Fusion of Membrane Vesicles Bearing Only the Influenza Hemagglutinin with Erythrocytes, Living Cultured Cells, and Liposomes*

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Membrane vesicles, bearing only the influenza viral hemagglutinin glycoprotein, were reconstituted following solubilization of intact virions with Triton X-100. The viral hemagglutinin glycoprotein was separated from the neuraminidase glycoprotein by agarose sulfonamidic acid column. The hemagglutinin glycoprotein obtained was homogenous in gel electrophoresis and devoid of any neuraminidase activity. A quantitative determination revealed that the hemolytic activity of the hemagglutinin vesicles was comparable to that of intact virions. Incubation of fluorescently labeled hemagglutinin vesicles with human erythrocyte ghosts (HEG) or with liposomes composed of phosphatidylcholine/cholesterol or phosphatidylcholine/cholesterol/gangliosides, at pH 5.0 but not at pH 7.4, resulted in fluorescence dequenching. Very little, if any, fluorescence dequenching was observed upon incubation of fluorescently labeled HA vesicles with neuraminidase or glutaraldehyde-treated HEG or with liposomes composed only of phosphatidylcholine. Hemagglutinin vesicles were rendered non-hemolytic by treatment with NH$_4$OH or glutaraldehyde or by incubation at 85°C or low pH. No fluorescence dequenching was observed following incubation of non-hemolytic hemagglutinin vesicles with HEG or liposomes. These results clearly suggest that the fluorescence dequenching observed is due to fusion between the hemagglutinin vesicles and the recipient membranes. Incubation of hemagglutinin vesicles with living cultured cells, i.e., mouse lymphoma S-49 cells, at pH 5.0 as well as at pH 7.4, also resulted in fluorescence dequenching. The fluorescence dequenching observed at pH 7.4 was inhibited by lysosomotropic agents (methylamine and ammonium chloride) as well as by EDTA and Na$_2$As, indicating that it is due to fusion of hemagglutinin vesicles taken into the cells by endocytosis.

Studies on the biological activity of isolated viral envelope glycoproteins are of crucial importance for the elucidation of the as yet unknown, initial steps of virus-membrane fusion, virus penetration, and infection.

Reconstituted Sendai virus envelopes have been shown to be as fusogenic as intact virions (1). Fusion of viral envelopes with cell membranes necessitated the presence of the two viral envelope glycoproteins, namely the hemagglutinin/neuraminidase and the fusion polyopeptides (1, 2).

In Sendai virus that belongs to the paramyxovirus group, the hemagglutinin binding activity and the neuraminidase are located on the same polyopeptide, the hemagglutinin/neuraminidase glycoprotein, thus preventing studies on the function of the neuraminidase itself in the membrane fusion process (2). On the other hand, in influenza that belongs to the orthomyxovirus group, these activities are located on two different glycoproteins, the hemagglutinin and the neuraminidase (3). The hemagglutinin glycoprotein mediates binding to cell surface receptors and is required for promoting virus-membrane fusion (3). However, the question of whether the neuraminidase glycoprotein also participates in the fusion process is still debatable. From studies using reconstituted viral envelopes, Huang et al. (4) have suggested that the neuraminidase glycoprotein is required for allowing virus-membrane fusion. Reconstituted envelopes bearing both glycoproteins, namely the hemagglutinin and neuraminidase, were fusogenic, whereas those bearing only the hemagglutinin glycoprotein were inactive (4). Recently, White et al. (5) have transformed simian CV-1 cells with plasmids containing the gene for the influenza hemagglutinin glycoprotein. Transformed cells which expressed the hemagglutinin glycoprotein underwent a process of cell-cell fusion at low pH values. These experiments clearly showed that the hemagglutinin glycoprotein by itself is sufficient for promoting membrane fusion. However, the possibility that the process of cell-cell fusion is different from virus-membrane fusion cannot be excluded. Furthermore, such transformed cells may possess a low level of endogenous neuraminidase activity, allowing fusion in the presence of the hemagglutinin glycoprotein only.

In the present study, we have prepared membrane vesicles bearing only the influenza virus hemagglutinin glycoprotein. Hemagglutinin vesicles prepared by this method possessed hemolytic as well as fusogenic activity comparable to that expressed by intact virions. Using a fluorescence dequenching method, we have shown that such hemagglutinin vesicles are able to fuse with erythrocyte membranes and living cultured cells, as well as with phospholipid vesicles lacking virus receptors.

MATERIALS AND METHODS

Chemicals—N-Acetylamino lactose, N-acetylaminoacetic acid, phosphatidylcholine (PC), (Type V-E), cholesterol, gangliosides (bovine brain, Type II), and octyl glucoside were purchased from Sigma.

1. The abbreviations used are: PC, phosphatidylcholine; HEG, human erythrocyte ghosts; R$_{19}$, octadecyl rhodamine B chloride; RIVE, reconstituted influenza virus envelopes; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium.

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SM-2 Bio-beads (20–50 mesh) were obtained from Bio-Rad, Triton X-100 (scintillation grade) was from Koch Light Laboratory Ltd. (Great Britain), neuraminidase (Vibrio cholera) was from Boehringer-werke (Federal Republic of Germany), and octadecyl rhodamine B chloride (Rho) was obtained from Molecular Probes (Junction City, OR).

**Virus-Influenza** (A/Pu strain) was isolated from the allantoic fluid of fertilized chicken eggs (6). The viral hemagglutinating units were determined essentially as previously described (7).

**Cells—Human blood, type O, Rh**, recently outdated, was washed three times in PBS, pH 7.4, and the final pellet obtained was suspended in PBS to give 40% (v/v). The washed erythrocytes were suspended in PBS, pH 7.4, as described before (8). Human erythrocyte ghosts (HEG) were obtained following hemolysis of the human erythrocytes with 40 volumes of 5 mM phosphate buffer, pH 8.0 (9). After three washings with the same buffer, the final pellet of white HEG was suspended in PBS, pH 7.4, to give 4 mg of protein/ml.

Mouse lymphoma S-49 cells were grown in DME medium + 10% horse serum, as described before (10). Prior to use, the cells were washed twice with DME without serum.

**Preparation of Reconstituted Hemagglutinin Vesicles—Influenza virosomes (10 mg) were solubilized by 2% (w/v) of Triton X-100 in a final volume of 1 ml of PBS, pH 7.4, as described before for Sendai virosomes (11).** The viral glycoproteins, present in the clear supernatant obtained after removal of the viral nucleocapsid (100,000 × g, 30 min), were separated by a column of agarose-sulfanilic acid according to Huang (13). Following the coupling process, the agarose beads were introduced into a short column (0.5 cm in diameter) to give a packed volume of 3 ml. The column was washed first with buffer A (50 mM Tricine-NaOH, pH 6.8, 20 mM CaCl₂, and 2% Triton X-100), and then with a solution containing 800 μg of phospholipids (PC: cholesterol, 2:1, mol/mol, 2 mg/ml) in 600 μl of the same buffer and then applied to the agarose-sulfanilic acid column. Following the coupling process, the agarose beads were introduced into a short column (0.5–1 cm in diameter) to give a packed volume of 3 ml. The column was washed first with buffer A (50 mM Tricine-NaOH, pH 6.8, 20 mM CaCl₂, and 2% Triton X-100), and then with a solution containing 800 μg of phospholipids (PC: cholesterol, 2:1, mol/mol, 2 mg/ml) in 600 μl of the same buffer and then applied to the agarose-sulfanilic acid column. Following the introduction of the solubilized viral envelopes, 6 fractions of 2 ml were collected. The column was washed with 10 ml of the same buffer and then with a buffer containing 0.1 M Na₂CO₃/NaHCO₃, pH 9.1, and 2% Triton X-100. Fractions of 2 ml were collected and their pH was immediately adjusted to 6.5-7.0 with 0.1 M acetic acid. The detergent (Triton X-100) was removed from the various fractions by direct addition of SM-2 Bio-beads, keeping the ratio of Bio-beads:Triton X-100 (w/w) at 7:1, as described before (11). Briefly, 280 mg of methanol-washed SM-2 Bio-beads were added to each of the three 2-ml fractions. After 2–3 h of incubation at 20 °C with vigorous shaking, an additional portion of 280 mg of SM-2 Bio-beads was added. Following an additional incubation period of 12–14 h, the turbid suspension obtained was separated from the Bio-beads and centrifuged (100,000 × g, 30 min). The pellet obtained was suspended in 300–500 μl of PBS, pH 7.4. Electrophoresis analysis revealed that the hemagglutinin glycoprotein was present mainly in the second and third fractions of the flow-through of the first buffer. The neuraminidase glycoprotein was present mainly in the second and third fractions of the second buffer, and it was found to be contaminated with the hemagglutinin glycoprotein. About 10% of the viral protein was recovered in the various fractions following removal of the Triton X-100 by SM-2 Bio-beads. The amount of Triton X-100 remaining in the hemagglutinin vesicles was found to be 0.025–0.035% (w/v) by the use of [3H]Triton X-100.

**Preparation of Fluorescently Labeled, Intact Influenza Virions or Hemagglutinin Vesicles—Intact virosomes or hemagglutinin vesicles were labeled with Rho, essentially as described before for Sendai virus (14, 15). Briefly, 2–3 μl of 1.25 mg/ml of ethanolic solution of Rho was rapidly injected into 250 μl of PBS, pH 7.4, containing 400 μg of viral protein. After 15 min of incubation at room temperature in the dark, the viral preparations were washed with 60 volumes of PBS (Eppendorf centrifuge, 15 min). Under such conditions, the Rho was inserted into the viral membranes at self-quenching surface density (about 3 mol % of total viral phospholipids), and its decrease was shown to be proportional to the fluorescence dequenching.

**Preparation of Liposomes—Large, unilamellar vesicles of the following composition: PC, PC:cholesterol (1:0.5, mol/mol), PC:cholesterol:gangliosides (1:0.5:0.3, mol/mol) were prepared by removal of the detergent from the octyl glucoside solution of the appropriate lipids (detergent:lipid, 10:1, mol/mol) as described before (16).**

**Fluorescence Measurements—Fluorescent influenza virosomes or hemagglutinin vesicles (5 μg of each) were incubated with HEG, liposomes, or living cultured cells, in a final volume of 200 μl of PBS, pH 7.4. Following 10 min of incubation at 4 °C, the pH of the medium was adjusted to the desired value by the addition of 50 μl of sodium acetate (0.5 M), and the suspension obtained was then incubated at 37 °C. At the end of the incubation period, a volume of 1 ml of PBS, pH 7.4, was added to the reaction mixture, and the degree of fluorescence (excitation at 560 nm, emission at 590 nm) of each sample was estimated before and after solubilization with 0.1% Triton X-100.** The extent of fluorescence obtained in the presence of the detergent was considered to represent 100% dequenching, i.e., infinite dilution of the probe (14, 15). All fluorescence measurements were carried out with a Perkin-Elmer MFP-4 spectrofluorimeter. Virus preparations were also incubated under the same experimental conditions, in the absence of recipient membranes.

The extent of fluorescence dequenching was calculated as described before (17).

**Determination of the Degree of Hemolysis—Various amounts of either intact virosomes or hemagglutinin vesicles were incubated with 2.5% (v/v) human erythrocytes for 10 min at 4 °C, in a final volume of 800 μl at pH 7.4. At the end of the incubation period, 200 μl of 0.5 M sodium acetate, pH 5.0, were added, and the suspension was further incubated for 15 min at 37 °C. At the end of the incubation period, the degree of hemolysis was estimated at 540 nm, as previously described (7).**

All the experiments described in the present work have been repeated at least three times. However, the data given represent results from one individual experiment. Quantitative differences between independent experiments never exceeded ±5%.

**Protein and Lipid Determinations—Protein was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard. Lipid concentration was estimated by the method of Stewart (19) with PC as a standard.**

### RESULTS

**Characterization and Hemolytic Activity of the Hemagglutinin Vesicles—**The gel electrophoresis pattern seen in Fig. 1A shows that the hemagglutinin vesicles obtained by the present method contain only the hemagglutinin glycoprotein. Under reducing conditions, this glycoprotein appears as the hemagglutinin 1 and hemagglutinin 2 polypeptides (Fig. 1A, c). Neither the neuraminidase glycoprotein which is present in

**Fig. 1. Gel electrophoresis analysis and electron microscopy observations of influenza hemagglutinin vesicles.** In A, intact influenza virosomes (a), RIVE (b) (50 μg of protein each), hemagglutinin vesicles (c), and neuraminidase vesicles (d) (15 μg of protein each) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% acrylamide) according to Laemmli (31). RIVE were prepared as described before for the preparation of reconstituted Sendai virus envelopes (11). The following viral polypeptides can be identified: NA, neuraminidase; NP, nucleoprotein; HA, hemagglutinin 1; HA2, hemagglutinin 2; M, matrix protein. In B, hemagglutinin vesicles were negatively stained with phosphotungstic acid, and prepared for electron microscopy as previously described (11). Magnification × 21,800. C, enlargement of the hemagglutinin vesicles seen in the middle of B (× 54,800).
the reconstituted influenza virus envelopes (RIVE) (Fig. 1A, b) nor the viral nucleoprotein or matrix protein polypeptides can be detected in the hemagglutinin fraction. On the other hand, the neuraminidase fraction (Fig. 1A, d) is contaminated by the hemagglutinin glycoprotein.

Electron microscopic observations revealed that the hemagglutinin fraction consists of closed membrane vesicles with spikes extending from their surfaces (arrows in Fig. 1B), thus resembling envelopes of intact virions.

When identical amounts of viral proteins were studied, it has been observed that the hemagglutinin vesicles possessed hemolytic activity with a degree slightly higher than that expressed by intact virions or RIVE (Table I, see also Fig. 2). Addition of a large excess of external phospholipids greatly inhibited the hemagglutinin hemolytic activity, resulting in the formation of non-hemolytic hemagglutinin vesicles at a protein:l lipid molar ratio of 1:74 (Table I). Electron microscopic observations revealed that most of the hemagglutinin vesicles formed in the presence of a large excess of phospholipids showed the appearance of viral spikes on their surface (not shown).

These preliminary observations confirm previous reports (29) showing that, even upon addition of a large excess of phospholipids, all the vesicles formed contained influenza viral glycoproteins. However, the possibility that the hemagglutinin vesicle preparations contained vesicles with a varied lipid:protein ratio cannot be excluded.

The hemagglutinin vesicles were practically devoid of any

neuraminidase activity (see also Fig. 1A). Conversely, a high degree of neuraminidase activity and a negligible degree of hemolytic activity were detected in the neuraminidase vesicles (Table I).

The hemolytic activity of the hemagglutinin vesicles was dependent upon the amount of protein used (Fig. 2, A and C). Similar to the activity of intact virions, that of the hemagglutinin vesicles was expressed only between pH 5.0 and 5.5 (Fig. 2B). Very little hemolysis, if any, was induced by the hemagglutinin vesicles at pH 6.0 and above.

**Fusion of Hemagglutinin Vesicles with Human Erythrocyte Ghosts and Phospholipid Vesicles**—It has been well established that fusion of fluorescently labeled enveloped virions with recipient membranes results in fluorescence dequenching (14, 15). Indeed, the results in Fig. 3A show that incubation of fluorescently labeled hemagglutinin vesicles with human erythrocyte ghosts resulted in fluorescence dequenching, the extent of which was dependent on the amount of the erythrocyte membranes present in the incubation mixture. Very little increase in the fluorescence dequenching was observed upon incubation at pH 7.4, whereas a significant increase was obtained at pH 5.0 (Fig. 3B). The pH profile of the fluorescence dequenching was similar to that observed for the virus and vesicle hemolytic activities (Fig. 2B). A high increase in the degree of fluorescence was obtained between pH 5.0 and 5.5. It was also clear from the results in Fig. 3A (A and B) that the hemagglutinin vesicles behaved exactly as intact virions in their ability to undergo fluorescence dequenching.

Support for the view that the fluorescence dequenching observed reflects a process of membrane fusion was obtained from the results summarized in Fig. 4. Very little fluorescence dequenching was obtained upon incubation of hemagglutinin vesicles or intact virions, either at pH 5.0 or pH 7.4, with neuraminidase- or glutaraldehyde-treated HEG. Treatment with neuraminidase removes membrane sialic acid residues which are known to serve as receptors for influenza virus particles (3). In addition, glutaraldehyde-treated membranes are resistant to virus-membrane fusion but allow lipid-lipid exchange processes (20).

As opposed to interaction with biological membranes such as HEG, fusion of enveloped viruses with phospholipid vesicles

**TABLE I**

**Influenza hemagglutinin vesicles: Characterization and hemolytic activity**

| Virus preparation | Protein:lipid (molar ratio) | NA activity | Hemolysis pH 7.4 | Hemolysis pH 5.0 |
|------------------|----------------------------|-------------|-----------------|-----------------|
| Intact virions   | 1:22                       | 168         | 3               | 50              |
| RIVE             | 1:3                          | 136         | 5               | 75              |
| HA vesicles      | 1:2.6                       | 0           | 0               | 87              |
| HA vesicles + 300| 1:4.4                       | ND*         | 0               | 40              |
| 7 µg of PL      | HA vesicles + 800           | 1:74        | ND              | 0               |
| 13 µg of PL     | NA vesicles                | 1:3         | 340             | 0               |

* ND, not determined.
The presence of sialoglycolipids (gangliosides) in the liposome virions, the hemagglutinin vesicles fused at pH 5.0, but not at pH 7.4, with liposomes composed of PC:cholesterol and lactating virus receptors (Fig. 1). This was inferred from the increase in fluorescence dequenching which was observed upon incubation with the PC:cholesterol liposomes (Fig. 5). The presence of sialoglycolipids (gangliosides) in the liposome bilayer further increased the extent of fluorescence dequenching obtained following incubation with fluorescently labeled hemagglutinin vesicles (Fig. 5). Fusion of hemagglutinin vesicles, similarly to fusion of intact virions, required the presence of cholesterol in the liposomes. A low extent of fluorescence dequenching was observed at either pH 5.0 or 7.4 with liposomes composed of only PC (Fig. 5).

Treatment of intact influenza virions with hydroxyamine or glutaraldehyde or with incubation at 85°C or at low pH values inactivates their hemolytic and fusogenic activities (22, 23). It has been well established that virus-induced hemolysis reflects a process of virus-membrane fusion (24). The same treatment rendered the hemagglutinin vesicles non-hemolytic (Table II), indicating that hemolysis induced by these vesicles is due to the activity of the viral hemagglutinin glycoprotein. These results show that the fluorescence dequenching observed upon incubation with HEG or with liposomes, results from the same process, namely fusion between the hemagglutinin vesicles and the recipient membranes.

From the results in Table II it is also clear that the non-hemolytic, fluorescently labeled hemagglutinin vesicles as well as the intact virions failed to undergo a process of fluorescence dequenching upon incubation with either HEG or with liposomes composed of PC: cholesterol or PC:cholesterol:gangliosides.

Fusion of Vesicles with Living Cultured Cells—The results in Fig. 6A show that incubation of fluorescently labeled hemagglutinin vesicles with living cultured cells such as mouse lymphoma 5-49 also resulted in fluorescence dequenching. An increase in the degree of fluorescence dequenching was observed following incubation at pH 5.0, as well as at pH 7.4. This is in contrast to the observation with HEG or with liposomes with which a high degree of fluorescence dequenching was observed only at pH 5.0. Very little fluorescence dequenching was observed upon incubation of non-hemolytic, treated hemagglutinin vesicles with lymphoma cells, indicating that the fluorescence dequenching observed at both pH values is due to fusion of the hemagglutinin vesicles with the recipient cells.

The results in Fig. 6B and Table III show that addition of lysosomotropic agents such as methylamine and ammonium chloride (3) greatly reduced the fluorescence dequenching observed upon incubation of hemagglutinin vesicles with lymphoma cells at pH 7.4, but had practically no effect on that observed at pH 5.0. This may indicate that the fluorescence dequenching observed at pH 7.4 is due to fusion with membranes of intracellular organelles such as endosomes or lysosomotropic agents such as methylamine and ammonium chloride (3) greatly reduced the fluorescence dequenching observed upon incubation of hemagglutinin vesicles with lymphoma cells at pH 7.4, but had practically no effect on that observed at pH 5.0. This may indicate that the fluorescence dequenching observed at pH 7.4 is due to fusion with membranes of intracellular organelles such as endosomes or
Fusion of Influenza Hemagglutinin Vesicles

TABLE II
Inactivation of the influenza hemagglutinin glycoprotein fusogenic activity

For inactivation of the intact influenza virions or hemagglutinin (HA) vesicles' fusogenic activity, 300 μg of viral protein in 200 μl were treated as follows: For heat and glutaraldehyde inactivation, a virus suspension in PBS, pH 7.4, was incubated at 85 °C for 30 min and in 0.1% glutaraldehyde for 30 min at 37 °C, respectively. Inactivation by low pH was performed essentially as described before (22) by incubating a virus suspension in sodium acetate (0.5 M, pH 5.0) for 30 min at 37 °C. For treatment with NH₄OH, a virus suspension in 1 M NH₄OH, pH 6.5, was incubated for 30 min at 37 °C as previously described (23). At the end of the incubation, the virus in the various systems was washed twice with 10 volumes of PBS, pH 7.4, resuspended in 200 μl of PBS, and labeled with Rhodamine Chol, cholesterol; gang, gangliosides.

| Virus preparation treatment | Human erythrocytes | HEG | PChol | PChol gang
|-----------------------------|-------------------|-----|-------|-------------|
|                             | % hemolysis       | %DQ | %DQ   | %DQ         |
| None                        | 95                | 40  | 23    | 40          |
| NH₄OH                      | 5                 | 11  | 7     | 12          |
| Glutaraldehyde             | 3                 | 8   | 2     | 6           |
| 85 °C                      | 5                 | 6   | 2     | 8           |
| Low pH                     | 4                 | 10  | 11    | 13          |

Fig. 6. Fusion of hemagglutinin vesicles with living, cultured cells. In A, fluorescently labeled hemagglutinin vesicles (5 μg of viral protein) (a) or hemagglutinin vesicles treated with NH₄OH (b), glutaraldehyde (c), incubated at low pH (d) or at 85 °C (e) (as described in the legend to Table II), were incubated with mouse lymphoma S-49 cells (5 × 10⁶ cells) in a final volume of 200 μl of DMEM, at pH 7.4 (without serum), for 10 min, after which 50 μl of sodium acetate (0.5 M), adjusted to pH 5.0 (□) or pH 7.4 (■), were added. Following 30 min of incubation at 37 °C, the extent of fluorescence dequenching was determined. In B, hemagglutinin vesicles were incubated with mouse lymphoma S-49 cells before (a) or after (b) the cells were treated with methylamine, at either pH 5.0 (□) or pH 7.4 (■). Mouse lymphoma S-49 cells, suspended in DMEM (without serum), at pH 7.5 were incubated for 30 min at 37 °C with 50 mM methylamine. After two washings with 10 volumes of the same medium, the cells were suspended in the same medium which contained 50 mM methylamine to give 2.5 × 10⁶ cells/ml. Hemagglutinin vesicles were then incubated with the treated cells, as described for A.

TABLE III
Interaction of hemagglutinin vesicles with lymphoma S-49 cells:
Effect of lysosomotropic agents and inhibitors of endocytosis

Mouse lymphoma S-49 cells were treated with methylamine (NH₄CH₃), ammonium chloride (NH₄Cl), sodium azide (NaN₃) (50 mM each) or EDTA (5 mM) as described for methylamine in the legend to Fig. 6B. All other experimental conditions were as described in the legend to Fig. 6B.

| Cells incubated with | pH of incubation | %DQ |
|---------------------|-----------------|------|
| None                | 7.4             | 30   |
| NH₄CH₃             | 7.4             | 12   |
| NH₄Cl              | 7.4             | 14   |
| EDTA                | 7.4             | 17   |
| NaN₃               | 7.4             | 15   |

somess, whereas that at pH 5.0 is due to fusion with the cell plasma membrane (3). Further support for this view was obtained from the experiments (Table III) showing that incubation of hemagglutinin vesicles with cells incubated with inhibitors of endocytosis such as EDTA or NaN₃ (25) also resulted in a low degree of fluorescence dequenching.

DISCUSSION

Reconstituted envelopes of fusogenic virions are not only an excellent tool for the elucidation of the molecular mechanism of virus-membrane interaction and fusion but also can be used as a vehicle for microinjection of macromolecules into living cells (1). However, in order to serve as an efficient biological carrier, reconstituted envelopes should possess a high fusogenic activity comparable to that expressed by intact virions. The results of this work clearly show that the hemagglutinin vesicles prepared by the present method are highly hemolytic and fusogenic. Based on quantitative measurements, it appears that the fusogenic activity of the present hemagglutinin vesicles is high, indicating that very little inactivation occurs during their isolation and reconstitution. An apparent 50% reduction in the hemolytic activity of the hemagglutinin vesicles was calculated when its activity was compared to that of the hemagglutinin glycoprotein present in intact virions (Fig. 2C). Our calculations were based on the assumption that the hemagglutinin glycoproteins consist of about 20% of the total viral proteins (32). However, it is noteworthy that the hemagglutinin vesicles were formed by reconstitution and, therefore, it is expected that about 50% of their glycoproteins will be facing the intravesicular space. Taking this into consideration, a 50% reduction in the hemolytic activity is not surprising. From our results, it appears that the hemagglutinin vesicles contained only the hemagglutinin glycoprotein and were practically devoid of the neuraminidase glycoprotein and neuraminidase activity.

Huang et al. (26) and Wharton et al. (27) have solubilized influenza virions or treated the influenza virus glycoproteins with the detergent octyl glucoside. This detergent was used also by others for solubilization of influenza virions and different enveloped viruses (3). In our work, we have solubilized and treated influenza virions with Triton X-100 in the same manner as we used this detergent for the preparation of highly fusogenic, reconstituted Sendai virus envelopes (11). Previously (14), it has been shown that treatment of Sendai virions with octyl glucoside caused inactivation of their hemolytic and fusogenic activities. We have found that solubilization of influenza virions with octyl glucoside instead of
Triton X-100 caused the complete inactivation of the viral fusogenic activity (not shown). Electron microscopic and gel electrophoresis studies revealed that the RIVE prepared by the use of octyl glucoside were identical to those obtained using Triton X-100; namely, they contained a high amount of viral spikes and were composed of the hemagglutinin and neuraminidase glycoproteins. The use of Triton X-100 was avoided by other groups because it is difficult to remove by conventional methods due to its low critical micellar concentration (28). However, it seems that, by the method previously described (11) (that is, by the direct addition of SM-2 Biobeads to the detergent-solubilized virus envelopes), most of the Triton X-100 can be removed. Our results also demonstrated that the hemolytic and fusogenic activities of the hemagglutinin vesicles, similarly to those of intact virions, were expressed only at low pH and were inhibited by conditions which destroy fusogenic activity. This clearly proves that hemolysis and fusion were induced by the viral hemagglutinin glycoprotein and not by the residual amounts of detergent left in this preparation.

In a previous attempt to prepare hemagglutinin vesicles (4, 12), the influenza envelope glycoproteins were applied to an agarose-sulfanilic acid column at pH 5.5. It has been well established that incubation of influenza virus glycoproteins at such low pH causes the rapid inactivation of its biological activity (22). To avoid such an inactivation, we have solubilized influenza virions by Triton X-100 in a medium whose pH was kept at 6.8. The separation of the viral glycoproteins by the agarose column was performed at the same pH.

Furthermore, in experiments performed in our laboratory, we have shown that the agarose-sulfanilic acid column used for the separation of the influenza hemagglutinin and neuraminidase glycoproteins absorbs a large percentage of the virus envelope phospholipids (not shown). A certain amount of phospholipids is required to allow expression of the fusogenic activity of the viral glycoprotein (29). Therefore, in our experiments, the column was washed with a detergent solution containing lipid molecules, thus saturating it with external lipids.

Based on previous observations (14, 15), it should be inferred that the fluorescence dequenching observed reflects a process of virus-membrane fusion. Our results show that incubation of hemagglutinin vesicles with glutaraldehyde-treated erythrocyte ghosts or, conversely, incubation of glutaraldehyde-treated vesicles with non-treated erythrocyte ghosts resulted in a very low degree of fluorescence dequenching. This further supports the view that the fluorescence dequenching observed is due to membrane fusion and not to lipid-lipid exchange processes.

The correlation between the fusion processes and fluorescence dequenching was further emphasized by the observation that treatment of the hemagglutinin vesicles as well as intact virions with hydroxylamine, or preincubation at 85 °C or low pH, significantly reduced the ability of the hemagglutinin vesicles to undergo a process of fluorescence dequenching. All of these treatments have been shown to affect the fusogenic activity of the influenza virions (22, 23).

Fusion of the hemagglutinin vesicles with living cultured cells—as opposed to fusion with erythrocyte ghosts or with liposomes—was observed not only at pH 5.0 but also at pH 7.4. It is conceivable that, at physiological pH, the hemagglutinin vesicles, similarly to intact virions, are taken into intracellular organelles such as endosomes or lysosomes by endocytic activity (3). Subsequent to endocytosis, the hemagglutinin vesicles fuse with membranes of intracellular organelles whose pH was found to be as low as 5.0 (3). This assumption is supported by the results showing that the fluorescence dequenching observed at pH 7.4, but not at pH 5.0, was inhibited by the lysosomotropic agents methylamine or ammonium chloride. Also EDTA and NaN₃, which are known to inhibit endocytosis (25), strongly suppressed the fluorescence dequenching observed at pH 7.4 but not at pH 5.0.

Thus, in their fusogenic ability, the hemagglutinin vesicles, prepared by the method described in the present work, behave in the same manner as intact virions, despite the fact that they are devoid of any neuraminidase activity.

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