

## ROLE OF $Ca^{++}$ IN VIRUS-INDUCED MEMBRANE FUSION

### $Ca^{++}$ Accumulation and Ultrastructural Changes Induced by *Sendai* Virus in Chicken Erythrocytes

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#### ABSTRACT

Some of the ultrastructural (freeze-etching technique), morphological, and biochemical effects of *Sendai* virus interaction with chicken erythrocytes have been studied under fusogenic (in the presence of  $CaCl_2$ ) and nonfusogenic (in the presence of ethyleneglycol-bis-*N,N'*-tetraacetic acid, [EGTA]) conditions. The following phenomena occur, irrespective of the presence of  $CaCl_2$  or EGTA: (a) binding of iodinated virus particles to chicken erythrocytes at 4°C and their partial release from the cells at 37°C; (b) gradual incorporation of the viral envelope and viral M-protein into plasma membrane, as visualized in the protoplasmic and exoplasmic fracture (P and E, respectively) faces of the membrane; and (c) virus-dependent transient clustering of intramembrane particles at 4°C, which is reversible after transferring the cells back to 37°C.

The following virus-induced phenomena occur only in the presence of  $CaCl_2$ : (a) rounding of cells followed by their fusion; (b) transient decrease in the density of intramembrane particles; and (c) the virus induces uptake of  $^{45}CaCl_2$  by chicken erythrocytes. The uptake is specific as it is inhibited by  $LaCl_3$ , and no accumulation of [ $^{14}C$ ]glucose-1-phosphate ([ $^{14}C$ ]G-1-P) could be observed under the  $^{45}CaCl_2$  uptake conditions.

The data show that fusion of virus with plasma membrane is a  $Ca^{++}$ -independent process and, as such, it should be distinguished from the virus-induced membrane-membrane and cell fusion processes. The latter is absolutely dependent on the rise of intracellular  $Ca^{++}$ , as reflected by the fact that  $Ca^{++}$ -induced rounding of chicken erythrocytes always precedes fusion (Volsky, D. and A. Loyter. 1977. *Biochim. Biophys. Acta* **471**:253-259).

**KEY WORDS** *Sendai* virus · membrane fusion ·  $Ca^{++}$  uptake · chicken erythrocytes

Many biological processes that involve membrane fusion, such as secretion of macromolecules from intact cells (24, 25, 45), release of neurotransmitters and neurohypophysial hormones (11), or

differentiation of myoblasts (42), are dependent upon the presence of  $Ca^{++}$  ions. In addition, induction of cell fusion, either by viruses of the paramyxovirus group (31, 44) or by nonviral fusogenic agents (1), usually demands  $Ca^{++}$  also. Despite the extensive research in this field, the role of  $Ca^{++}$  in promoting membrane fusion is still ob-

scure (15). However, some insight into the involvement of  $\text{Ca}^{++}$  in membrane fusion was obtained from experiments with phospholipid vesicles.  $\text{Ca}^{++}$  alone was shown to induce fusion of liposomes containing negatively charged phospholipids, mainly phosphatidylserine (33, 34). In these systems,  $\text{Ca}^{++}$  was shown to increase the transition temperature of the phospholipids and to promote domain separation in the phospholipid bilayer (33, 34). It was suggested that, in the case of exocytosis, similar changes in the physical state of membrane lipids resulting from the increase in intracellular  $\text{Ca}^{++}$  may lead to fusion of secretory vesicles with plasma membrane (34, 35).

In the last few years, we have used chicken erythrocytes as a model system for studying the mechanism of virus-induced cell fusion and the role of  $\text{Ca}^{++}$  in this process (43, 44, 47). It may be assumed that the combination of virus and  $\text{Ca}^{++}$  is essential for inducing cell fusion for the following reasons:

(a)  $\text{Ca}^{++}$  ions may increase the amount of virus particles that are specifically attached to the cell membrane.

(b) Attachment of virus to the cell membrane may expose some of the membrane components (proteins or phospholipids) to which  $\text{Ca}^{++}$  could not otherwise bind. Binding of  $\text{Ca}^{++}$  to these specific sites may be essential for the development of the fusion process. Indeed, it has recently been shown that *Sendai* virus stimulates  $\text{Ca}^{++}$  exchange in Lattree cells (30, 36).

(c) The virus may promote the entry of  $\text{Ca}^{++}$  into the cells. The intracellular  $\text{Ca}^{++}$  may cause cell fusion either directly, by affecting the cell membrane (35, 47), or indirectly, by causing a dissociation of the cell microtubules (40). In this context, it should be mentioned that fusion of chicken erythrocytes can be induced by the combination of  $\text{Ca}^{++}$  and the ionophore A-23187 without the addition of virus (3, 47). Moreover, an influx of  $\text{Ca}^{++}$  is usually associated with the membrane fusion process during exocytotic events such as release of the neurohypophysial hormone (11) or extrusion of secretory granules in mast cells (10).

In the present communication, we have used the freeze-etching technique in order to reveal the ultrastructural changes induced in the chicken erythrocyte membrane by *Sendai* virus. This includes the comparison of the membrane alterations induced by the virus, in a system containing  $\text{Ca}^{++}$  or EGTA, and a study of the successive

stages in the incorporation of viral particles into the plasma membrane. In addition, by using  $^{125}\text{I}$ -virus, we have studied the binding of *Sendai* virus to chicken erythrocytes in the presence of  $\text{Ca}^{++}$  or EGTA. Finally, the possibility that *Sendai* virus may promote  $\text{Ca}^{++}$  accumulation in chicken erythrocytes was also investigated.

The freeze-etching nomenclature used throughout the present work is based on the recent proposition of uniform freeze-etching nomenclature, as outlined by Branton et al. (9).

## MATERIALS AND METHODS

### Cells

Chicken blood was collected from the necks of decapitated chickens into an Erlenmeyer flask containing heparin (100 U/ml). The final sediment was suspended in Solution K to give 5% (vol/vol) ( $3.5 \times 10^8$  cells/ml) or 40% (vol/vol) concentration.

Hepatoma tissue culture (HTC) cells, subclone GM 22-5, were grown as described previously (22). The cells were washed twice in Solution A and finally suspended in Solution A to give 10% (vol/vol) (about  $2 \times 10^7$  cells/ml).

### Salt Solutions

The medium used for the suspension of chicken erythrocytes contained 135 mM KCl, 5.4 mM NaCl, 0.8 mM  $\text{MgCl}_2$  in 20 ml Tricine-NaOH, pH 7.4 (Solution K). The medium used for the suspension of HTC contained 160 mM NaCl and 20 mM Tricine-NaOH buffer, pH 7.4 (Solution A).

### Virus

*Sendai* virus was isolated and its hemagglutinin titer (expressed in hemagglutination units, HAU) determined as previously described (43).

### Virus-Induced Fusion of Chicken Erythrocytes

Fusion of chicken erythrocytes by *Sendai* virus was performed essentially as described before (43), except that the cold preincubation step with the virus was omitted. Chicken erythrocytes suspended in Solution K, 2.5% (vol/vol), were incubated for 5 min at 37°C with gentle shaking (in a New Brunswick shaker [New Brunswick Scientific, New Brunswick, N. Y.] at 100 rpm).  $\text{CaCl}_2$  was then added to the final concentration of 20 mM and, after another 10 min at 37°C, 2,000 HAU/ml of the virus were added (zero time). This resulted in immediate agglutination followed by massive fusion within 10–15 min of incubation at 37°C. The extent of fusion and the dimensions of the polykaryons were found to be unaffected by the omission of the cold preincubation step.

### *Labeling of Sendai Virus with $^{125}\text{I-Na}$*

The method reported before for iodination of bovine serum albumin (BSA) (26) was used with slight modifications for iodination of *Sendai* virus. Iodination under the present conditions did not affect the various viral activities.<sup>1</sup> In short, 10 mg of the viral protein (200,000 HAU) were labeled with 1 mCi of  $^{125}\text{I-Na}$  in the presence of 30  $\mu\text{g}$  of chloramine T. The reaction was terminated with sodium metabisulphate and cold KI. Free  $^{125}\text{I-Na}$  and chloramine T were removed by Sephadex G-25. Iodination resulted in about  $3.6 \times 10^6$  cpm/mg viral protein.

### *Binding of $^{125}\text{I-Sendai}$ Virus to Chicken Erythrocytes*

Chicken erythrocytes, 2.5% (vol/vol), were preincubated with  $^{125}\text{I-Sendai}$  virus at 4°C for 10 min and then transferred to 37°C for further incubation (for details, see legend to Fig. 1). During the incubation period, duplicate samples of 0.1 ml of the virus-cell suspension were withdrawn and layered on top of 1.5-ml cushions of 0.3 M sucrose in Eppendorf polystyrene tubes. After 3-min centrifugation at 12,000 rpm (Eppendorf centrifuge), the supernate was discarded and the tubes were washed with 1 ml of cold Solution K. After additional centrifugation (15 s at 12,000 rpm), the tip of the tube, containing the packed cells, was cut off into a scintillation vial and counted in an Auto-Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

The method described above was found to be satisfactory for separation of unbound  $^{125}\text{I-Sendai}$  virus and chicken erythrocytes. Less than 1% of the free  $^{125}\text{I-virus}$  passed through the sucrose cushion, while either chicken erythrocytes or erythrocyte ghosts readily pelleted under these conditions.

### *Hydrolysis of Chicken Erythrocytes' Sialic Acid by Neuraminidase*

Chicken erythrocytes were desialized essentially as described before (44). 30  $\mu\text{l}$  of neuraminidase (*Vibrio cholerae*, 500 U/ml (Behringwerke AG, Marburg/Lahn, Federal Republic of Germany)) were added to 2 ml, 20% (vol/vol), chicken erythrocytes in 50 mM acetate buffer, pH 5.8, and the mixture was incubated for 2 h at 37°C. The cells were then washed three times in Solution K.

### *Measurement of $^{45}\text{Ca}^{++}$ Uptake*

$\text{Ca}^{++}$  accumulation in chicken erythrocytes was measured essentially as described before (47). Cells in Solution K, 20% (vol/vol), were preincubated with *Sendai* virus (2,000 HAU/ml) for 10 min at 4°C, in the presence of  $^{45}\text{Ca}^{++}$  (0.03  $\mu\text{Ci/ml}$  cell suspension) and a

specified amount of carrier  $\text{CaCl}_2$ . The virus-cell suspension was then transferred to 37°C for further incubation, during which duplicate samples were withdrawn, centrifuged, and  $^{45}\text{Ca}^{++}$  was estimated in their supernate.  $\text{Ca}^{++}$  accumulation in the cells was calculated by subtracting the amount of  $^{45}\text{CaCl}_2$  found in the supernate of cells incubated with the virus from that of cells incubated without the virus (47). Binding of  $^{45}\text{Ca}^{++}$  by virus alone was found to be below the experimental error under the conditions used. Washing of the cells after  $\text{Ca}^{++}$  introduction resulted in release of intracellular  $\text{Ca}^{++}$  (and hemoglobin) to the medium at each wash. Therefore the washing was omitted for kinetic studies.  $^{45}\text{Ca}^{++}$  accumulation of HTC cells in the presence of *Sendai* virus was measured as in chicken erythrocytes, except that 10% cell suspension (vol/vol) and 4,000 HAU/ml of the virus were used.

### *Antiviral Glycoprotein Antibody*

The *Sendai* virus glycoproteins were obtained after solubilization of the virus in ether and Tween-20 according to Hosaka et al. (18). The envelope glycoproteins were injected into rabbits and the antiserum was collected as described by Sato et al. (41). The antibody prevented virus-induced agglutination, hemolysis, and fusion of intact erythrocytes.

### *Freeze-Etching and Electron Microscopy*

Cells were fixed at a given temperature by addition of glutaraldehyde (Ladd Research Industries, Inc. Burlington, Vt.) to a final concentration of 1% (vol/vol), and then freeze-etching was performed as previously described (13). Micrographs were obtained in a Phillips EM 300 operating at 80 kV. In freeze-fracture illustrations, the encircled arrowhead indicates the direction of shadow.

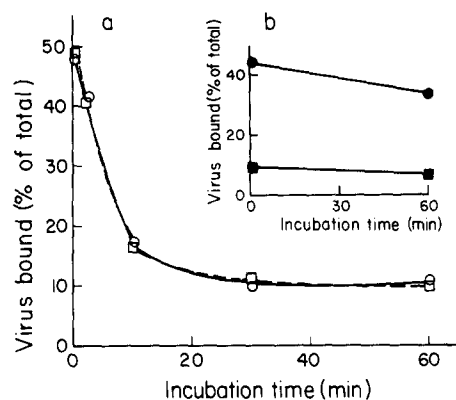
All reagent chemicals used were commercially obtained and were of analytical grade. The ionophore A-23187 was a generous gift from Eli Lilly & Co. (Indianapolis, Ind.) and was dissolved in 95% ethanol (0.3–3 mg/ml).  $^{45}\text{Ca}^{++}$  carrier free (2 mCi/ml) was obtained from New England Nuclear (Boston, Mass.).  $^{125}\text{I-Na}$  (100 mCi/ml) and [ $^{14}\text{C}$ ]glucose-1-phosphate (4 mCi/mmol) were purchased from Radiochemical Centre Ltd. (Amersham, England).

## RESULTS

### *Effect of $\text{Ca}^{++}$ and Anti-Sendai Antiserum on the Binding and the Elution of Sendai Virus from Chicken Erythrocytes*

Fig. 1 shows that when 2,000 HAU of  $^{125}\text{I}$ -labeled *Sendai* virus were added in the cold to a

<sup>1</sup> Wolf, D., and A. Loyter. Manuscript in preparation.



**FIGURE 1** Release of <sup>125</sup>I-Sendai virus from chicken erythrocytes during their incubation at 37°C. (a) <sup>125</sup>I-Sendai virus (2,000 HAU/ml, 400 cpm/HAU) was added to chicken erythrocytes (2.5% vol/vol) suspended in Solution K containing either 20 mM CaCl<sub>2</sub> (○—○) or 2 mM EGTA (□—□). The cells were incubated for 10 min at 4°C, at the end of which duplicate samples were withdrawn for virus binding measurements, as described in Materials and Methods (zero time). The systems were then immediately transferred to 37°C for further incubation with gentle shaking. No fusion was observed in the system containing EGTA, while extensive fusion could be observed after 15 min of incubation at 37°C in the presence of CaCl<sub>2</sub>. (b) Untreated (●—●) or neuraminidase-treated (desialized) erythrocytes (■—■) (2.5% vol/vol) were incubated with <sup>125</sup>I-Sendai virus (2,000 HAU) for 10 min at 4°C and further incubated as described in Fig. 1a, except that the viral antibody (see Materials and Methods) was added to the virus-agglutinated untreated cells in the cold (●—●).

suspension of 2.5% (vol/vol) of chicken erythrocytes, about 50% of the total amount of the virus was bound to the cells (Fig. 1a). Binding of virus is specific, as neuraminidase-treated cells lost almost all their binding capacity (Fig. 1b). Fig. 1a also shows that 60–65% of the bound virus is released from the cells during the first 10 min of incubation at 37°C. (In other experiments, 45–50% of the bound virus was released during the same period.) Addition of Ca<sup>++</sup> did not affect the binding capacity nor the elution kinetics of the virus. The absence of Ca<sup>++</sup>, however, had a profound effect on the development of fusion. In experiments shown in Fig. 1 and throughout the present work, fusion was completely prevented when chicken erythrocytes were incubated with Sendai virus in the presence of EGTA (see also references 31, 43, and 49). Interestingly, when antiviral antiserum was added in the cold to virus-

agglutinated chicken erythrocytes, it inhibited the release of the virus at 37°C (Fig. 1b). Under these conditions, both the hemolytic and the fusion activities of the virus were blocked (not shown).

#### *Ultrastructural Changes Induced in Chicken Erythrocyte Membranes during Virus-Cell Interaction*

Incubation of chicken erythrocytes with 20 mM CaCl<sub>2</sub> for 10 min at 37°C did not cause any changes in the normal distribution pattern of the intramembrane particles (Fig. 2a). Addition of virus, which caused an immediate agglutination of the cells, induced an apparent reduction in the density of the intramembrane particles within the first 2 min, as was observed in the replica of the protoplasmic fracture (P) and exoplasmic fracture (E) faces of these cells (Fig. 2b and c). Quantitative estimation of the particles' density in the P- and E-fracture faces of those cells (Fig. 3) shows the significance of the observed phenomenon: The number of the intramembrane particles in the P-fracture face decreased from more than 4,200 particles/μm<sup>2</sup> at zero time to less than 2,900 particles/μm<sup>2</sup> after addition of the virus.

An important observation was the virus-dependent induction of thermotropic separation of the intramembrane particles. Cells that were first incubated with the virus from 1 to 5 min at 37°C (Figs. 2b and c) and then transferred to the cold showed a high degree of the intramembrane particles' clustering in both the P- and E-fracture faces of the fractured membranes (Fig. 2d and e). Complete redistribution of the intramembrane particles occurred when the cells were transferred back from the cold to 37°C for several minutes, showing that the virus-induced thermotropic separation of the intramembrane particles is fully reversible (not shown).

Observations by phase microscopy revealed that cell fusion was initiated between 3–5 min of incubation and was completed after 8–10 min (scheme in Fig. 5). A survey of the freeze-fractured membranes showed that formation of polyerythrocytes was accompanied by gradual increase in the density of the intramembrane particles in the P-fracture face (Fig. 3), reaching about 5,000 particles/μm<sup>2</sup> after 6–8 min of incubation (Figs. 2 and 3). Interestingly, the intramembrane particles of the fused cells were found to be refractile to the thermotropic separation. The

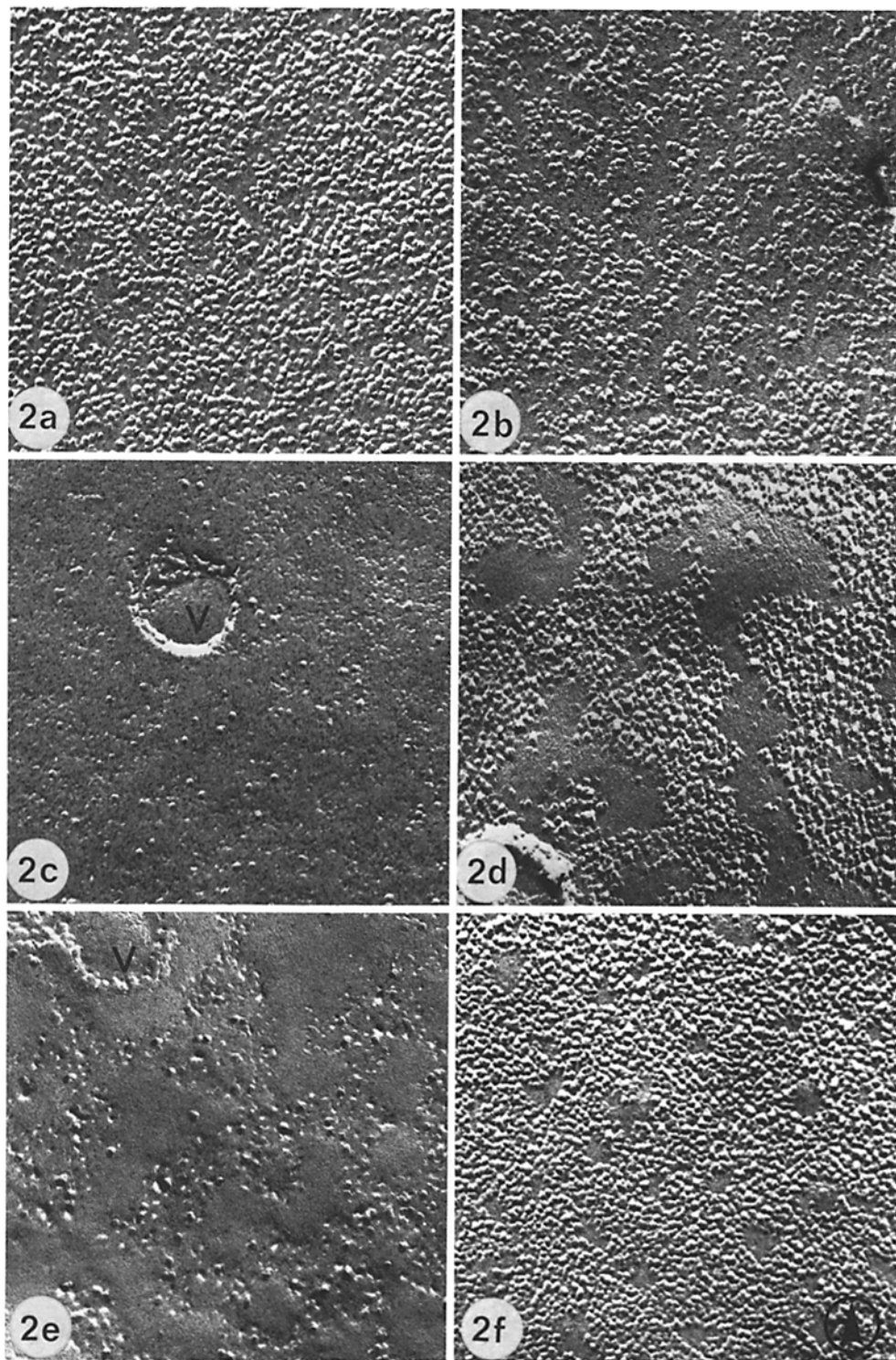
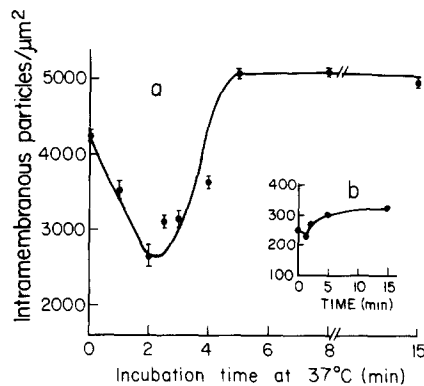


FIGURE 2 Virus-induced thermotropic separation of intramembrane particles in freeze-fractured chicken erythrocytes. Chicken erythrocytes were fused by *Sendai* virus (2,000 HAU/ml) in the presence of  $\text{CaCl}_2$  (20 mM), as described in Materials and Methods. (a) Replica of P-fracture face of cells preincubated before addition of the virus for 10 min at 37°C (control). (b) Replica of P-fracture face of cells after 2-min incubation with the virus at 37°C. (c) Same as b but showing the E-fracture face. (d and e) P- and E-fracture faces, respectively, of cooled membranes of fusing cells. 0.5 ml of cell suspension from the system described in b were transferred to 14 ml of cold Solution K containing EGTA (2 mM). After 5 min at 4°C, cells were fixed and freeze-fractured. Note the large smooth areas devoid of intramembrane particles in d and characteristic circular arrangement of the particles in e. (f) The P-fracture face of cooled polyerythrocyte membrane obtained after 10-min incubation of cells with virus at 37°C and then transferred to 4°C, before fixation, using the procedure described in d. V, virus particle. (a-f)  $\times 100,000$ .



**FIGURE 3** Changes in intramembrane particles' density during virus-induced fusion of chicken erythrocytes. Experimental conditions as described in Materials and Methods and the legend to Fig. 2. Briefly, the cells were incubated with *Sendai* virus (2,000 HAU/ml) at 37°C. At each time-point indicated, samples were removed and immediately fixed with glutaraldehyde (1%, vol/vol, final concentration) at the same temperature. Each point represents an average of the particles' density calculated from 8 to 10 micrographs. For each set of numbers, the statistical error mean  $\pm$ SEM was calculated. The counting on individual micrographs was performed by random sampling of at least 30% of the total area of a micrograph of P-fracture face and the entire micrograph area of the E-fracture face. Thus, for each number presented in this figure, approx. 6,000–18,000 particles were counted on the P-fracture face, and about 2,000–5,000 particles on the E-fracture face. In most cases, micrographs of a final magnification of 150,000 were used. (a and b) Intramembrane particles' density of P- and F-fracture faces, respectively.

intramembrane particles of polyerythrocytes appeared highly dense and evenly distributed, both at 37°C (not shown) and at 4°C (Fig. 2*f*), thus showing the dependency of the thermotropic separation upon the incubation time with the virus at 37°C (see also Fig. 5).

The initial reduction in the intramembrane particles' density seen in the fusing systems (Figs. 2, 3, and 5) could not be detected in the P fracture face of cells incubated with the virus in the presence of EGTA (Fig. 4*a*). However, when these virus-agglutinated cells were transferred to the cold after 3–5 min of incubation at 37°C, clustering of the intramembrane particles was induced (Fig. 4*b*), although it was less pronounced than the clustering observed in the presence of  $Ca^{++}$  (cf. Fig. 2*d* with Fig. 4*b*).

Fig. 5 summarizes the effect of antiviral antibody on virus-induced fusion, alteration in the

intramembrane particles' density, and thermotropic separation of the particles. When the antiviral antiserum was added at 37°C, immediately after induction of agglutination by the virus (zero time of incubation), it inhibited both the development of membrane fusion and the reduction in the intramembrane particles' density. The particles in the P fracture face of these cells did not show thermotropic separation at any point along the incubation period at 37°C. When the antibody was added after 2-min incubation at 37°C, it inhibited formation of polyerythrocytes but preserved the virus-dependent, cold-induced clustering of the intramembrane particles. These nonfusing cells exhibited thermotropic separation of the intramembrane particles, even after prolonged incubation at 37°C (Fig. 5), as opposed to fused cells in which the particles could be clustered in the cold only during the first stages (initial 1–5 min) of the fusion process (Figs. 2 and 5). When the antibody was added after membrane fusion was started (5 min of incubation at 37°C), it did not affect any of the virus-induced changes seen in chicken erythrocytes.

#### *Microscope Evidences for the Fusion of Sendai Virus Envelope with the Chicken Erythrocyte Membrane in the Presence and Absence of $Ca^{++}$*

Fig. 6*a* shows what probably is the first step in the interaction between *Sendai* virus and the plasma membrane of chicken erythrocytes. The intimate association between the virus envelope and its receptor on the surface of the membrane causes folding of the cell membrane, thus creating a small depression. Indeed, a virus particle that has been fused and became incorporated into the membrane can be visualized (Fig. 6*b*) when the P-fracture face of the chicken erythrocyte is examined. Fig. 6*c* shows a side view of a membrane in which an early stage of virus-cell interaction can be seen. The virus penetrates the membrane through the edge of its envelope in a narrow and restricted region. Two consecutive steps in the process of virus fusion with the cell membrane are seen in Fig. 6*d* and *e* which show the E-fracture face of the membrane. In Fig. 6*d*, the contours of the virus particle can still be identified, although most of the particle has already fused with the cell membrane. In Fig. 6*e*, only the remnants of the virus particle can be seen after its outer membrane

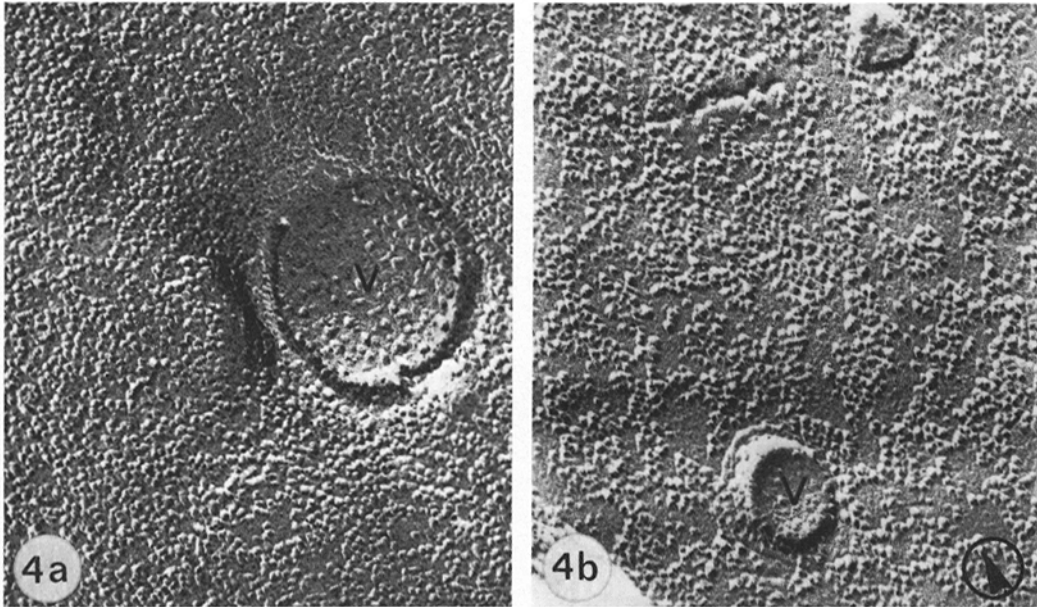


FIGURE 4 Thermotropic separation of intermembrane particles in chicken erythrocytes incubated with *Sendai* virus in the presence of EGTA. Chicken erythrocytes were incubated with *Sendai* virus (2,000 HAU/ml) essentially as described in Materials and Methods for the fusion system, except that the  $\text{CaCl}_2$  was omitted and EGTA (2 mM) was added. No fusion was observed. (a) P-fracture face of cell membranes obtained after 2-min incubation with the virus at 37°C. (b) Membranes' P-fracture face from the system described in a but cooled at 4°C for 5 min before fixation. V, virus particle. (a and b)  $\times 100,000$ .

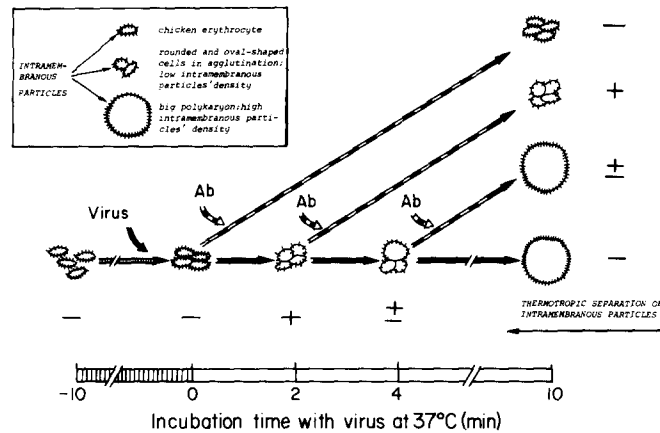
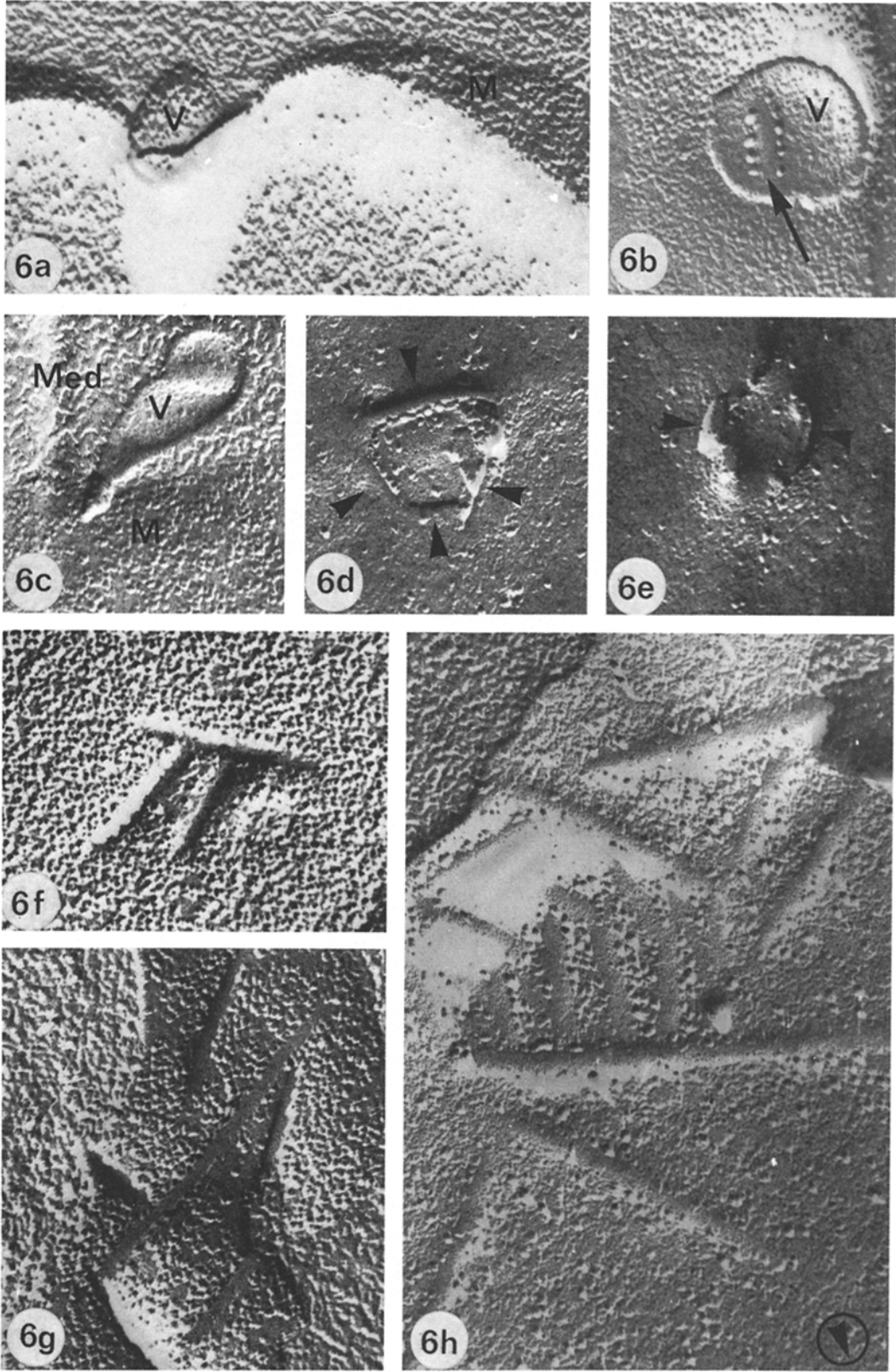


FIGURE 5 Effect of antiviral antiserum on virus-induced fusion of chicken erythrocytes and on cold-induced clustering of their intramembrane particles. The figure presents a schematic summary of an experiment combining observations in phase and electron microscopy. Experimental conditions and the procedure of cooling the cells for testing the thermotropic separation of intramembrane particles were as described in the legend to Fig. 2. At each time-point indicated in the scheme, the incubation mixture was divided into four parts: The first was incubated further at 37°C; the second was fixed for freeze-etching at 37°C; the third was cooled at 4°C before fixation; and to the last part the antiviral antibody was added at 37°C. The system containing the virus antibody was incubated for another 10 min at 37°C and then fixed either immediately at 37°C or after cooling at 4°C, as described in the legend to Fig. 2. Ab, antiviral antiserum.





has been completely fused with and melted into the E-fracture face of the cell membrane.

The isolated internal protein of *Sendai* virus (M-protein) appears in the electron microscope as aggregates of long filaments and rods which can be as long as 0.5–1  $\mu\text{m}$  (16). Fig. 6*f* and *h* probably represent these filaments (M-protein) after or during their incorporation into the chicken erythrocyte membrane. The P-fracture face is interrupted by depressions of various lengths (Fig. 6*f* and *g*), while the complementary long protrusions in the E-fracture face can be seen in Fig. 6*h*. In addition, a rearrangement in the internal part of the viral envelope, which could lead to the integration of the filamentous M-protein into the cells' plasma membranes, can be distinguished in Fig. 6*b* (arrow). The freeze-fractured membranes containing the rod structures were more abundant in an EGTA-containing system as compared to a  $\text{CaCl}_2$  system. The significance of this observation is not yet clear. It should be stressed that very few or none of the above described signs of virus incorporation could be distinguished in fractured membranes of cells incubated for more than 4–5 min after addition of the virus at 37°C, irrespective of the development of membrane fusion (in the presence of  $\text{CaCl}_2$ ) or lack of membrane fusion (EGTA) in the system.

#### *Virus-Induced $\text{Ca}^{++}$ Accumulation in Chicken Erythrocytes and HTC Cells*

Table I shows that *Sendai* virus stimulated the uptake of  $\text{Ca}^{++}$  ions into chicken erythrocytes, while under the same conditions movement of glucose-1-phosphate was not affected. Low concentration of  $\text{La}^{+++}$  (0.07 mM) completely blocked the virus-induced  $\text{Ca}^{++}$  uptake (Table I).  $\text{La}^{+++}$  is known to possess a high affinity for  $\text{Ca}^{++}$ -binding sites, and was shown to replace  $\text{Ca}^{++}$  in several biological systems (50).

About 60% of the total amount of  $\text{Ca}^{++}$  introduced in chicken erythrocytes after incubation

with *Sendai* virus for 30 min at 37°C could be released either by hypotonic hemolysis or addition of Triton X-100 (1% vol/vol) to the cells. The remaining  $\text{Ca}^{++}$  was found to be tightly associated with the membranes (not shown). (See also reference 47.)

Fig. 7*a* shows that  $\text{Ca}^{++}$  uptake was highly dependent on virus and  $\text{Ca}^{++}$  concentrations in the medium. Incubation of the cells with the virus also induced a certain degree of hemolysis (numbers in brackets in Fig. 7*a*). However, an inverse relationship was detected between the amount of  $\text{Ca}^{++}$  accumulated and the degree of hemolysis when the cells were incubated with a fixed amount of *Sendai* virus and increasing concentrations of  $\text{Ca}^{++}$  (Fig. 7*a*). Kinetic studies revealed that about 50% of the maximal  $\text{Ca}^{++}$  was accumulated during the first 10 min, during which the degree of virus-induced hemolysis was negligible (Fig. 7*b*).

Essentially, the same results were obtained by incubation of HTC cells with *Sendai* virus and  $\text{Ca}^{++}$  (Table II). As can be seen in this table, a similar, though smaller, rise in intracellular  $\text{Ca}^{++}$  concentration per 1,000 HAU of the virus was observed in HTC cells as compared to chicken erythrocytes.

#### *Is the Rise in Intracellular $\text{Ca}^{++}$ Essential for Virus-Induced Fusion of Chicken Erythrocytes?*

Fusion could be promoted by *Sendai* virus even in the presence of EGTA, provided the cells were first preconditioned with intracellular  $\text{Ca}^{++}$ . The latter can be achieved by incubating chicken erythrocytes with  $\text{CaCl}_2$  and the ionophore A-23187, followed by extensive washings as previously described (47). As can be seen in Table III, a high degree of fusion was obtained after a short period of incubation (2–3 min) at 37°C when low amounts of virus were incubated with these  $\text{Ca}^{++}$ -

FIGURE 6 Successive steps in the incorporation process of virus particles into chicken erythrocyte membranes in the presence and absence of  $\text{Ca}^{++}$ . Chicken erythrocytes were incubated with *Sendai* virus at 37°C in the presence of  $\text{CaCl}_2$  or EGTA, as described in the legend to Figs. 2 and 4 respectively. Freeze-fracturing was performed 1–5 min after the virus addition. (a) Whole virus particle is seen on the P-fracture face of the membrane. (b) Internal part of the viral envelope with rearrangement of its proteins (arrow) – P-fracture face. (c) Deformed viral particle during penetration of the membrane – P-fracture face. (d and e) Remnants of the viral envelope (arrowheads) on the E-fracture face of the membrane. (f and g) Long depressions on the P-fracture face. (h) Complementary protrusions on the E-fracture face. V, viral particle; M, membrane; Med, surrounding medium. (a–h)  $\times 100,000$ .

TABLE I  
Specificity of Virus-Induced  $Ca^{++}$  Uptake by Chicken Erythrocytes: Effect of G-1-P and  $La^{+++}$

System	$[^{14}C]G-1-P$ uptake	$^{45}Ca$ uptake
	$\mu mol/l$ ml 20% erythrocytes	$\mu mol/l$ ml 20% erythrocytes
Exp I		
$^{45}CaCl_2$ (2 mM)		0.12
$^{45}CaCl_2$ (2 mM) + virus		0.47
$^{45}CaCl_2$ (2 mM) + G-1-P (2 mM)		0.11
$^{45}CaCl_2$ (2 mM) + G-1-P (2 mM) + virus		0.45
$^{45}CaCl_2$ (2 mM) + G-1-P (10 mM) + virus		0.42
$^{14}C-G-1-P$ (2 mM)	0.14	
$^{14}C-G-1-P$ (10 mM)	0.10	
$^{14}C-G-1-P$ (2 mM) + $CaCl_2$ (2 mM)	0.03	
$^{14}C-G-1-P$ (10 mM) + $CaCl_2$ (10 mM)	0.10	
$^{14}C-G-1-P$ (2 mM) + $CaCl_2$ (2 mM) + virus	0.03	
$^{14}C-G-1-P$ (10 mM) + $CaCl_2$ (10 mM) + virus	0.13	
Exp II		
$^{45}CaCl_2$ (2 mM) + $LaCl_3$ (0.01 mM)		0.08
$^{45}CaCl_2$ (2 mM) + $LaCl_3$ (0.01 mM) + virus		0.48
$^{45}CaCl_2$ (2 mM) + $LaCl_3$ (0.07 mM)		0.10
$^{45}CaCl_2$ (2 mM) + $LaCl_3$ (0.07 mM) + virus		0.13

$Ca^{++}$  accumulation in chicken erythrocytes in the presence of *Sendai* virus was assayed after 30-min incubation at 37°C, as described in Materials and Methods. 1,200 HAU/ml of the virus were used and a given amount of glucose-1-phosphate (Exp I) or  $LaCl_3$  (Exp II).  $[^{14}C]G-1-P$  accumulation in chicken erythrocytes was measured after 30-min incubation at 37°C using the  $Ca^{++}$  uptake method and  $Ca^{++}$  uptake system, in the presence of nonradioactive  $CaCl_2$  at a given concentration and 1,200 HAU/ml of the virus (Exp I).

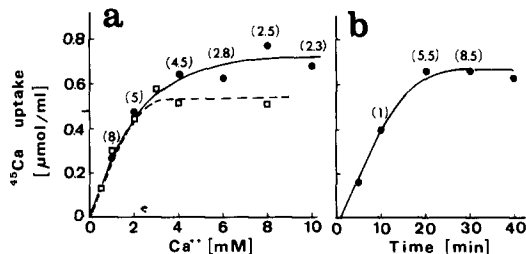


FIGURE 7 *Sendai* virus-induced  $Ca^{++}$  uptake by chicken erythrocytes. For experimental conditions, see Materials and Methods. (a)  $Ca^{++}$  concentration curve.  $^{45}Ca$  accumulation in chicken erythrocytes was measured after 30 min. Cells were incubated at 37°C with 600 ( $\square$ - $\square$ ) or 1,200 ( $\bullet$ - $\bullet$ ) HAU/ml of *Sendai* virus. (b) Time curve at 37°C. Cells were incubated with 1,200 HAU/ml of the virus and 2 mM of  $^{45}CaCl_2$ . Numbers in brackets in a and b show the extent of lysis (in percentage) in a system.

treated cells in the presence of EGTA. No fusion was observed with untreated cells under the same conditions.

#### DISCUSSION

The present work is an attempt to study some of the effects of *Sendai* virus action on chicken

erythrocytes and to correlate them with the cells' ability to undergo fusion.

The results of this work, as well as some of our previous reports (23, 27, 37, 47), are summarized in a scheme presented in Fig. 8. This scheme represents our view of the sequence of some of the virus-induced structural changes which eventually lead to membrane fusion.

The first noticeable result of the *Sendai* virus interaction with chicken erythrocytes is binding of the virus to the cells, thus causing their immediate agglutination. Subsequently, the envelopes of the viral particles that remained attached to the cell surface (Fig. 1) fuse with the cells' plasma membranes at 37°C, thereby allowing integration of viral proteins into the membrane of the recipient cell. Incorporation of viral antigens into membranes of cells undergoing fusion has been shown previously by using antiviral antibodies (7, 49) and by identifying specific viral proteins among the various proteins of fused plasma membranes (21, 32). Attachment of whole virus particles to the P- and E-fracture faces of plasma membranes has been also demonstrated before by use of the freeze-etching technique (5, 6).

TABLE II  
Comparison of the Sendai Virus-Induced Ca<sup>++</sup> Uptake by Chicken Erythrocytes and HTC

Cells	No. of cells in 1 ml 10% suspension	Intracellular	Ca <sup>++</sup> in the incubation medium		Maximal Ca <sup>++</sup> uptake	
		Ca <sup>++</sup>	with virus		$\mu M/1,000$ HAU	mol Ca <sup>++</sup> /cell/HAU
		$\mu M$	$mM$			
Chicken erythrocytes	$7 \times 10^8$	40*	0.5	620	$0.83 \times 10^{-19}$	
			2.0	2,100	$3.15 \times 10^{-19}$	
HTC	$0.2 \times 10^8$	10‡	0.5	110	$5.50 \times 10^{-19}$	
			2.0	175	$8.75 \times 10^{-19}$	

<sup>45</sup>Ca<sup>++</sup> accumulation experiments were performed as described in Materials and Methods. Maximal Ca<sup>++</sup> uptake was measured in chicken erythrocytes after 30-min incubation at 37°C with 1,200 HAU of the virus per 1 milliliter 20% cells (vol/vol), and in HTC cells after 15-min incubation at 37°C, using 4,000 HAU of the virus per 1 milliliter 10% cells (vol/vol).

\* This number has been reported for human erythrocytes (39).

‡ Number reported for HeLa cells and monkey kidney cells grown in culture (8).

TABLE III  
Effect of Intracellular Ca<sup>++</sup> on Virus-Induced Fusion of Chicken Erythrocytes

	Virus (HAU)	CaCl <sub>2</sub>	EGTA	Cells fused	Time to fusion
		$mM$	$mM$	% of total	min at 37°C
(A) Untreated fresh cells					
	800	—	2	0	
	800	20	—	20-30	50-60
	2,400	—	2	0	
	2,400	20	—	80-100	15
(B) Ca <sup>++</sup> and ionophore-treated cells					
	800	—	2	80-100	2-3
	800	20	—	80-100	2-3

(A) Sendai virus and chicken erythrocytes (2.5% vol/vol) were preincubated for 10 min at 4°C in the presence or absence of CaCl<sub>2</sub> and then transferred to 37°C for further incubation. (B) Chicken erythrocytes (2.5% vol/vol) were incubated with CaCl<sub>2</sub> (1 mM) and ionophore A-23187 (3  $\mu g/ml$ ) for 10 min at 37°C. The treatment was followed by four consecutive washings in Solution K containing EGTA (1 mM). Finally, the cells were resuspended in Solution K to give a final concentration of 2.5% (vol/vol) (=Ca<sup>++</sup> and ionophore-treated cells). Then Sendai virus and CaCl<sub>2</sub> or EGTA were added, and the mixture was incubated for 10 min at 4°C and then transferred to 37°C.

The present work shows that, in addition to the virus envelope proteins, the M-protein of the virus also is probably integrated into the plasma membrane of chicken erythrocytes (Fig. 6). It should be noted that during the preparation of this manuscript a work was published showing, by the freeze-etching technique, fusion of Sendai virus particles with the human erythrocyte membrane (20). The electron micrographs presented in that publication are similar to those shown in the present work. The authors suggested that the long rods (seen in membranes in which the virus was incorporated) are part of the virus envelope (20). We propose, however, that the long structures are

filaments of viral M-protein since similar structures were seen in electron micrographs of pure M-protein (16).

Neither binding nor incorporation of the virus into the cell membrane requires Ca<sup>++</sup> (Figs. 1 and 6). Recent electron microscope studies in our laboratory, with ferritin conjugated antibodies, clearly showed the incorporation of Sendai virus antigens into membranes of chicken erythrocytes which had been incubated with the virus at 37°C in the presence of EGTA (2 mM).<sup>2</sup> Incorporation

<sup>2</sup> Volsky, D. J., N. Zakai, and A. Loyter. Unpublished results.

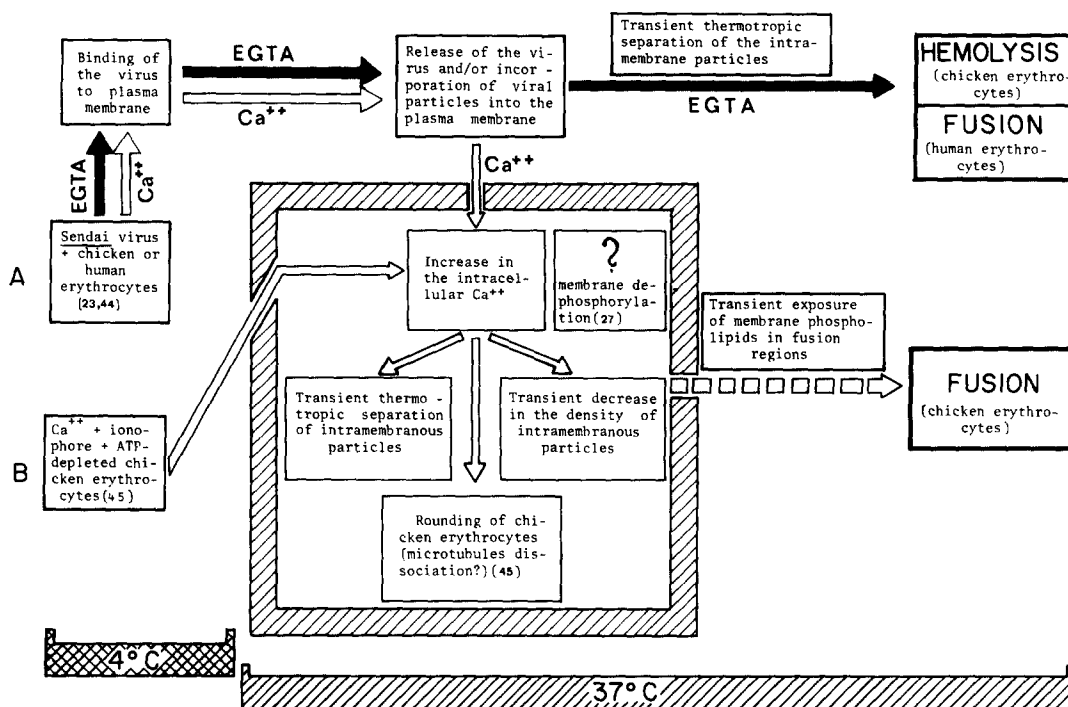


FIGURE 8 A summary of some morphological and ultrastructural changes participating in the development of membrane fusion induced by (A) *Sendai* virus in chicken and human erythrocytes, and (B)  $\text{Ca}^{++}$  and an ionophore in ATP-depleted chicken erythrocytes. Black arrows refer to systems containing EGTA, while white arrows refer to systems containing  $\text{CaCl}_2$ .

of viral particles into cell membranes, in the absence of  $\text{Ca}^{++}$ , can also be inferred from the fact that *Sendai* virus is able to induce rapid and high hemolysis of erythrocytes in the presence of EDTA or EGTA (31, 43). Leakage of hemoglobin was suggested to take place through pores that exist in the incorporating virus (4).

Since the presence of  $\text{Ca}^{++}$  ions is essential for virus-induced membrane fusion of chicken erythrocytes (31, 43, 44), we suggest that a distinction between the requirements for virus-membrane fusion and membrane-membrane fusion should be made (Fig. 8). Indeed, recently Maeda et al. showed that high concentrations of mono- and disaccharides such as glucose or sucrose strongly blocked *Sendai* virus-induced membrane fusion, although they did not affect the incorporation of virus particles into the cell's plasma membrane (29).

Interaction of *Sendai* virus with chicken erythrocytes promoted transient thermotropic separation and transient decrease in the density of intramembrane particles. Kinetic studies as well as experiments with antiviral antibody (Fig. 5) re-

vealed a close correlation between the first stages of the virus-cell interaction, development of membrane fusion, and the above two phenomena. It may be speculated that the cold-induced clustering of the intramembrane particles might result from a virus-induced partial dissociation of the erythrocyte spectrin, or from the integration of viral envelope lipids into the cell's membrane. Partial removal of spectrin has been shown to release the intramembrane particles from their restriction and to allow their lateral movement with respect to one another (12).<sup>3</sup> The decrease in the intramembrane particles' density might also be due to the virus-induced removal of spectrin, as suggested above, assuming that some of the intramembrane particles are firmly attached to spectrin (45).

On the other hand, the incorporation of virus envelope lipids may create an area of rigid phospholipids from which intramembrane particles would be excluded (28). In this context, it should be noted that the enrichment of the cholesterol

<sup>3</sup> Volsky, D. J., and A. Loyter. *Biochim. Biophys. Acta*. Manuscript submitted for publication.

content of hen erythrocytes increased fusion of the cells by *Sendai* virus (17). This might be due, as suggested by the authors, to an increased phase separation of the lipid bilayer (17). A lack of thermotropic separation of intramembrane particles in fused cells, which indicates that the alteration induced in the plasma membranes during the first steps of virus-membrane interaction is reversible, may result from an increase in density of the particles in these cells (Figs. 2 and 3).

Howe (6, 7) reported that a vast clustering of intramembrane particles occurred during the interaction of *Sendai* virus with intact human erythrocytes at 37°C. However, neither we (Fig. 2) nor others were able to repeat this observation (20). Thus, the previous observations on clustering of the intramembrane particles may be due also to the thermotropic separation of the particles, as the authors cooled the cells before subjecting them to the freeze-etching process (6, 7).

Ca<sup>++</sup> is required for virus-induced fusion of chicken erythrocytes or of other nucleated cells but not for fusion of intact human erythrocytes or human erythrocyte ghosts, which can be fused by *Sendai* virus, even in the presence of high concentrations of EDTA (37). We suggest that these differences arise from the fact that chicken erythrocytes as well as other nucleated cells contain cell microtubules that mammalian erythrocytes generally lack (14). Cell microtubules, which are organized in the form of a "marginal band," stabilize the oval shape of chicken erythrocytes (14). Rounding of chicken erythrocytes in the presence of Ca<sup>++</sup> and an ionophore (3, 47) may be due to the disorganization of the marginal bands, as was suggested before (47). Ca<sup>++</sup> was found to cause depolymerization of microtubules and to inhibit their assembly (40).

As the rounding of chicken erythrocytes always precedes fusion of these cells by *Sendai* virus (44), nonviral fusogenic agents (1), or Ca<sup>++</sup> and an ionophore (3, 47), we suggest that the dissociation of microtubules is a prerequisite for the induction of cell fusion in nucleated cells. Indeed, colchicine (10<sup>-5</sup>-10<sup>-4</sup> M) strongly stimulated virus-induced fusion of chicken erythrocytes (not shown), and its presence was found to be essential for promoting fusion of erythrocytes with cells grown in monolayers (48).

It is conceivable, therefore, that *Sendai* virus increases the permeability of cell membrane to Ca<sup>++</sup>, thus raising its intracellular concentration and thereby affecting the state of the cells' micro-

tubules. This possibility is strengthened by three observations in the present work: (a) *Sendai* virus stimulated uptake of Ca<sup>++</sup> by chicken erythrocytes and HTC cells; (b) membrane fusion could be induced by *Sendai* virus in the presence of EGTA only in Ca<sup>++</sup>-loaded chicken erythrocytes that are already partially rounded; and (c) a transient decrease in the density of intramembrane particles (Fig. 3) was promoted by the virus in the presence of CaCl<sub>2</sub> but not in the presence of EGTA.

As La<sup>+++</sup> or pretreatment of virus particles with La<sup>+++</sup> (not shown)<sup>4</sup> prevented Ca<sup>++</sup> accumulation, it is possible that Ca<sup>++</sup> is introduced into the cells via the virus particles. This view could explain the fact that Ca<sup>++</sup> is required for cell-cell fusion but not for the fusion of the viral envelope with the cell membrane.

It has been proposed before that membrane fusion takes place between the lipid bilayers of two adjacent cells (2, 38, 51). Induction of fusion by viruses or by other fusogenic agents should therefore promote a sequence of events which eventually will lead to the exclusion of intramembrane particles from the fusion area, concomitant with the exposure of membrane phospholipids in that region.

The decrease in the intramembrane particles' density and their thermotropic separation, observed in the present work during the first stages of the fusion process, may indicate an increase in the freedom of movement by membrane proteins with respect to one another, a step that is required for their removal from the fusion area.

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Preliminary results of this work were presented at the Sixth European Congress on Electron Microscopy (Jerusalem, September 1976) (46).

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