Kinetics of Sendai Virus Envelope Fusion with Erythrocyte Membranes and Virus-Induced Hemolysis†

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ABSTRACT: The kinetics of fusion of the Sendai virus envelope with erythrocyte membranes were studied by spin-label electron spin resonance (ESR). The virion envelope lipid bilayer was labeled with nitroxide derivatives of phosphatidylcholine and phosphatidylethanolamine. As the virus envelope fuses with the erythrocyte membrane, the viral lipids diffuse into the cellular lipid bilayer and the spin-label spectrum changes from that characteristic of virus to that of the erythrocyte. The ESR spectral changes can be quantitatively related to the fraction of spin-labeled virus fused with the erythrocyte membrane. The fusion process was found to follow the form of a first-order reaction with a half-time of 7 ± 1 min at 37 °C that is independent of virus concentration. The kinetics of the viral hemolytic activity were studied by spin-label ESR as changes in erythrocyte bilayer fluidity induced by hemolysis. The half-time of the hemolytic reaction is similar to that of envelope fusion and is independent of virus concentration at submaximal levels of hemolysis, suggesting that envelope fusion is the rate-limiting step in hemolysis under conditions of active hemolysis. However, virions with very low hemolytic activity, prepared by harvesting virus early after infection, lyse erythrocytes much more slowly, even though they fuse with erythrocyte membranes as efficiently as normal virus. Different strains of Sendai virus vary in their envelope-fusing activity, and these differences are correlated with their ability to fuse erythrocytes with each other.

Enveloped viruses such as influenza and parainfluenza virus possess a membrane or envelope which has a lipid bilayer derived from the host plasma membrane upon virus assembly by budding at the cell surface. However, the proteins associated with the viral membrane are determined by the viral genome. Spin-label electron spin resonance (ESR[1]) methods have been used to investigate the interaction between enveloped viruses and erythrocyte membranes as a model for the early events in virus infection (Lyles & Landsberger, 1976, 1977, 1978; Maeda et al., 1975, 1977; Landsberger et al., 1978).

Parainfluenza viruses such as Sendai virus have two types of glycoprotein "spikes" on the outer surface of their membrane-like envelope. One glycoprotein (HN) has hemagglutinating and neuraminidase activities and is responsible for virus attachment to and elution from host cellular receptors (Scheid et al., 1972; Scheid & Choppin, 1973, 1974; Tozawa et al., 1973). The other glycoprotein (F) is responsible for membrane fusing and hemolytic activity associated with parainfluenza virions and is required for infectivity (Homma & Ohuchi, 1973; Scheid & Choppin, 1974, 1976). Penetration of enveloped viruses may involve fusion of the viral envelope with host cell membranes [reviewed by Dales (1973)]. Sendai virus envelopes have been observed to fuse with erythrocyte membranes (Howe & Morgan, 1969; Bächi et al., 1973), providing a convenient model for virus penetration.

The activities associated with the Sendai virion envelope can be modified according to the conditions of virus cultivation. Sendai virus grown in tissue culture cells such as Madin–Darby bovine kidney (MDBK) cells is released with the F glycoprotein in the form of an inactive precursor F_0, lacking infectivity, and hemolytic and cell-fusing activities (Scheid & Choppin, 1974). In vitro cleavage of F_0 to F with trypsin restores infectivity and hemolytic and cell-fusing activities. Early harvest egg-grown virions (harvested 24 h after infection) lack hemolytic activity, even though the F protein is in the active form and the virus is infectious and can cause cell fusion (Homma et al., 1976).

The kinetics of fusion of Sendai virus envelopes with erythrocyte membranes are examined in this communication. The virion envelope is prelabeled with spin-label derivatives of phospholipids, whose ESR spectra reflect the motional freedom or "fluidity" of the lipid fatty acyl chains (Hubbell & McConnell, 1971; Jost et al., 1971). As the virus envelope fuses with the erythrocyte membrane, there is rapid mixing of the viral and cellular membrane components (Bächi et al., 1973), including phospholipids (Kornberg & McConnell, 1971; Devaux & McConnell, 1972). Since the fluidity of the viral lipid bilayer differs from that of the erythrocyte membrane, the spin-label spectrum changes as the bilayers fuse from that characteristic of the virus to that of the cell membrane.

In the intact erythrocyte, phosphatidylcholine derivative spin-labels exist in a more rigid environment than the phosphatidylethanolamine derivatives. Upon osmotic (Tanaka & Ohnishi, 1976) or virus-induced (Lyles & Landsberger, 1977) hemolysis, the region of the bilayer probed by the phosphatidylcholine spin-label derivative becomes more fluid whereas that of the phosphatidylethanolamine label becomes more rigid, such that the spectra of the two labels are identical. These changes in erythrocyte bilayer fluidity have been used in the present study to monitor continuously the kinetics of the hemolytic action of Sendai virus.

Materials and Methods

Virus. Sendai virus strains Fushimi, MN, Z, Obayashi, and RU and the X-31 strain of influenza virus were grown in embryonated eggs and purified as described by Lyles & Landsberger (1977). Unless otherwise noted, all experiments were performed with the RU strain of Sendai virus harvested 48 h after infection.

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[1] Abbreviations used: ESR, electron spin resonance; MDBK cells, Madin–Darby bovine kidney cells; PBS, phosphate-buffered saline; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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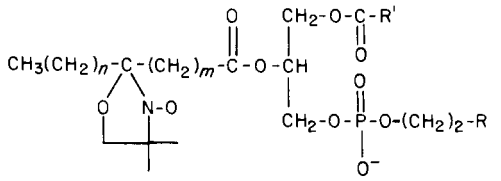
Erythrocytes. Human and sheep erythrocytes were obtained from freshly drawn blood and washed as described by Lyles & Landsberger (1977). Unless otherwise noted, all experiments were carried out on human erythrocytes. Hemoglobin-free erythrocyte ghosts were prepared as described by Steck (1974). Erythrocyte membranes were labeled by incubation of intact erythrocytes with [^{32}P]orthophosphate (Bennett & Branton, 1977). Labeled erythrocytes were lysed, pelletted, and resealed by incubation in isotonic saline. Resealed ghosts were purified by centrifugation on dextran gradients (Steck, 1974).

Spin-Labeling. The spin-labels used were nitroxide derivatives of phosphatidylcholine and phosphatidylethanolamine prepared as described previously by Boss et al. (1975) and Lyles & Landsberger (1977):



PC5: R = $^{+}N(CH_3)_3$, $m = 3$, $n = 10$
PC7: R = $^{+}N(CH_3)_3$, $m = 5$, $n = 10$
PC12: R = $^{+}N(CH_3)_3$, $m = 10$, $n = 5$
PE12: R = $^{+}NH_3$, $m = 10$, $n = 5$

The erythrocytes were spin-labeled by incubation with sonicated vesicles of these spin-labels followed by exhaustive washing (Lyles & Landsberger, 1977). Sheep erythrocyte incubations were carried out in the presence of 1 mM EDTA to inhibit the action of the membrane lecithinase on the spin-labels (Kramer et al., 1974).

Purified virions in 0.9% NaCl and 10 mM Tris-HCl (pH 7.4) (Tris-buffered saline) were pelletted by centrifugation at 27000*g* for 1 h and then resuspended in the same buffer containing spin-label vesicles (1.0 mg/mL, 0.03 mL/mg of viral protein) and bovine serum albumin (2 mg/mL, Sigma type V, essentially fatty acid free) and incubated at 37 °C for 4 h. Labeled virus was purified from excess spin-label vesicles by centrifugation on potassium tartrate gradients (Landsberger et al., 1971) and dialyzed overnight against Tris-buffered saline. Protein concentrations were estimated by the method of Lowry et al. (1951) using bovine serum albumin (Sigma type V) as the standard. The amount of spin-label incorporated into the virus was quantitated by adding an aliquot of the virus to 1% sodium dodecyl sulfate ($NaDodSO_4$) and comparing the ESR spectral intensity to standards of known concentrations of spin-label in 1% $NaDodSO_4$. Phospholipid concentrations were determined by the method of Raheja et al. (1973) on the extract of viral lipids (Folch et al., 1957). In all of these experiments, the spin-label constituted 3 to 4% of the total viral phospholipids.

Fusion of Spin-Labeled Viruses with Erythrocyte Membranes. Spin-labeled viruses (0.02–0.1 mg/mL, usually 5 or 10 mL) were incubated with unlabeled erythrocytes or erythrocyte ghosts (1% v/v, 8×10^7 cells/mL) on ice for 10 min to allow virus attachment. The suspension was centrifuged at 1000*g* for 5 min at 4 °C for erythrocytes or at 12000*g* for 15 min at 4 °C for erythrocyte ghosts, and the pellet was taken up in an aqueous ESR sample cell. The ESR spectrum was recorded as a function of time at 37 °C with a Varian E12 spectrometer interfaced to a T.I. 980A computer (Lenard et al., 1976). The Varian variable temperature apparatus was preequilibrated at 37 °C measured with a thermocouple. Temperature equilibration in the sample cell was complete in less than 1.0 min. The first data points were collected after 2 or 3 min at 37 °C. Only the outermost peaks of the spectrum, which define the splitting parameter $2A_{zz}'$ (cf. Figure 1), were recorded, expanded to the fullest possible extent. The data for each time point were collected in less than 1 min, and relative $2A_{zz}'$ values could be estimated to the nearest 0.1 G by triangulation.

The parameter $2A_{zz}'(t)$, recorded as a function of time, appears to follow the form of a first-order rate equation

$$2A_{zz}'(t) = 2A_{zz}'(0) - [2A_{zz}'(0) - 2A_{zz}'(\infty)](1 - e^{-kt}) \quad (1)$$

where $2A_{zz}'(0)$ and $2A_{zz}'(\infty)$ are the initial and final values of $2A_{zz}'$, respectively. The parameters k, $2A_{zz}'(\infty)$, and $2A_{zz}'(0)$ were determined by a nonlinear least-squares fit of eq 1 using the Fortran program CURFIT (Bevington, 1969).

According to the data presented in Figure 2, the apparent $2A_{zz}'(t)$ is proportional to the fraction of spin-label in the cell membrane bilayer, which is interpreted to be the proportion of spin-labeled virions that have fused with the erythrocytes [$\alpha_f(t)$]

$$\alpha_f(t) = \frac{2A_{zz}'(t) - 2A_{zz}'(0)}{2A_{zz}'(\text{cell}) - 2A_{zz}'(\text{virus})} \quad (2)$$

where $2A_{zz}'$(cell) and $2A_{zz}'$(virus) are determined on separate samples of spin-labeled cells and spin-labeled virus alone, respectively. Combining eq 1 and 2

$$\alpha_f(t) = \alpha_f(\infty)(1 - e^{-k_f t}) \quad (3)$$

where $\alpha_f(\infty)$ is the value of α_f at infinite time and k_f is the rate constant for the fusion process.

Virus-Induced Hemolysis. The kinetics of the bilayer fluidity changes accompanying virus-induced hemolysis (Lyles & Landsberger, 1977) were determined by a protocol identical with that described above, except that unlabeled virus was added to spin-labeled erythrocytes. Apparent rate constants (k_h) and extents (α_h) of hemolysis were determined in a manner analogous to eq 1 and 2. The kinetics of hemolytic reactions with $k_h > 0.1$ min^{-1} were also measured by continuously monitoring the change in spectral amplitude at a constant magnetic field at the extremum of the low-field peak of the erythrocyte ghost spectrum (Figure 1), which in most cases gave equivalent results to monitoring $2A_{zz}'$, but was more accurate for the faster reactions. For hemolytic reactions, α_h was calculated from $2A_{zz}'$ of intact erythrocytes and erythrocyte ghosts

$$\alpha_h(t) = \frac{2A_{zz}'(t) - 2A_{zz}'(\text{erythrocyte})}{2A_{zz}'(\text{ghost}) - 2A_{zz}'(\text{erythrocyte})} \quad (4)$$

and corresponds to the fraction of cells that have undergone a bilayer structural change upon hemolysis (Lyles & Landsberger, 1977).

Attachment at 0 °C of Radiolabeled Sendai Virus to Erythrocyte Ghosts. Egg-grown Sendai virus grown in the presence of [^{3}H]leucine (5–50 μg of protein, $\sim 5 \times 10^3$–5×10^4 cpm) (Scheid & Choppin, 1974) was incubated with resealed erythrocyte ghosts prelabeled with [^{32}P]phosphate (1% v/v, $\sim 10^4$ cpm) in a final volume of 0.5 mL for 10 min on ice and then centrifuged through a 4.0-mL dextran T500 (Pharmacia) gradient (0–8% w/v) over a 0.5-mL 60% (w/w) sucrose cushion at 100000*g* for 2 h. Fractions of ~0.4 mL were collected and dissolved in 3.5 mL of Aquasol (New England Nuclear) for liquid scintillation counting. The fraction of virus that was unattached was corrected for contamination by virus attached to co-sedimenting ghosts determined by the ^{32}P label (5–10% of the unattached virus).

Fusion at 37 °C of Radiolabeled Sendai Virus with Erythrocytes. Sendai virus grown in MDBK cells in the

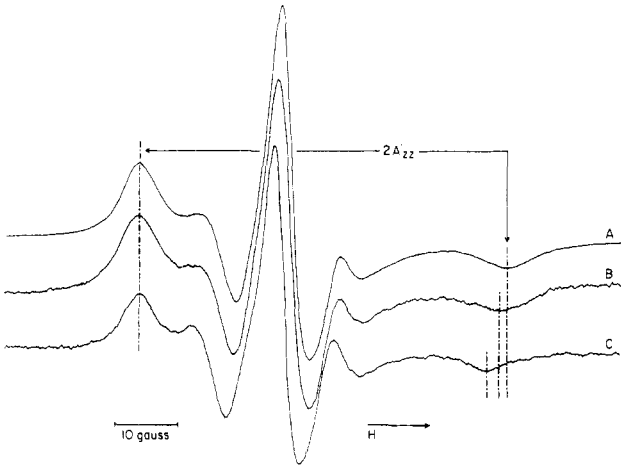


FIGURE 1: ESR spectra of PC7 spin-label incorporated into Sendai virus envelopes and erythrocyte ghosts. (A) PC7 Sendai virus; (B) PC7 Sendai virus (1.0 mg of protein) + erythrocyte ghosts (0.1 mL packed) incubated at 37 °C for 1.0 h; (C) PC7 erythrocyte ghosts. Spectra were recorded at 37 °C. Vertical lines are drawn through the outermost peaks of the spectra. The splitting of the outermost peaks ($2A_{zz}'$) is defined for spectrum (A).

presence of [5,6-^{3}H]uridine (2 μCi/mL, 40–50 Ci/mmol, New England Nuclear) and [^{32}P]phosphate (1.0 μCi/mL, New England Nuclear) was either activated with trypsin (Scheid & Choppin, 1974) or mock incubated without trypsin. Virus aliquots (700 cpm ^{3}H, 1500 cpm ^{32}P, 50 μg of protein) were incubated with a 1% (v/v) suspension of intact erythrocytes of Tris-buffered saline in a final volume of 0.5 mL for 10 min on ice. The cells and attached virus were centrifuged at 1000*g* for 10 min at 4 °C, and an aliquot of the supernatants was removed for liquid scintillation counting; the pellets were washed, resuspended in 0.45 mL of buffer, incubated at 37 °C for 1 h, and then centrifuged. An aliquot of the supernatants and several pellets were taken for liquid scintillation counting. The remaining pellets were washed once and incubated at 37 °C for 30 min in 0.45 mL of buffer containing Pronase (Sigma) (100 μg/mL). The cells were centrifuged, an aliquot of the supernatants was counted, and the pellets were washed once and counted. The ^{32}P cpm/^{3}H cpm ratios were corrected to reflect the ratio of viral lipids to RNA by subtracting from the experimental ^{32}P/^{3}H ratio the ^{32}P/^{3}H ratio of the original virus multiplied by the fraction of ^{32}P of the original virus that could not be extracted by organic solvents (0.1–0.13) (Folch et al., 1957).

Results

Quantitation of Fusion of Sendai Virus Envelopes with Erythrocyte Membranes. Figure 1 shows the ESR spectra of the PC7 spin-label incorporated into the lipid bilayer of egg-grown Sendai virus (Figure 1A) and erythrocyte ghosts (Figure 1C). The distance between the outermost peaks of the spectrum ($2A_{zz}'$, defined in Figure 1) is greater when the spin-label is incorporated into the virus envelope than into the erythrocyte membrane, indicating that the viral lipid bilayer is more rigid than that of the erythrocyte ghost (Landsberger et al., 1971). When spin-labeled Sendai virus is incubated at 37 °C for 1 h with unlabeled erythrocyte ghosts (Figure 1B), the viral envelope fuses with the erythrocyte membrane (Howe & Morgan, 1969; Bächi et al., 1973) and the viral lipids (and spin-label) are diluted by the large excess of cellular lipids, so that the spin-label spectrum changes from that of the virus to that of the erythrocyte bilayer. The final spectrum is intermediate between the two, suggesting that only a fraction of the spin-labeled virus has fused with the erythrocyte membrane. The apparent $2A_{zz}'$ of the composite spectrum is related to the fraction of spin-label transferred to the erythrocyte membrane by envelope fusion. The spectra of the PC7 label in virus (Figure 1A) and in erythrocyte ghosts (Figure 1C) were added together in varying ratios of spectral intensity, and the apparent $2A_{zz}'$ of the computer-averaged spectrum is shown to be a linear function of the fraction of spin-label in the erythrocyte in Figure 2. Thus, the parameter α_f (eq 2, Materials and Methods) is interpreted to represent the fraction of the spin-labeled virus that has fused with the erythrocyte. The two components that make up the composite spectrum, i.e., unfused virus and the fused virus–erythrocyte membrane complex, can be resolved by treating the preparation with Pronase (100 μg/mL) to remove attached but unfused virus and separating the two membranes by potassium tartrate density gradient centrifugation. Proteolytic digestion has no detectable effect on the spin-label spectrum of the virus envelope or the erythrocyte ghost (Landsberger et al., 1971; and unpublished experiments). The spin-label spectra of the recovered unfused virus and erythrocyte membrane-fused virus complex are the same as that of the original spin-labeled virus and erythrocyte membrane, respectively. Spin-labeled virus or erythrocyte ghosts incubated alone at 37 °C for 1 h undergo no detectable spectral change. Spin-labeled erythrocyte ghosts incubated with unlabeled virus undergo no detectable spectral change (Lyles & Landsberger, 1977) except at the highest virus concentrations used in this study (1.0 mg/0.1 mL of packed cells), where a small increase in $2A_{zz}'$ is observed, probably due to the incorporation of viral lipids into the cellular membrane to ~10% of the cellular lipids.

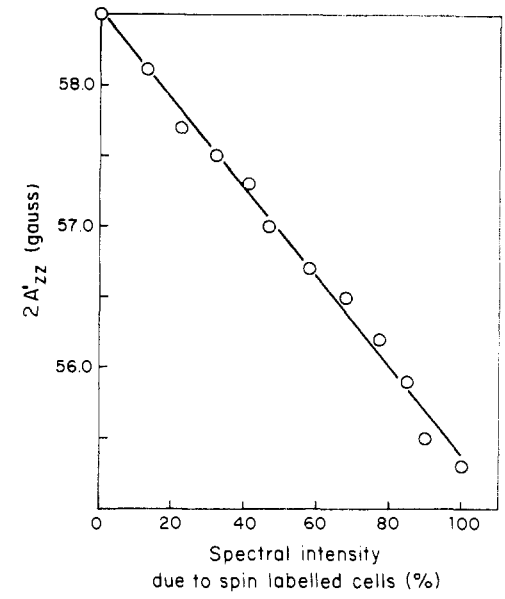


FIGURE 2: Dependence of $2A_{zz}'$ on relative proportion of spin-label spectrum contributed by labeled virus vs. labeled erythrocyte ghosts. Spectra (A) and (C) in Figure 1 were computer summed with different weighting factors by adjusting the spectrometer receiver gain, and the apparent $2A_{zz}'$ was determined.

Kinetics of Viral Envelope Fusion. The kinetics of the change in $2A_{zz}'$ when spin-labeled viruses fuse with unlabeled erythrocyte membranes are shown in Figure 3. Egg-grown Sendai virus fuses rapidly with the erythrocyte membrane. Influenza virus (also grown in eggs) undergoes no significant change in spin-label spectrum upon incubation with erythrocyte ghosts, suggesting the absence of passive transfer of spin-label from virus envelopes to cell membranes. Likewise, Sendai virus grown in MDBK cells, which has the F glycoprotein largely in the form of its inactive precursor F_0, has little envelope-

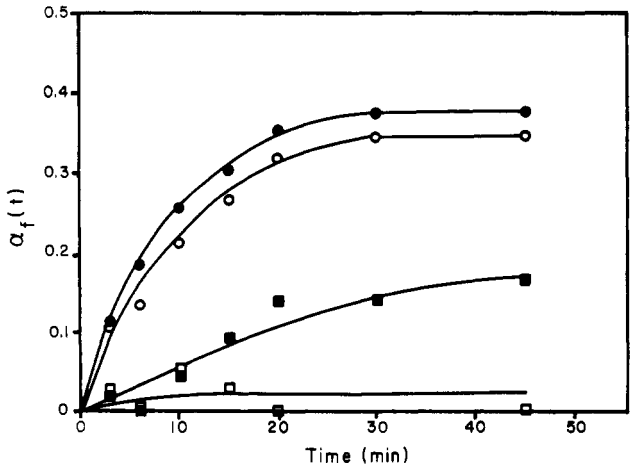


FIGURE 3: Kinetics of viral envelope fusion with erythrocyte membranes. (○) PC7 Sendai virus (grown in eggs) + human erythrocyte ghosts; (□) PC7 influenza virus + human erythrocyte ghosts; (●) PC7 Sendai virus (grown in MDBK cells, trypsin activated) + sheep erythrocytes; (■) PC7 Sendai virus (grown in MDBK cells, unactivated) + sheep erythrocytes. Labeled virus (1.0 mg of protein) was incubated with unlabeled erythrocyte membranes (0.1 mL packed) at 37 °C, and $2A_{zz}'$ was recorded as a function of time. α_f was calculated according to eq 2.

fusing activity, whereas in vitro cleavage of F_0 to F with trypsin enables MDBK-grown Sendai virus to fuse with erythrocyte membranes. The experiments with MDBK-grown Sendai virus were carried out on sheep erythrocytes, because the spectral splitting of PC7 labeled, MDBK-grown Sendai virus (54.5 G) is significantly different from that of sheep erythrocytes (59.3 G for PC7) but not from that of human erythrocyte ghosts (55.0 G). The term $2A_{zz}'$(cell) in the equation for α_f (eq 2) was obtained by incubating PC7-labeled sheep erythrocytes with unlabeled virus to account for the spectral changes in the sheep erythrocyte membrane due to virus-induced hemolysis (Lyles & Landsberger, 1977). These corrections were small (10–20% hemolysis of sheep erythrocytes with trypsin-treated virus).

The fusion of spin-labeled virions with erythrocyte membranes appears to follow the form of a first-order reaction (eq 3). When ln $[\alpha_f(\infty) - \alpha_f(t)]$ is plotted as a function of t (Figure 4A), a straight line is obtained, the slope of which gives the rate constant of the reaction, k_f, and the intercept is ln $\alpha_f(\infty)$, where $\alpha_f(\infty)$ is the maximum or plateau value of α_f. For the fusion of egg-grown Sendai virus with human erythrocyte ghosts and MDBK-grown Sendai virus with intact sheep erythrocytes, $k_f = 0.1$ min^{-1} at 37 °C, which corresponds to a reaction half-time of ~7 min ($t_{1/2} = \ln 2/k$). The same kinetics are observed with virus labeled with PC5 or PC12 (data not shown). Similar slopes and intercepts are obtained for the fusion with erythrocyte ghosts of egg-grown Sendai virus harvested early (24 h) or late (48 h) after infection. Early harvest, egg-grown Sendai virus has very low hemolytic activity (Homma et al., 1976), which from Figure 4A is clearly not due to the lack of ability to fuse with the erythrocyte membrane. The kinetics of envelope fusion with intact human erythrocytes or ghosts were assayed by using early harvest virus and were found to be the same. Early harvest Sendai virus does not cause a change in the fluidity of the lipid bilayer of human erythrocytes at low virus concentrations (Lyles & Landsberger, 1977). At the high virus concentrations used in these fusion experiments, some change in the spectrum of spin-labeled intact erythrocytes does occur due to the residual hemolytic activity of early harvest virus. This spectral change was taken into account in the calculation of α_f (eq 2) as described above for sheep erythrocytes.

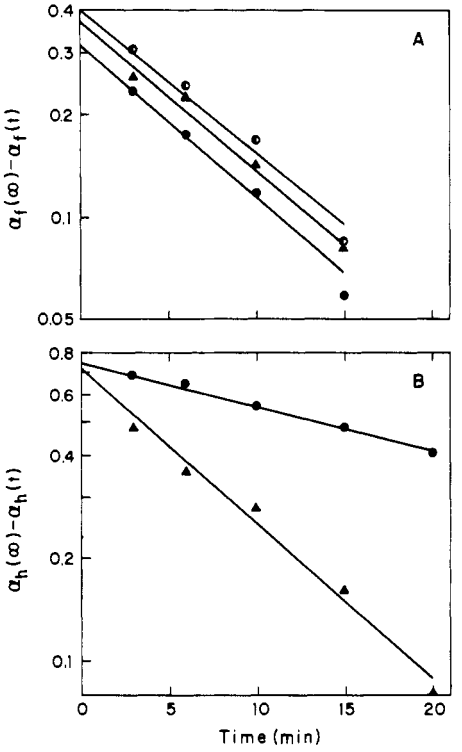


FIGURE 4: Semilogarithmic plots of Sendai virus envelope fusion with human erythrocyte membranes and of hemolysis-induced change in erythrocyte lipid bilayer fluidity. (A) Envelope fusion: spin-labeled virus grown in eggs (1.0 mg) added to unlabeled cells (0.1 mL packed). (◐) PE12 Sendai virus (early harvest) + intact erythrocytes; (●) PC7 Sendai virus (early harvest) + erythrocyte ghosts; (▲) PC7 Sendai virus (late harvest) + erythrocyte ghosts. (B) Virus-induced hemolysis: unlabeled virus added to spin-labeled cells. (●) Sendai virus (early harvest, 1.0 mg) + PE12 erythrocytes (0.1 mL packed); (▲) Sendai virus (late harvest, 87 μg) + PC12 erythrocytes (0.1 mL packed). Virus + cells were incubated at 37 °C as in Figure 3. (A) $\alpha_f(t)$ and $\alpha_f(\infty)$ were calculated as described under Materials and Methods. (B) $\alpha_h(t)$ and $\alpha_h(\infty)$ were calculated according to eq 4. The concentration of late harvest virus in (B) was chosen to give a final ESR spectral change similar to that of early harvest virus.

Kinetics of Virus-Induced Hemolysis. The kinetics of the ESR spectral changes resulting from a structural change in erythrocyte membranes due to viral hemolytic activity for both early and late harvest Sendai virus are shown in Figure 4B. Equivalent results are obtained with both PC12 and PE12, even though the environment of PC12 becomes more fluid and that of PE12 becomes more rigid. The half-time for the hemolysis-induced change in erythrocyte bilayer fluidity due to early harvest Sendai virus ranges from 25 to 36 min with different virus preparations. The extent of the hemolysis-induced spectral change parallels the amount of hemoglobin released by late harvest virus (Lyles & Landsberger, 1977). However, in the case of early harvest Sendai virus, the extent of this spectral change $[\alpha_h(\infty)]$ is 74%, even though the total hemoglobin released was only 26%. Similarly, it has been observed that Ca^{2+} can inhibit virus-induced hemoglobin release without completely preventing the associated erythrocyte bilayer fluidity change (Lyles & Landsberger, 1977). Hemolysis by an amount of late harvest Sendai virus necessary to give an equivalent final ESR spectral change (less than one-tenth the amount of early harvest virus) has a half-time similar to that of envelope fusion, i.e., 8.0 min.

Effect of Virus Concentration, Virus Attachment, and Temperature. The half-time for envelope fusion with erythrocyte membranes is independent of virus concentration over

Table I: Concentration Dependence of Sendai Virus Envelope Fusion with Human Erythrocyte Ghosts

virus added (mg of protein per 0.1 mL of packed cells)	virus attached at 0 °C[a]	virus fused at 37 °C[b]	fraction of attached virus that fused	$t_{1/2}$[b] (= ln 2/k_f) (min)	N
0.2	0.15	0.072 ± 0.006	0.48	6.8 ± 1.3	4
0.3	0.20	0.10 ± 0.02	0.50	6.7 ± 0.7	5
0.5	0.30	0.19 ± 0.03	0.63	7.4 ± 0.9	5
1.0	0.58	0.29 ± 0.05	0.50	7.0 ± 1.1	5

[a] Determined by using egg-grown Sendai virus labeled with [^{3}H]-leucine. Average of two experiments. [b] Mean ± standard deviation determined in N independent experiments using egg-grown Sendai virus labeled with PC7.

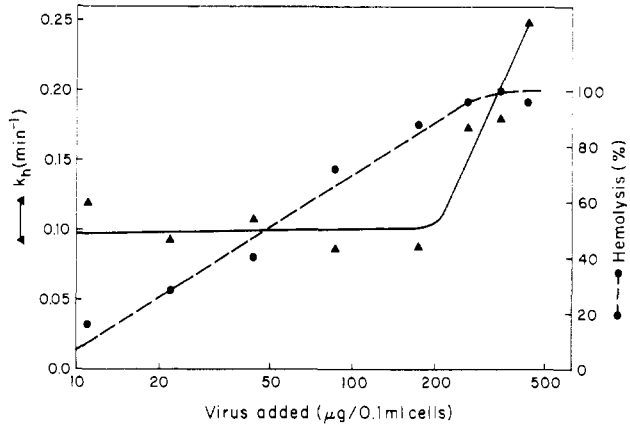


FIGURE 5: Dependence of rate and extent of the hemolysis-induced change in erythrocyte lipid bilayer fluidity on virus concentration. PC12 erythrocytes were incubated with Sendai virus (late harvest) at 37 °C. The extent of hemolysis was determined from $2A_{zz}'$ measured after 60 min, which has been shown to correspond to the fraction of hemoglobin released (Lyles & Landsberger, 1977).

the range of concentrations examined (Table I), providing support for the assumption that envelope fusion follows the form of a first-order reaction. The fraction of attached virus that fuses with the erythrocyte membrane also does not appear to depend upon virus concentration. The concentration dependence of the hemolytic reaction appears to be more complex (Figure 5). The extent of the ESR spectral change accompanying hemolysis [$\alpha_h(\infty)$] depends upon virus concentration, of course, whereas the rate constant for this change is independent of virus concentration to the point where practically all of the cells are hemolyzed, beyond which the rate constant is strongly dependent upon virus concentration.

The kinetics of envelope fusion are the same whether determined by using a cell pellet with virus attached (as above) or whether the cells are allowed to remain in suspension, as shown in Figure 6. In this experiment, spin-labeled virus was allowed to attach to erythrocyte ghosts at 0 °C. Aliquots were incubated at 37 °C, the suspensions were pelleted at 4 °C, and the ESR spectra were taken at room temperature, where the rate of envelope fusion is sufficiently slow so that an accurate determination of $2A_{zz}'$ could be made. However, if the virus and erythrocyte ghosts were preincubated at 37 °C before being added together, so that both attachment and fusion occurred at 37 °C, the rate of envelope fusion was reduced to $t_{1/2}$ = 11 min and $\alpha_f(\infty)$, the total fraction of virus fused, was decreased.

The temperature dependence of the rate of envelope fusion with the erythrocyte membrane is shown as an Arrhenius plot in Figure 7. The apparent activation energy calculated from the slope of the straight line is 26 kcal/mol. The fraction of

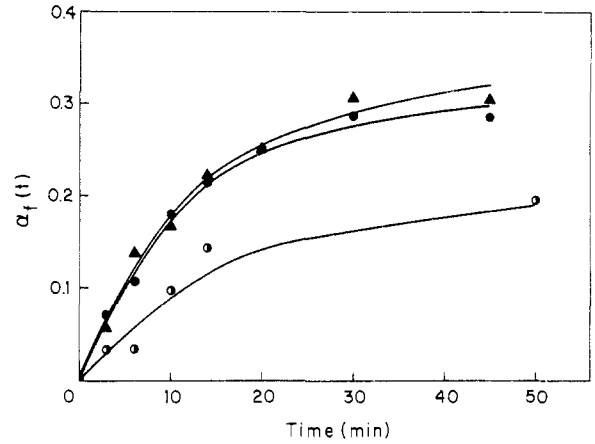


FIGURE 6: Effect of preattachment of Sendai virus on the rate of envelope fusion with human erythrocyte membranes. (▲) PC7 Sendai virus (0.1 mg/mL) + erythrocyte ghosts (1% v/v) were incubated for 10 min at 0 °C and centrifuged, and the pellet was incubated at 37 °C; (●) aliquots of PC7 Sendai virus (0.1 mg/mL) + erythrocyte ghosts (1%) were incubated for 10 min at 0 °C, incubated the specified length of time at 37 °C, and centrifuged at 4 °C, and the ESR spectrum was recorded at room temperature (23 °C); (◑) PC7 Sendai virus (0.1 mg/mL) + erythrocyte ghosts (1%) were mixed at 37 °C, incubated the specified length of time at 37 °C, and centrifuged at 4 °C, and the ESR spectrum was recorded at room temperature (23 °C). Incubations were carried out in 5 mL in a thin-walled 50-mL Erlenmeyer flask to facilitate temperature equilibration, which required about 0.5 min.

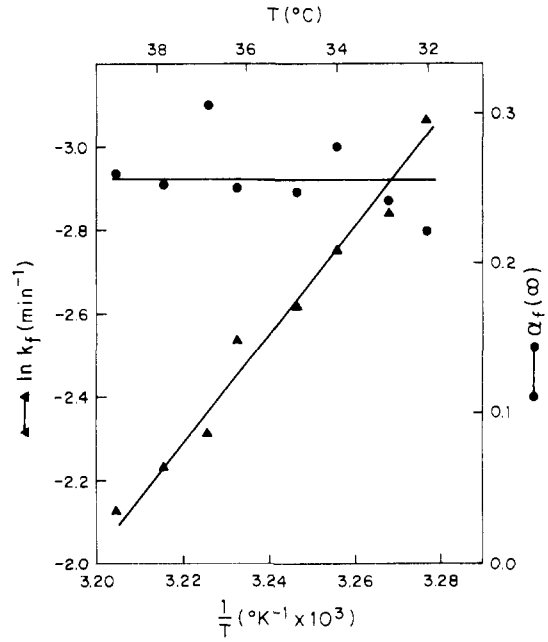


FIGURE 7: Temperature dependence of the rate k_f and extent $\alpha_f(\infty)$ of virus envelope fusion with erythrocyte membranes. PC7 Sendai virus (late harvest) + erythrocyte ghosts (1.0 mg/0.1 mL of packed cells) were incubated at the specified temperature in a Varian variable temperature accessory.

spin-labeled virus that eventually fuses with the erythrocyte [$\alpha_f(\infty)$] does not display a strong temperature dependence. The rate constant for envelope fusion displays a pH optimum between 7.4 and 8.0.

The ability of different strains of Sendai virus to fuse with erythrocyte ghosts is compared in Table II. The rate constant, k_f, and extent of envelope fusion, $\alpha_f(\infty)$, both follow the order Z > Fushimi ≃ RU > MN > Obayashi. The same order is observed when the abilities of the different strains to cause fusion between erythrocytes are compared (Figure 8). The

Table II: Envelope Fusion of Different Strains of Sendai Virus with Erythrocyte Ghosts

virus strain	virus fused[a] $[\alpha_f(\infty)] \times$ (mg/0.1 mL of cells)	$t_{1/2}$[a] $(=\ln 2/k_f)$ (min)	N
Z	0.39 ± 0.11	5.1 ± 0.9	14
Fushimi	0.29 ± 0.09	5.7 ± 1.8	3
RU	0.29 ± 0.05	7.0 ± 1.1	5
MN	0.15[b]	6.3[b]	2
Obayashi	<0.1[c]	[c]	2

[a] PC7-labeled virus (1.0 mg) incubated at 37 °C with 0.1 mL of packed human erythrocyte ghosts. Mean ± standard deviation of N determinations. [b] Approximate values. The PC7-labeled MN strain undergoes spectral changes at 37 °C in the absence of erythrocyte ghosts, which must be taken into account in the calculation of $\alpha_f(t)$. [c] Spectral changes were too small to make an accurate determination.

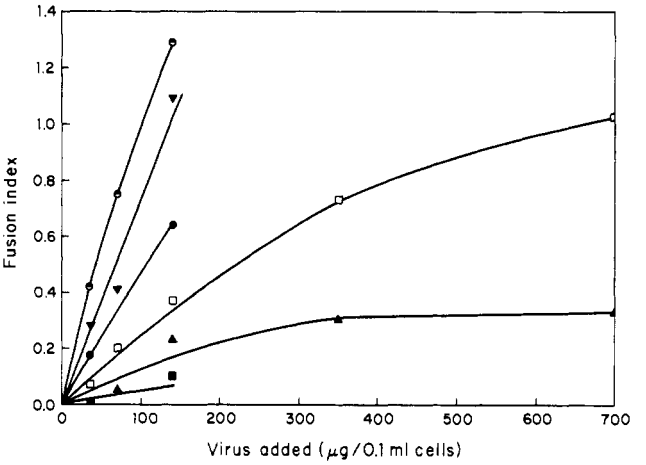


FIGURE 8: Ability of different strains of Sendai virus to cause human erythrocytes to fuse with each other. (◓) Z, (▼) Fushimi, (●) RU, (□) MN, and (▲) Obayashi all incubated with intact erythrocytes; (■) Ru + erythrocyte ghosts. The fusion index was determined after incubation of virus and cells at 37 °C for 1 h, diluting with buffer to give a 0.1% suspension of cells, and then counting the cell number (both individual cells and fused cells) in a hemacytometer. The fusion index was calculated according to Okada & Tadokoro (1962): fusion index = (cell number without virus/cell number with virus) − 1.0.

extent of Sendai virus-induced fusion of erythrocyte ghosts with each other is much less than that of the fusion of intact erythrocytes with each other (Peretz et al., 1974; and Figure 8), even though the viral envelope fuses with ghosts as efficiently as intact cells (Figure 4A). Thus, cell-to-cell fusion is affected by host factors which do not appear to be significant in virus-to-cell fusion.

It has been suggested that the Sendai viral F glycoprotein can catalyze the exchange of phospholipids between the virus envelope and cell membrane without causing envelope fusion (Maeda et al., 1977) on the basis of the observation that PC12-labeled virus which elutes from erythrocytes contains less spin-label than that of the original virus. This problem has been reexamined by using Sendai virus grown in MDBK cells radiolabeled with [^{32}P]phosphate and [^{3}H]uridine (Table III). The following viral populations were examined: (1) unattached virus, (2) eluted virus, released into the supernatant after incubation at 37 °C for 1 h, (3) virus which remains attached but does not fuse, released into the supernatant by treatment of the pellet from fraction (2) with Pronase (100 μg/mL, 37 °C, 30 min), and (4) virus which has fused, the pellet from fraction (3). The quantity of fraction (4) may be underestimated because of the losses to fractions (2) and (3), although such losses do not appear to be too large since 21.5% of the input labeled lipids are recovered in this fraction, which is close to the 29% measured by the spin-label approach (Table I). The preferential incorporation of viral lipids over viral RNA into erythrocytes appears as an increase in ^{32}P/^{3}H ratio in the reaction pellet. Upon examination of the different populations of virions, the following order of ^{32}P/^{3}H ratios is seen: eluted virus and unattached virus (1.38) < input virus (1.79) < virus attached but not fused ≃ virus fused (~4). These differences are probably due to the well-documented heterogeneity of parainfluenza virions [e.g., Kingsbury et al. (1970)], suggesting that virions containing less lipid relative to RNA (i.e., those that have smaller envelopes) elute more readily. Similar variations in the biological activities of parainfluenza viruses depending on the size of the virion have been observed previously [e.g., Granoff & Henle (1954a,b)]. This heterogeneity makes it difficult to attribute any of these differences to the activity of the F glycoprotein. While it is difficult to rule out lipid transfer in the absence of fusion, the data presented in Table III suggest that this does not occur. The similarity between the ^{32}P/^{3}H ratios of virus which remains attached but not fused and that which has fused, and the observation of similar ^{32}P/^{3}H ratios for unactivated and trypsin-activated MDBK-grown Sendai virions, suggest that the F glycoprotein does not catalyze the exchange of lipids between virions and cellular membranes in the absence of fusion.

Discussion

The interaction of Sendai virus with erythrocytes provides an appropriate, convenient model for the early events in virus infection. The virus particle attaches to host cellular receptors through the hemagglutinin, a glycoprotein on the outer surface (Choppin & Compans, 1975). Virus attachment results in an increase in the fluidity of the plasma membrane of avian or amphibian erythrocytes, which appears to be mediated by microtubules and requires the cross-linking of the cellular

Table III: Fusion of MDBK-Grown Sendai Virus Labeled with [^{32}P]Phosphate and [^{3}H]Uridine with Human Erythrocytes[a]

virus fraction	^{32}P cpm/^{3}H cpm[b]			% of total ^{32}P cpm[b]		
	untreated	trypsin activated	N	untreated	trypsin activated	N
input virus	1.92	1.79		100	100	
unattached at 0 °C	1.27 ± 0.11	1.38 ± 0.11	6	37.8 ± 2.4	45.1 ± 2.1	6
attached at 0 °C	2.31	2.13	calcd	62.2	54.9	calcd
eluted at 37 °C	[c]	[c]		5.0 ± 1.0	7.9 ± 1.9	6
uneluted at 37 °C	3.82	4.12	2	57.2[d]	47.0[d]	calcd
released by Pronase (attached but not fused)	[c]	[c]		44.6 ± 2.4	25.5 ± 1.0	4
fused	3.29 ± 0.39	3.92 ± 0.61	4	12.6[d]	21.5[d]	calcd

[a] Sendai virus (50 μg) labeled with ^{3}H and ^{32}P was incubated with 0.5 mL of a 1% (v/v) human erythrocyte suspension. [b] Mean ± standard deviation of N determinations; calcd = calculated. ^{32}P cpm was corrected to reflect the percent of ^{32}P in viral lipids as described under Materials and Methods. [c] ^{3}H cpm released into supernatants was too dilute for accurate determinations. [d] Since recovery of the pellet material was not quantitative, these fractions were calculated from ^{32}P cpm released into the supernatants.

receptors (Lyles & Landsberger, 1976, 1977, 1978). Parainfluenza virus penetration appears to involve fusion of the viral envelope with the cellular plasma membrane [discussed by Scheid & Choppin (1976)]. Mammalian erythrocytes were used in the present study to investigate virus envelope fusion and virus-induced hemolysis, since virus attachment does not induce a structural change in these erythrocytes (Lyles & Landsberger, 1978). The change in the apparent $2A_{zz}'$ of the ESR spectrum as spin-labeled virus fuses with unlabeled cells (Figure 1) is proportional to the amount of spin-label transferred to the cell by envelope fusion (Figure 2). Fusion of the Sendai virus envelope with erythrocyte membranes follows the form of a first-order reaction with a half-time of 7 min at 37 °C, which is independent of virus concentration (Table I). Allowing the virus to attach at low temperature and starting the fusion reaction by incubation at 37 °C simplify the kinetics of envelope fusion (to first order) by making them independent of virus attachment. If both attachment and fusion of virus envelopes with cell membranes occur at 37 °C, the kinetics of the fusion process appear to be more complex (Figure 6).

Spin-label electron spin resonance has also been used by Maeda et al. (1975, 1977) to study fusion of the Sendai virus envelope with erythrocyte membranes by using a different approach involving incorporation of large amounts of spin-label into the viral envelope (PC12 was ~25% of viral lipids), resulting in an ESR spectrum characterized by strong spin–spin exchange interaction. These data are not readily interpretable in terms of the fraction of viral envelopes fused with the erythrocyte membrane. Furthermore, the spectral changes occurring in the cell membrane as a result of virus-induced hemolysis (Lyles & Landsberger, 1977) can lead to an error of 10–20% in the measurements of Maeda et al. (1975, 1977). Nonetheless, the data can be analyzed to be consistent with a half-time of the fusion process of ~5–7 min. The dependence of envelope fusion on cleavage of the F glycoprotein (Maeda et al., 1977) and its independence of virus concentration (Maeda et al., 1975) were demonstrated.

The kinetics of fusion of the Sendai viral envelope with cell membranes provide information on the relationship between envelope fusion and the other biological activities of the F glycoprotein, i.e., hemolysis and cell–cell fusion. The low fusing activity of MDBK-grown Sendai virus indicates that envelope fusion with cell membranes does depend upon proper cleavage of the F glycoprotein (Figure 3). The presence of viral hemolytic activity does not appear necessary for envelope fusion to occur, since egg-grown virus harvested early fuses with erythrocyte membranes as effectively as virions harvested late (Figure 4).

The rate constant for the change in erythrocyte bilayer fluidity induced by the hemolytic action of late harvest Sendai virus is similar to that of the envelope fusion reaction and is independent of virus concentration at low virus concentrations (Figure 5). However, if the extent of hemolysis approaches 100%, the rate constant increases markedly with increasing virus concentrations. Analysis of the kinetic data of Granoff & Henle (1954a) and of Bratt & Clavell (1972) in a manner analogous to eq 3 also suggests that the rate constant for hemoglobin release is independent of virus concentration at submaximal levels of hemolysis. These data are consistent with a model of hemolysis in which only a fraction of virus particles are hemolytic (Sagik & Levine, 1957; Clavell & Bratt, 1972), and hemolysis follows rapidly after fusion of one of these particles with the erythrocyte membrane. Hemolysis may occur because of ionic permeability changes in the part of the membrane derived from the virus envelope (Howe & Morgan, 1969; Homma et al., 1976), leading to hemolysis by a colloid osmotic mechanism. Thus, the rate of hemolysis will be limited by the rate of envelope fusion unless there is more than one hemolytic particle per cell, at which point the probability of one hemolytic particle fusing with the erythrocyte membrane increases with increasing virus concentration.

The nature of the hemolytic activity of Sendai virus harvested early (24 h) after infection differs in several respects from that of Sendai virus harvested at the usual 48-h post-infection. The number of hemolytic particles in preparations of early harvest Sendai virus is greatly reduced (Homma et al., 1976), taking over 10 times more virus to achieve the same change in the erythrocyte bilayer fluidity as late harvest virus (Figure 4). Furthermore, envelope fusion is not the rate-limiting step in hemolysis by early harvest virus. The rate constant for hemolysis by early harvest virus is about one-fourth that of late harvest virus, whereas the rates of envelope fusion with erythrocyte membranes are the same for both early and late harvest virions (Figure 4). Finally, the changes in erythrocyte bilayer fluidity detected by spin-label ESR are more sensitive to the low hemolytic activity of early harvest Sendai virus than is hemoglobin release, presumably because ionic changes occur inside the erythrocyte following fusion with early harvest virus that are sufficient to alter bilayer structure without causing hemoglobin loss (Tanaka & Ohnishi, 1976; Lyles & Landsberger, 1977). Although it has been suggested that the membrane-fusing activity of Sendai virus involves an increase in the fluidity of the erythrocyte bilayer by some mechanism in addition to its hemolytic activity, on the basis of the effects of high concentrations of early harvest virus on PC12-labeled erythrocytes (Maeda et al., 1977), it is likely that these results are due to the residual hemolytic activity of early harvest Sendai virus. As shown here, these changes in erythrocyte bilayer fluidity, induced by early harvest Sendai virus, although differing quantitatively from hemoglobin release, do not differ qualitatively from the effects of virus-induced hemolysis, i.e., PC12 becomes more fluid while PE12 becomes more rigid, and no effects are observed with spin-labeled erythrocyte ghosts. Furthermore, these bilayer fluidity changes are probably not involved in membrane fusion. They occur much more slowly than envelope fusion of early harvest virus; late harvest virus, which causes changes in bilayer fluidity more rapidly and to a much greater extent, does not differ significantly from early harvest virus in its membrane-fusing activity.

Sendai virus will induce the fusion of intact erythrocytes, but not of erythrocyte ghosts, with each other (Peretz et al., 1974; Figure 8). This difference may be due to alterations in the protein spectrin underlying the erythrocyte membrane upon hemolysis (Sekiguchi & Asano, 1978; Lalazar & Loyter, 1979). In contrast, fusion of the Sendai viral envelope occurs as efficiently with ghosts as with intact erythrocytes (Figure 4). Thus, cell-to-cell membrane fusion appears to be influenced by cellular factors to which envelope-to-cell fusion is not sensitive. Factors which do affect the process of envelope fusion, such as variation among different strains of Sendai virus (Table II), are reflected in the cell-fusing activity (Figure 8).

The kinetic data presented here are consistent with a central role for envelope fusion in the other biological activities associated with the Sendai viral F glycoprotein. The sensitivity of hemolytic and cell-fusing activities to viral and host factors to which envelope fusion is not sensitive emphasizes that these processes are very different expressions of the same glycoprotein.

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References

Bächi, T., Aguet, M., & Howe, C. (1973) *J. Virol. 11*, 1004–1012.

Bennett, V., & Branton, D. (1977) *J. Biol. Chem. 252*, 2753–2763.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 237–241, McGraw-Hill, New York.

Boss, W. F., Kelley, C. J., & Landsberger, F. R. (1975) *Anal. Biochem. 64*, 289–292.

Bratt, M. A., & Clavell, L. A. (1972) *Appl. Microbiol. 23*, 454–460.

Choppin, P. W., & Compans, R. W. (1975) in *Comprehensive Virology* (Fraenkel-Conrat, H., & Wagner, R. R., Eds.) Vol. 4, pp 95–178, Plenum Press, New York.

Clavell, L. A., & Bratt, M. A. (1972) *Virology 49*, 195–204.

Dales, S. (1973) *Bacteriol. Rev. 37*, 103–135.

Devaux, P., & McConnell, H. M. (1972) *J. Am. Chem. Soc. 94*, 4475–4481.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Granoff, A., & Henle, W. (1954a) *J. Immunol. 72*, 322–328.

Granoff, A., & Henle, W. (1954b) *J. Immunol. 72*, 329–339.

Homma, M., & Ohuchi, M. (1973) *J. Virol. 12*, 1457–1465.

Homma, M., Shimizu, K., Shimizu, Y. K., & Ishida, N. (1976) *Virology 71*, 41–47.

Howe, C., & Morgan, C. (1969) *J. Virol. 3*, 70–81.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.

Jost, P., Libertini, L. J., Hebert, V. C., & Griffith, O. H. (1971) *J. Mol. Biol. 59*, 77–98.

Kingsbury, D. W., Portner, A., & Darlington, R. W. (1970) *Virology 42*, 857–871.

Kornberg, R. D., & McConnell, H. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2564–2568.

Kramer, R., Jungi, B., & Zahler, P. (1974) *Biochim. Biophys. Acta 373*, 404–415.

Lalazar A., & Loyter, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 318–322.

Landsberger, F. R., Lenard, J., Paxton, J., & Compans, R. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2579–2583.

Landsberger, F. R., Lyles, D. S., & Choppin, P. W. (1978) in *Negative Strand Viruses and the Host Cell* (Mahy, B. W. J., & Barry, R. D., Eds.) pp 787–800, Academic Press, London.

Lenard, J., Tsai, D. K., Compans, R. W., & Landsberger, F. R. (1976) *Virology 71*, 389–394.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lyles, D. S., & Landsberger, F. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3497–3501.

Lyles, D. S., & Landsberger, F. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1918–1922.

Lyles, D. S., & Landsberger, F. R. (1978) *Virology 88*, 25–32.

Maeda, T., Asano, A., Ohki, K. Okada, Y., & Ohnishi, S. (1975) *Biochemistry 14*, 3736–3741.

Maeda, T., Asano, A., Okada, Y., & Ohnishi, S. (1977) *J. Virol. 21*, 232–241.

Okada, Y., & Tadokoro, J. (1962) *Exp. Cell Res. 26*, 108–118.

Peretz, H., Toister, Z., Laster, Y., & Loyter, A. (1974) *J. Cell Biol. 63*, 1–11.

Raheja, R. K., Kaur, C., Singh, A., & Bhatia, I. S. (1973) *J. Lipid Res. 14*, 695–697.

Sagik, B. P., & Levine, S. (1957) *Virology 3*, 401–416.

Scheid, A. S., & Choppin, P. W. (1973) *J. Virol. 11*, 263–271.

Scheid, A. S., & Choppin, P. W. (1974) *Virology 57*, 475–490.

Scheid, A. S., & Choppin, P. W. (1976) *Virology 69*, 265–277.

Scheid, A. S., Caliguiri, L. A., Compans, R. W., & Choppin, P. W. (1972) *Virology 50*, 640–652.

Sekiguchi, K., & Asano, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1740–1744.

Steck, T. L. (1974) in *Methods in Membrane Biology* (Korn, E. D., Ed.) Vol. 2, pp 245–281, Plenum Press, New York.

Tanaka, K., & Ohnishi, S. (1976) *Biochim. Biophys. Acta 426*, 218–231.

Tozawa, H., Watanabe, M., & Ishida, N. (1973) *Virology 55*, 242–253.