. However, the REV virosomes, although possessing hemagglutination activity, did not exhibit any fusion activity towards a biological target membrane (Figure 3), perhaps as a result of the exposure of the HA to ether.

$C_{12}E_8$ reconstitution of influenza virus envelopes

In agreement with observations of Metsikkö *et al.* (1986) on VSV and Vainstein *et al.* (1984) on Sendai virus, reconstitution of influenza virus envelopes by solubilization of the viral membrane in the Triton-like detergent, $C_{12}E_8$, and subsequent detergent removal using Bio-Beads SM-2 produced an essentially quantitative incorporation of spike proteins into a single population of virosomes of uniform buoyant density. Figure 2D shows that the spike proteins are present on both leaflets of the virosomal membrane. This suggests that the protein molecules were incorporated *during* the actual formation of the lipid bilayers. It would appear that this is a prerequisite not only for an efficient protein incorporation, but perhaps also for functional reconstitution. Insertion of viral spike protein molecules into pre-formed vesicles, if this can be achieved at all, may well result in a non-functional conformation of the proteins in the membrane (Metsikkö *et al.*, 1986). For example, the virosomes produced by slow dialysis of octylglucoside from solubilized VSV envelopes by Eidelman *et al.* (1984) have the same protein to lipid ratio as the viral envelope, but they do not exhibit pH-dependent cell–cell fusion activity (Metsikkö *et al.*, 1986). Like the REV virosomes in our study (Figure 3) the reconstituted VSV envelopes in the study of Eidelman *et al.* (1984) fuse only with negatively charged

liposomes. Previously, we have demonstrated that fusion of influenza virus with negatively charged cardiolipin liposomes deviates in several aspects from the biological fusion activity of the virus (Stegmann *et al.*, 1986). Similar observations have been done by Chejanovsky *et al.* (1986), who demonstrated that reconstituted vesicles, containing the binding protein, but not the fusion protein, of Sendai virus fuse efficiently with negatively charged liposomes at low pH. Clearly, this fusion reaction differs from the biological fusion activity of Sendai virus.

Reconstitution employing a detergent with a low c.m.c. has the disadvantage that inevitably some detergent is left in the reconstituted virosomes (Vainstein *et al.*, 1984; Metsikkö *et al.*, 1986). The estimated amount of residual $C_{12}E_8$ in our virosome preparation is 7.5 mol% on the basis of total viral lipids. Yet, it can be excluded that the residual detergent is responsible for the observed fusion activity of the virosomes. Firstly, virosomes pre-incubated briefly at low pH in the absence of target membranes rapidly lose all their fusion and hemolytic activity. Furthermore, at neutral pH virosomes display neither fusion nor hemolytic activity. This suggests that the residual detergent is not present in the external half of the virosomal membrane, but locked in the inner leaflet due to a very slow rate of transbilayer movement.

Functional activity of $C_{12}E_8$ virosomes

The $C_{12}E_8$ influenza virosomes fuse efficiently with erythrocyte ghosts (Figure 6). The pH dependence of this fusion reaction is identical to that of fusion of the intact virus (Figure 7). Previously, we have shown this specific pH dependence to be an important characteristic of the biological fusion activity of the virus, the pH dependence of non-physiological fusion of the virus with negatively charged cardiolipin liposomes being distinctly different (Stegmann *et al.*, 1986). The fusion activity of the $C_{12}E_8$ virosomes toward erythrocyte ghosts at the optimum pH is comparable to that of the virus activity (Figure 6), although the initial rate of fusion was somewhat lower for the virosomes (Figure 7). This may be due to the symmetrical spike distribution in the virosomes (Figure 2D), resulting in a reduced spike density relative to that on the intact virus. In this respect it should also be noted that for the measurement of the fusion activity of the intact virus we employed an assay based on the dilution of octadecyl rhodamine B from the viral into the ghost membrane, since the RET probes cannot be inserted into intact virus particles (Hoekstra *et al.*, 1984). Therefore the small differences in the initial fusion rates of virosomes and intact virus may also be the result of differences between the two assays employed.

Pre-incubation at low pH of the virosomes alone produces a rapid irreversible loss of fusion activity toward biological target membranes (Figure 8). Also in this respect the virosomes closely resemble the intact virus. We have presented evidence (Stegmann *et al.*, 1986) indicating that the inactivation is due to the occurrence of the well-documented conformational change in the viral HA, exposing the hydrophobic N terminus of the HA_2 subunit (Skehel *et al.*, 1982; Doms *et al.*, 1985). In the absence of target membranes this conformational change presumably results in a clustering of the HA molecules (Junankar and Cherry, 1986) and a concomitant irreversible loss of fusion activity. The results in Figure 8 indicate that the conformational change also occurs in HA reconstituted in virosomes. Moreover, the observation that during a low-pH pre-incubation virosomal fusion activity is lost much more rapidly than that of the intact virus (Figure 8) suggests that the HA spikes in the virosomes are highly mobile. This enhanced mobility might be due to the lack of internal viral proteins.

The $C_{12}E_8$ virosomes display hemolytic activity similar to that of the intact virus (Figure 5). Importantly, no hemolytic activity is observed at neutral pH, indicating that it is not the residual detergent in the virosomes that causes the hemolysis. On the other hand, it cannot be excluded that, once pH-dependent fusion of the virosomes with the erythrocyte membrane has occurred, residual detergent facilitates the process of hemolysis.

Future prospects

Functionally active virosomes reconstituted from influenza virus envelopes are ideally suited for delivery of foreign substances into cells. The preliminary results in Table I show that the virosomes have the capacity to fuse with target membranes of nucleated cells, either intracellularly at the level of endosomal or lysosomal compartments after internalization through receptor-mediated endocytosis or directly at the level of the cellular plasma membrane induced by a brief lowering of the pH in the external medium. The size of the virosomes and the method of their preparation allow efficient entrapment of water-soluble compounds, including substances of low mol. wt as the reconstitution procedure does not involve dialysis. Moreover, membrane-associated compounds can be introduced either into the endosomal/lysosomal membranes or directly into the plasma membrane (Van Meer *et al.*, 1985). The HA glycoprotein is quite resistant to proteolytic enzymes and the virosomes retain their fusion activity for weeks during storage in the cold. Furthermore, the complete loss of fusion activity that is induced by a pre-incubation at low pH, while the receptor-binding activity remains unaffected under these conditions (Yewdell *et al.*, 1983) provides an excellent non-fusogenic control virosome preparation).

Materials and methods

Chemicals

N-Rh-PE, *N*-NBD-PE, DOPE, DOPC and cardiolipin (CL) were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Disialoganglioside (G_{Dla}) was from Supelco, Inc. (Bellefonte, PA), octadecyl rhodamine B chloride from Molecular Probes (Junction City, OR). Octaethyleneglycol mono (*n*-dodecyl)ether ($C_{12}E_8$) was obtained from Nikko Chemicals (Tokyo, Japan), octaethyleneglycol mono(*n*-[1-^{14}C]dodecyl)ether ([^{14}C]$C_{12}E_8$) from CEA-Saclay (Gif-sur-Yvette, France), Bio-Beads SM-2 from BioRad (Richmond, CA), sodium cholate and deoxycholate from Calbiochem (La Jolla, CA). Octylglucoside was from Boehringer (Mannheim, FRG).

Virus, liposomes and erythrocyte ghosts

The X-47 recombinant strain of influenza virus was propagated in the allantoic cavity of embryonated eggs, purified, handled and stored essentially as in Stegmann *et al.* (1985). Viral phospholipid phosphate was determined, after extraction of membrane lipids (Folch *et al.*, 1957) by phosphate analysis (Böttcher *et al.*, 1961). Protein was determined according to Peterson (1977). Liposomes (large unilamellar vesicles) were prepared by reverse phase evaporation (Szoka and Papahadjopoulos, 1978) with several modifications (Wilschut *et al.*, 1980) and sized by extrusion through polycarbonate filters with a pore size of 0.2 μm (Olson *et al.*, 1979). After extrusion, any larger or multilamellar liposomes were removed by centrifugation (Szoka and Papahadjopoulos, 1978) and phospholipid phosphate of the liposomes in the supernatant was determined by phosphate analysis (Böttcher *et al.*, 1961). Erythrocyte ghosts were prepared essentially as described by Steck and Kant (1974).

Fusion and hemolysis

For the RET fusion assay (Struck *et al.*, 1981) 0.6 mol% each of *N*-NBD-PE and *N*-Rh-PE were incorporated in the membrane of the virosomes as described in the text. Measurements were carried out at 37°C, under continuous stirring, in a final volume of 1.4 ml 135 mM NaCl, 15 mM sodium citrate, 10 mM Mes, 5 mM Hepes (fusion buffer), set to various pH values. Erythrocyte ghosts (final concentration, 50 μg/ml membrane protein) or cardiolipin liposomes (final concentration, 5 μM phospholipid phosphorus) were present in the cuvette. A small volume of the virosome preparation (35 μl) was injected to a final virosomal phospholipid concentration of 2.5 or 5 μM, as indicated. The increase in fluorescence, due to dilution of the fluorophores into the ghost or liposomal membrane upon fusion, was recorded continuously at excitation and emission wavelengths of 465 and 530 nm, respectively, with an SLM-8000 fluorimeter (SLM/

Aminco, Urbana, IL). For calibration of the fluorescence scale, the initial residual fluorescence of the virosomes was set to zero and the fluorescence at infinite probe dilution to 100%. The latter value was determined by addition of Triton X-100 (0.5% v/v) to the virosomes and subsequent correction of the fluorescence intensity for sample dilution and for the effect of Triton on the quantum yield of the *N*-NBD-PE (Struck *et al.*, 1981). Relative to the fluorescence at infinite probe dilution the residual fluorescence of the virosomes was 30–35%.This value is to be expected at *N*-NBD-PE and *N*-Rh-PE concentrations in the membrane of 0.6 mol% each (Driessen *et al.*, 1985). The rate of fluorescence increase was calculated from tangents drawn to the steepest part of the fusion curve after elapse of the lag phase and taken as a measure of fusion activity.

Fusion of intact virus, based upon relief of self-quenching of octadecyl rhodamine B (Hoekstra *et al.*, 1984), was measured as described before (Stegmann *et al.*, 1986).

Hemolysis was determined by adding 25 μl virus or virosomes to 4×10^7 human A^+ erythrocytes in 0.975 ml buffer, followed by an incubation at 37°C for 30 min and neutralization with 1 M NaOH. Subsequently, the mixture was centrifuged for 10 min at 1350 *g*, the absorbance of the supernatant was determined at 541 nm and taken as a measure of the extent of hemolysis and corrected for autohemolysis in the absence of virus. Maximal hemolysis was determined after lysis of the erythrocytes in distilled water.

Reconstitution

For reconstitution by dialysis of octylglucoside, virus at a concentration of 5 mg/ml protein (1.25 mM membrane phospholipid) was solubilized in 45 mM of octylglucoside in 145 mM NaCl, 2.5 mM Hepes, pH 7.4 (dialysis buffer). After an incubation for 10 min at room temperature, the mixture was centrifuged for 20 min at 140 000 *g* in a Beckman SW 50.1 ultracentrifuge rotor and the supernatant transferred to a dialysis bag with a mol. wt cut-off at 15 000 (Spectrapor-2, Philadelphia, PA) and dialyzed for 48 h against four changes of 1500 vols of dialysis buffer, first at room temperature and, after the first change, at 4°C. Slow dialysis was performed essentially according to Eidelman *et al.* (1984).

For the reverse-phase evaporation (REV) method of reconstitution, 10 mg/ml viral protein was extracted with 60 mM octylglucoside as above. The supernatant was adjusted with NaCl/Hepes to 30 mM octylglucoside, 1 M NaCl, 5 mM Hepes, pH 7.4. Five μmol phospholipid (DOPC/DOPE; molar ratio, 1:1) was dissolved in 1 ml ether, 0.4 ml buffer was added and the two-phase mixture was sonicated in a bath sonicator until a stable emulsion was obtained. Upon removal of ether at 35°C under reduced pressure this mixture formed a gel. The viral supernatant (0.2–0.3 ml) was added to this gel and the gel was caused to collapse by vigorous vortexing. Residual ether was removed by further evaporation under reduced pressure, and residual detergent and NaCl were removed by dialysis against four changes of 1000 vols of dialysis buffer over a period of 48 h at 4°C.

Reconstitution with the detergent $C_{12}E_8$ was carried out according to Metsikkö *et al.* (1986). Briefly, a pellet of influenza virus (1.5 μmol of viral membrane phospholipid) was solubilized in 0.7 ml of 100 mM $C_{12}E_8$ in dialysis buffer for 10 min at room temperature. The mixture was centrifuged at 170 000 *g* for 30 min and 0.56 ml of the supernatant was added to 160 mg wet Bio-Beads SM-2 and shaken at 1400 r.p.m. in an Eppendorf shaker for 1 h at room temperature. To the clear suspension, 80 mg of wet Bio-Beads were added and shaking was continued for 8 min at 1800 r.p.m., yielding a turbid suspension. Routinely, the preparation was centrifuged on a discontinuous sucrose gradient [0.75 ml 40% (w/v) and 3.5 ml 5% (w/v) in 145 mM NaCl, 5 mM Hepes, pH 7.4] at 170 000 *g* for 90 min in a Beckman SW 50.1 rotor.

Analytical

Equilibrium density gradient centrifugation was performed on linear 20–60% (w/v) sucrose gradients in 0.5 M NaCl, 5 mM Hepes, pH 7.4, in a Beckman SW 41 rotor at 140 000 *g* for 48 h (Figure 1A, B) or 10–60% sucrose gradients in 145 mM NaCl, 5 mM Hepes, pH 7.4, in a SW 50.1 rotor at 170 000 *g* for 30 h (Figure 4A, B). Radioactivity was determined in Plasmasol (Packard) in an LKB liquid scintillation counter.

Electron microscopy

For cryo-electron microscopy a small drop of virosomes, incubated for the indicated time at the appropriate temperature and pH in fusion buffer, was applied to carbon-coated copper grids and frozen immediately in liquid ethane (Lepault *et al.*, 1983). Grids were then transferred to liquid nitrogen, into the pre-cooled stage of a Philips EM 400 electron microscope and examined at 80 kV (Booy *et al.*, 1985). For negative staining virosome samples were contrasted with 2% phosphotungstate on carbon-coated Formvar films.

Cells

BHK-21 (C 13) cells were maintained in Glasgows modificaton of Eagles minimal essential medium containing 10% tryptose phosphate broth and 5% fetal calf serum (Flow Laboratories, Irvine, UK). The day before experiments 10^6 cells were plated in plastic Petri dishes (diameter 35 mm). Experiments were performed with subconfluent monolayers of logarithmically growing cells. Before the addition of virosomes, cells were extensively washed with Hanks/Hepes buffer (137 mM NaCl, 5.4 mM KCl, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.41 mM $MgSO_4$, 0.4 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose buffered with 10 mM Hepes (pH 7.4) at 37°C to remove all traces of serum. Subsequently, virosomes labelled with *N*-NBD-PE and *N*-Rh-PE were allowed to bind to the cells at 4°C. After 1 h the cells were washed to remove unbound virosomes. After the experiment, cells were cooled to 4°C, scraped off the dishes with a rubber policeman and transferred to a fluorimeter, all at 4°C. *N*-NBD-PE fluorescence was measured before and after addition of Triton X-100, as described above, and corrected for the background fluorescence of the cells (1–2%).

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