# Mass Action Analysis of Kinetics and Extent of Fusion between Sendai Virus and Phospholipid Vesicles†

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ABSTRACT: The kinetics and extent of fusion between Sendai virus particles and liposomes were investigated with an assay for lipid mixing based on the relief of self-quenching of fluorescence. The measurements, which were carried out at pH 7.4 and 5.0, included liposomes of three compositions, cardiolipin (CL), CL/dioleoylphosphatidylcholine (CL/DOPC 1:1), and phosphatidylserine (PS). Liposomal lipid concentrations varied from 2.5 to 50 $\mu$M. In addition, the effect of low concentrations of the dehydrating agent poly(ethylene glycol) (PEG) on fusion between the virus and the liposomes at pH 7.4 was studied. The results were analyzed in terms of a mass action kinetic model which views the overall fusion reaction as a sequence of a second-order process of virus–liposome adhesion or aggregation, followed by the first-order fusion reaction itself. The fusion products were shown to consist of a single virus particle and several liposomes. Analytical solutions were found for the final extent of fusion and increase in fluorescence intensity following the fusion of fluorescently labeled virus particles with liposomes. The final extents of fluorescence intensity were explained by assuming an essentially irreversible binding of liposomes to inactive virus particles. The percents of active virus particles and the rate constants of fusion and aggregation were larger at pH 5 than at pH 7.4, increased when PEG was included in the medium, and varied with liposomal lipid composition according to the sequence CL > CL/DOPC > PS. At pH 5, the percents of Sendai virus particles capable of fusing with CL, CL/DOPC, and PS liposomes were 100, 80, and 30, respectively, indicating that the fusion activity is not merely a function of liposomal surface charge. The rate constant of fusion, $f$, exhibited the largest variation as a function of pH and liposome composition, varying from 1 to 0.005 $s^{-1}$, whereas the rate constant of aggregation varied from $1.8 \times 10^8$ to $0.7 \times 10^8$ $M^{-1} \cdot s^{-1}$. A small degree of reversibility was needed to simulate and predict the kinetics of fusion. This reversibility promoted the overall fusion rate at the later stages due to a certain degree of dissociation of liposomes bound to inactive virus particles.

Valuable insight into the mechanisms underlying fusion has been obtained by the employment of assays (Hoekstra et al., 1979, 1984; Wilschut et al., 1980; Vanderwerf & Ullman, 1980; Struck et al., 1981; Hoekstra, 1982; MacDonald & MacDonald, 1983) which allow continuous and sensitive monitoring of membrane fusion. Yet, these assays follow essentially the overall fusion reaction, which involves an initial binding step of adjacent membranes and the actual fusion reaction per se. Hence, to obtain a more detailed picture of membrane merging, a distinction between these sequential steps by quantifying their rate constants would be desirable. In conjunction with the above experimental approach, a theoretical model, which is based on a simple mass action kinetic model, has been developed. This kinetic model views the overall fusion reaction as a sequence of a second-order process of membrane adhesion followed by the first-order fusion itself (Nir et al., 1982, 1983; Bentz et al., 1983). Aggregation and fusion rate constants are obtained by varying the concentrations of interacting particles. At high concentrations, the fusion reaction per se determines the overall rate, whereas at low particle concentration the initial binding step will be essentially rate limiting. Thus, by determining the aggregation rate constant, a reflection is obtained of the balance of forces that mediates close apposition (Nir et al., 1980, 1983; Parsegian & Rand, 1983; Bentz et al., 1985). Similarly, the fusion rate constant provides a direct measure of the significance of parameters affecting the molecular events involved in the fusogenic destabilization of apposed bilayers.

In the case of fusion of Sendai virus with erythrocye membranes, we have recently established the experimental (Hoekstra et al., 1984, 1985a) and theoretical methodology (Nir et al., 1986b) to obtain the separate rate constants for virus attachment, detachment, and the actual fusion process. As shown in the preceding paper (Klappe et al., 1986), the ability of Sendai virus to fuse with liposomes is strongly dependent on the liposome composition and pH. The virus fuses very efficiently with pure negatively charged vesicles, whereas inclusion of the zwitterionic lipid phosphatidylcholine (PC)[1] in such bilayers strongly inhibits the fusion reaction. It is demonstrated here that the distinct ability of Sendai virus to fuse with liposomes of various compositions under a variety of conditions, i.e., as a function of pH and in the presence of absence of low amounts of the dehydrating agent poly(ethylene glycol), is primarily determined by the fusion rate per se, rather than the aggregation rate constant. The results are discussed in light of previous work on Sendai virus–erythrocyte membrane fusion and fusion of influenza virus with liposomes (Nir et al., 1986a,b).

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[1] Abbreviations: CL, cardiolipin; PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; LUV, large unilamellar vesicles; $R_{18}$, octadecylrhodamine B chloride; PEG, poly(ethylene glycol); Tris, tris(hydroxymethyl)aminomethane.

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## Experimental Procedures

*Materials.* Cardiolipin (CL, bovine heart), phosphatidylserine (PS, bovine brain), and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Octadecylrhodamine B chloride ($R_{18}$) was a product from Molecular Probes Inc. (Junction City, OR). Poly(ethylene glycol), molecular weight 8000, was purchased from Sigma (St. Louis, MO).

*Virus.* Sendai virus (Z strain) was grown in the allantoic cavity of 10-day-old embryonated chicken eggs. Allantoic fluid was harvested after 72 h, and the virus was purified and stored as previously described (Hoekstra et al., 1985a; Hoekstra & Klappe, 1986).

*Liposomes.* Large unilamellar vesicles of various compositions (see legends) with an average diameter of 0.1 $\mu$m were prepared by reverse-phase evaporation and subsequent extrusion through Unipore polycarbonate filters as previously described (Hoekstra et al., 1985b).

*Fusion Measurements.* Details of the $R_{18}$ assay have been described elsewhere (Hoekstra et al., 1984, 1985a). The principle of the assay relies on the relief of self-quenching of fluorescence upon fusion between $R_{18}$-labeled virus and liposomes. Fluorescently tagged virus was prepared by injecting 10 $\mu$L of an $R_{18}$ solution (2.2 mM in ethanol) into a suspension of Sendai virus (1 mg/mL). After incubation for 1 h in the dark, the virus was chromatographed on Sephadex G-75 to separate noninserted probe from the membrane-labeled (at a self-quenching concentration) virus. The amount of $R_{18}$-labeled virus was determined by protein measurement. One milligram of viral protein corresponds to 276 nmol of viral phospholipid (Hoekstra et al., 1984; Harmsen et al., 1985). For fusion measurements, the virus was suspended into a cuvette containing a buffer consisting of 130 mM NaCl/10 mM sodium acetate/10 mM sodium phosphate/10 mM Tris, adjusted to the appropriate pH. Fusion was initiated by injecting the liposomes into the cuvette with a Hamilton syringe. The final incubation volume was 2 mL, and the temperature was kept at 37 °C with a thermostated circulating water bath. As a measure of fusion, the increase of $R_{18}$ fluorescence, due to dilution of the probe into the liposomal target membrane upon virus–liposome fusion, was recorded continuously ($\lambda_{ex}$ = 560 nm; $\lambda_{em}$ = 590 nm) in a Perkin-Elmer MPF 43 fluorometer. The initial residual fluorescence intensity of the $R_{18}$-labeled virus was taken as the zero level, and the fluorescence at infinite probe dilution, determined after addition of Triton X-100 (1% v/v) and correction for sample dilution, was taken as 100%. The conditions of $R_{18}$ labeling are such that upon relief of fluorescence self-quenching the fluorescence of $R_{18}$ increases linearly with probe dilution.

## Theory

*Final Extents of Fusion and Fluorescence Increase.* The initial molar concentrations of liposomes and virus particles are denoted by $L_0$ and $V_0$, respectively. Previous studies (Stegmann et al., 1985; Nir et al., 1986a; Klappe et al., 1986; Amselem et al., unpublished results) as well as our current results show that the fusion products mostly consist of a single virus and any number of liposomes. Hence, we will focus here on this case. We take explicitly into account the difference in size between liposomes and virus particles. The ratio of liposomal lipid concentration, $L_s$, to viral lipid concentration, $V_s$, is related to the molar concentrations of particles by

$$L_0/V_0 = L_s[R_v^2 + (R_v - \delta_v)^2]/\{V_s[R_L^2 + (R_L - \delta_L)^2]\} = L_s n^2/V_s \quad (1)$$

in which $R_v$, $R_L$, $\delta_v$, and $\delta_L$ are virus radius, liposome radius, and their membrane thicknesses, respectively, and the number $n^2$ equals approximately $(R_v/R_L)^2$. We assume that a fraction $\alpha$ of virus particles is inactive, i.e., incapable of fusion with the liposomes at the given temperature and pH, whereas all liposomes can fuse, unless they are bound irreversibly to inactive virus particles. Let $L_f$ and $V_f$ denote the final concentrations of fused liposomes and virus particles, respectively. If the binding of liposomes to virus particles is reversible, then $L_f = L_0$ (Nir et al., 1986a), whereas in the extreme case of irreversible binding $L_f = (1 - \alpha)L_0$.

In our system, the virus particles are labeled. In the linear regime, where the increase in fluorescence intensity, $I$, varies linearly with the surface concentration of $R_{18}$ molecules according to $I = 1 - X$, where $X$ is the concentration relative to the initial stage, the final level of fluorescence is given by

$$I = (V_f/V_0)[1 - V_f n^2/(L_f + V_f n^2)] = (V_f/V_0)[L_f/(L_f + V_f n^2)] = (V_f/V_0)/(1 + V_f n^2/L_f) \quad (2)$$

We distinguish between two cases.

*Case 1: Reversible Binding.* We employ the following expression (Nir et al., 1986a) for the final extent of fusion of active virus particles:

$$V_f = V_0[1 - \exp(-L_0/V_0)] \quad (3)$$

with the modification that $V_0$ has to be multiplied by $1 - \alpha$, i.e.

$$V_f = V_0(1 - \alpha)\{1 - \exp(-L_0)/[V_0(1 - \alpha)]\} \quad (4)$$

The expression for $I$ is obtained by substituting $V_f$ from eq 4 in eq 2 and setting $L_f = L_0$.

*Case 2: Irreversible Binding.* Let $V^*$ denote the total concentration of virus particles associated with liposomes:

$$V^* = V_0[1 - \exp(-L_0/V_0)] \quad (5)$$

and

$$V_f = V_0(1 - \alpha)[1 - \exp(-L_0/V_0)] \quad (6)$$

The expression for $I$ is obtained by substituting $V_f$ from eq 6 in eq 2 and setting

$$L_f = (1 - \alpha)L_0$$

For $\alpha = 0$ (100% activity) and a 1:1 ratio of viral to liposomal lipid, the final levels of fluorescence are expected to be 20% higher in our case as compared with those calculated for the fusion of influenza virus with cardiolipin vesicles (Nir et al., 1986a), because in the latter case the radii of viral and liposomal particles are equal, whereas the mean radius of Sendai virus is 75 nm (Harmsen et al., 1985), i.e., 1.5 times larger than that of a liposome. Larger virus radii correspond to larger ratios of the number of liposomes to virus particles, and hence, relatively more virus particles have a chance to fuse. At larger lipid ratios (e.g., $L_s/V_s$ equals 4:1 or 8:1), the effect of size difference is insignificant because all active virus particles fuse under those conditions. Table I demonstrates that the reduction in $I$ values due to irreversible binding of liposomes to inactive virus particles becomes more pronounced at smaller values of the final extents, i.e., for smaller ratios of liposomal to viral lipid.

## Results

*Extent of Fusion.* The extent of fusion between Sendai virus and liposomes of various compositions can be deduced from the final levels of $R_{18}$ fluorescence, i.e., after incubation times of 3–24 h. In Tables I and II, the effect of vesicle composition and pH on the final extent of fusion is shown. In addition, we examined how the final levels of fusion were influenced by inclusion of the dehydrating agent PEG in the fusion medium. For each case, we show the dependence of the final

Table I: Experimental and Calculated Values of Final Levels of Fluorescence Intensity[a]

| liposome compn | liposomal lipid (μM) | medium | exptl % fluorescence | calcd % fluorescence: irreversible binding[c] | calcd % fluorescence: reversible binding[d] |
|---|---|---|---|---|---|
| | | | | (100)[b] | |
| CL | 2.5 | pH 5 | 50.6 | 44 | |
| | 10 | | 85.8 | 78 | |
| | 25 | | 84.7 | 90 | |
| | 50 | | 99 | 95 | |
| | | | | (60) | (60) |
| CL | 2.5 | pH 7.4 | 27.6 | 26.3 | 35.1 |
| | 10 | | 44 | 46.9 | 51.4 |
| | 25 | | 56.1 | 53.9 | 56.2 |
| | 50 | | 62.7 | 56.8 | 58 |
| | | | | (80) | (80) |
| CL/DOPC (1:1) | 2.5 | pH 5 | 29.7 | 35.1 | 40.2 |
| | 10 | | 61.1 | 62.5 | 65.3 |
| | 25 | | 67.1 | 71.9 | 73.4 |
| | 50 | | 80.9 | 75.8 | 76.6 |
| | | | | (30) | (20) |
| CL/DOPC (1:1) | 2.5 | pH 7.4 | 13.0 | 13.1 | 16.3 |
| | 10 | | 21.8 | 23.5 | 18.9 |
| | 25 | | 25 | 27 | 19.6 |
| | 50 | | 29.6 | 28.4 | 19.8 |
| | | | | (80) | (80) |
| CL | 2.5 | pH 7.4, | 37.6 | 35.1 | 40.2 |
| | 10 | 4% | 56.7 | 62.5 | 65.3 |
| | 25 | PEG | 69.7 | 71.9 | 73.4 |
| | 50 | | 77.5 | 75.8 | 76.6 |

[a] $R_{18}$-labeled Sendai virus particles were incubated with different liposomes for 3–24 h at 37 °C. The virus concentration was 20 μg of protein/2 mL, or 2.8 μM with respect to phospholipid. [b] When all virus particles are active, eq 4 or 6 reduces to eq 3. Numbers in parentheses indicate percent active virus. [c] See eq 6. [d] See eq 4.

Table II: Percent of Sendai Virus Fusion Activity and Rate Constants of Fusion ($f$), Aggregation ($C$), and Dissociation ($D$) as Functions of Liposomal Membrane Composition, pH, and PEG Concentration at 37 °C[a]

| liposome compn, pH | % PEG | % active virus[a] | $f$ ($s^{-1}$) | $C$ ($M^{-1}\cdot s^{-1}$) | $D$ ($s^{-1}$) |
|---|---|---|---|---|---|
| CL, 5 | | 100 | 1 | $1.8 \times 10^8$ | 0.001 |
| CL, 7.4 | 8 | 86 | 0.05 | $1.2 \times 10^8$ | 0.01 |
| CL, 7.4 | 4 | 80 | 0.03 | $1.2 \times 10^8$ | 0.025 |
| CL, 7.4 | | 55 | 0.018 | $9 \times 10^7$ | 0.02 |
| CL/DOPC, 5 | | 80 | 0.2 | $1.2 \times 10^8$ | 0.01 |
| CL/DOPC, 7.4 | 8 | 70 | 0.013 | $8 \times 10^7$ | 0.02 |
| CL/DOPC, 7.4 | 4 | 50 | 0.01 | $8 \times 10^7$ | 0.02 |
| CL/DOPC, 7.4 | | 30 | 0.009 | $8 \times 10^7$ | 0.02 |
| PS, 5 | | 30[b] | 0.03 | $5 \times 10^7$ | 0.04 |
| PS, 7.4 | 8 | 30 | 0.013 | $7 \times 10^7$ | 0.04 |
| PS, 7.4 | 4 | 30 | 0.006 | $7 \times 10^7$ | 0.04 |
| PS, 7.4 | | 25 | 0.005 | $7 \times 10^7$ | 0.04 |

[a] Each case in this table represents a summary of a comparison between calculated and experimental levels of final fluorescence for four ratios of liposomes to viruses. Examples of such a comparison are given in Table II. [b] The actual values in this case may vary between 20% and 40%, but the results are better described by assuming irreversible binding of liposomes to inactive virus particles. The uncertainty in other cases is ca. 10%.

level of $R_{18}$ fluorescence on the ratio of liposomal to viral lipid, which was varied from 0.89 to 17.8. Since the virus is labeled, it can be expected that the dilution of the $R_{18}$ probe and hence the fluorescence intensity will increase with the concentration of liposomes. However, except when cardiolipin liposomes were employed, the final levels of fluorescence increase did not approach 100%, even in the presence of a large excess of liposomes. Qualitatively, the obvious implication is that a fraction, $\alpha$, of the virus particles is inactive. Addition of unlabeled liposomes to 1:1 mixtures after long incubation times resulted in an increase in fluorescence intensity, indicating

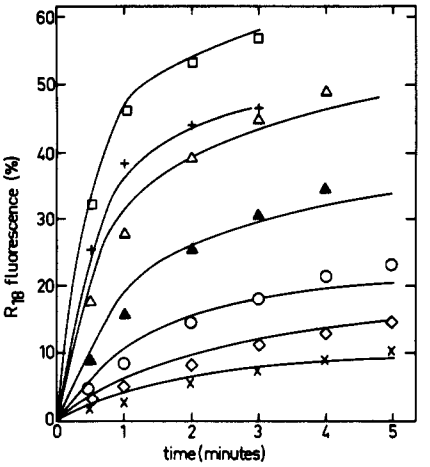


FIGURE 1: Effect of liposome concentration and composition on the kinetics of $R_{18}$ fluorescence increase upon fusion between $R_{18}$-labeled Sendai virus and liposomes. Fusion experiments were carried out at pH 5.0. The incubation temperature was 37 °C. Calculated values are given by data points. The viral concentration was 2.8 μM (phospholipid). The liposomal target membranes were: (i) CL vesicles at lipid concentrations of 2.5 (○), 10 (△), and 25 μM (□), (ii) CL/DOPC vesicles at 10 (▲) and 50 μM (+) lipid, and (iii) PS vesicles at 25 (×) and 50 μM (◇) lipid. Rate constants used in the calculations are given in Table II.

fusion of liposomes with the fusion products, whereas addition of unlabeled or labeled virus particles did not result in an increase in fluorescence intensity (Klappe et al., 1986). This is in accord with the model (Nir et al., 1986a) which predicts that fusion products consist mainly of a single virus and any number of liposomes. Table I indicates that the experimental results can be well explained and predicted by a model which assumes that (i) fusion products consist of a single virus and any number of liposomes, (ii) a fraction $\alpha$ of the virus particles is inactive, i.e., incapable of fusing with a particular system of liposomes at a given pH, and (iii) the inactive virus particles bind liposomes in an essentially irreversible manner so that practically all the liposomes are associated with virus particles, but only a fraction $(1 - \alpha)$ of them fuses.

The deduction from the final fluorescence levels is summarized in Table II in terms of $\beta = 1 - \alpha$, i.e., the fraction of active virus particles. At pH 5, all virus particles are active, i.e., capable of fusing with cardiolipin liposomes. In contrast, the activity of Sendai virus particles in fusing with PS liposomes is only ca. 30%, whereas with CL/DOPC (1:1) the activity is intermediate. At pH 7.4, the percent activity of CL or CL/DOPC is about half of the value at pH 5. Addition of PEG enhances the fusion activity, in particular when CL/DOPC liposomes are employed. In this case, the fusion activity at pH 7.4 increased from 30% to 50% or 70% at final PEG concentrations of 4% and 8% (w/v), respectively.

*Kinetics of Fusion.* The results in Figure 1 show the kinetics of the increase in $R_{18}$ fluorescence intensity as a function of liposome concentration (2.5–50 μM lipid) for a fixed concentration of virus particles. The liposomes used consisted of CL, CL/DOPC, or PS. The overall fusion rate increases with lipid concentration, and its dependence on liposome composition is according to the sequence CL > CL/DOPC > PS.

Figure 2 shows that the process of fusion is faster at pH 5 than at pH 7.4. Figure 3 illustrates that the overall fusion rate (at pH 7.4) increases significantly when PEG is present in the medium.

An analysis of these results in terms of the mass action kinetic model yields the rate constants of aggregation, deaggregation, and fusion, which are denoted by $C$, $D$, and $f$,

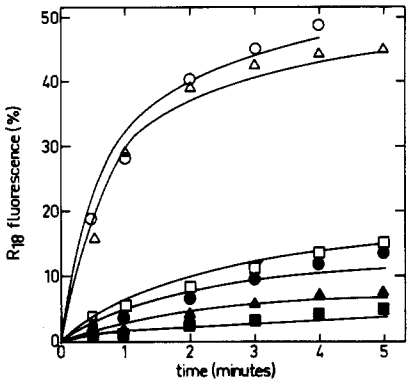


FIGURE 2: pH dependence of the kinetics of $R_{18}$ fluorescence increase upon virus–liposome fusion. The virus (2.8 $\mu$M phospholipid) was suspended in a medium of pH 7.4 (closed symbols) or 5.0 (open symbols), and fusion was initiated upon injection of the liposomes. The drawn lines indicate the fusion curves, obtained by continuous monitoring of $R_{18}$ fluorescence increase. Calculated values are given by data points. CL vesicles, 10 $\mu$M lipid (○, ●); CL/DOPC vesicles, 25 $\mu$M lipid (△, ▲); PS vesicles, 50 $\mu$M lipid (□, ■). The rate constants are given in Table II.

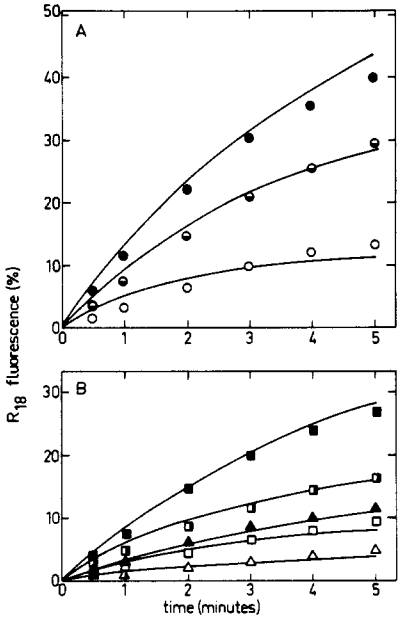


FIGURE 3: Effect of PEG on virus–liposome fusion. Fusion between $R_{18}$-labeled Sendai virus (2.8 $\mu$M lipid) and liposomes of various compositions was monitored (drawn lines) in the absence and presence of PEG at 37 °C. The pH of the fusion medium was 7.4. Data points represent the calculated values. (A) CL liposomes, 10 $\mu$M lipid: (○) 0%, (◒) 4%, and (●) 8% PEG (w/v). (B) CL/DOPC liposomes, 50 $\mu$M lipid: (□) 0%, (◨) 4%, and (■) 8% PEG (w/v). PS liposomes, 50 $\mu$M lipid: (△) 0% and (▲) 8% PEG (w/v). The rate constants employed in the calculations are indicated in Table II.

respectively. The essential features of the analysis and a presentation of the equations describing the formation of aggregation–fusion products are given in Nir et al. (1986a). In the current study, the programs account for the fact that in the present work the virus particles rather than the liposomes are labeled. In addition, the difference in size between liposomes and virus particles has been explicitly considered, as described under Theory. The overall fusion process is viewed as a sequence of two kinetic steps, aggregation, which is of second order in particle concentrations, followed by membrane destabilization and merging, which is a first-order process. As in the case of fusion between influenza virus and liposomes, there was no aggregation or fusion between liposomes or between virus particles, and the fusion products were assumed to consist of a single virus and several liposomes [cf. Klappe et al. (1986)]. The analysis takes explicitly into account the fraction of inactive virus particles, $\alpha$, which was determined from the final levels of fluorescence intensity increase (see Tables I and II). First, we determined the rate constant $C$ ($M^{-1}\cdot s^{-1}$) from the results with relatively low vesicle concentrations, where aggregation is rate limiting to the overall fusion process. Then the rate constant of fusion, $f$($s^{-1}$), was determined from fusion results in more concentrated suspensions. The rate constant of deaggregation, or dissociation, $D$ ($s^{-1}$), was determined from the results at the later, prefinal stages of the fusion reaction. As pointed out in the previous subsection, the final levels of fluorescence intensity were explained by assuming an essentially irreversible binding of liposomes to inactive virus particles. However, in the case of the kinetics of fluorescence increase, the results were explained by assuming a small degree of reversibility.

A comparison of experimental and calculated values of $R_{18}$ fluorescence intensity indicates that the mass action kinetic model can yield reasonable simulations and predictions of the results (see Figures 1–3). Table II gives the rate constants as a function of vesicle composition, pH, and PEG concentration.

The results in Figure 1 indicate an increase in $I$ values by a factor of 4–5 (at $t$ = 1 min) for a 20-fold increase in liposome concentrations. Hence, while aggregation has a significant effect on the overall fusion rate, it may be expected to constitute the rate-limiting step only at the lower liposome concentrations employed. Similarly, the initial linear increase in $I$ values with time, which was shown to occur when aggregation is the rate-limiting step (Nir et al., 1986a), is observed in a few cases only, e.g., at pH 7.4 with 2.5 $\mu$M CL/DOPC liposomes.

The results in Table II and Figures 1–3 indicate that the overall fusion process is generally faster in cases where the percent of active virus particles is larger. Indeed, the calculations also indicate that the percent of active virus affects the kinetics significantly. However, the final extents and rates do not always exhibit the same trend. For instance, at pH 7.4, the initial kinetics of fusion of Sendai virus with CL liposomes are faster than in the case of CL/DOPC liposomes in a medium containing 8% PEG, whereas the percents of active viruses are 55 and 70, respectively.

## Discussion

Recent studies (Gething et al., 1980; Wilson et al., 1981; Haywood & Boyer, 1982; Richardson & Choppin, 1983; White et al., 1983) emphasized the role of viral envelope proteins in affecting the fusion of viruses with biological membranes. The current results demonstrate that the lipid composition of the target membrane also affects the rate and extent of virus fusion. Conceivably, the source of this effect could be due to differences in the specific interactions between the viral glycoproteins and the lipids of the target membrane. Similar to the results of studies with influenza virus (Stegmann et al., 1985; Nir et al., 1986a), fusion of Sendai virus with liposomal target membranes does not require specialized receptors.

The presence of PEG in the medium promotes both the extent and the rate of fusion at neutral pH. The effect of PEG is presumably related to the necessity to overcome the repulsive hydration forces between apposed membranes before fusion can occur (Rand, 1981; Parsegian & Rand, 1983). Previous work (Hoekstra, 1982) revealed that $Ca^{2+}$- and $Mg^{2+}$-induced fusion of acidic phospholipid vesicles was dramatically enhanced in the presence of low concentrations of PEG; i.e., rapid and extensive fusion was induced at subthreshold cation con-

centrations. The effect of PEG was explained by an indirect, dehydrating action of the agent on the lipid bilayer. Recent work (MacDonald, 1985; Klose et al., 1985) evidently supports this notion, and it would appear that the present results on virus–liposome interaction can be explained similarly. After initial attachment, the interbilayer distance between viral and liposomal membranes, will be at least 100 Å [Harmsen et al., 1985; cf. Klappe et al. (1986)], a distance at which hydration forces are of minor significance (Parsegian & Rand, 1983). Indeed, the aggregation rate constants for the virus–liposome interaction are very similar, irrespective of the presence of PEG. Partial dehydration of the interbilayer phase, which is facilitated by the dehydrating action of PEG, should thus be reflected by an increase in the fusion rate constants upon addition of the agent. In fact, with increasing PEG concentration, this trend became apparent as shown in Table II, although the effect of pH is more pronounced.

The rate and extent of fusion of Sendai virus with CL liposomes are larger at pH 5 than at pH 7.4, as observed for influenza virus. However, we note that the fusion activity of Sendai virus with cells, e.g., erythrocyte ghosts, is most pronounced around pH 7.5 (Hoekstra et al., 1985a). The fusion products of both viruses consist of a single virus and several liposomes, at least for liposomal:viral lipid ratios of up to about 10:1. Future studies will test the generality of this statement with other virus particles.

The deductions from the final extents do not depend on the rate constants. These results indicate that for the same conformation of the viral proteins, which is dictated by pH, the percent of virus particles capable of fusing with liposomes depends on the lipid composition of the liposomes. It should be emphasized that there is a distinction between the percent of virus particles capable of fusing with liposomes of a certain composition, at a given pH and temperature, and the percent of virus particles that actually fuse. This distinction stems from the fact that the fusion products consist of a single virus and several liposomes. Thus, unless the number of liposomes exceeds significantly the number of virus particles (e.g., 4:1), there will be free, i.e., unfused, virus particles which are capable of fusing with liposomes, whereas other virus particles have fused with several liposomes.

The results in Figures 1–3 demonstrate that the mass action kinetic model can adequately simulate and predict the outcome of the kinetics of fusion of Sendai virus particles with liposomes. This model views the fusion process to consist of a second-order aggregation step followed by a sequence of first-order steps such as membrane destabilization and merging. One of the new elements emerging is that in the analysis of the kinetics of virus–vesicle fusion, where times of interest might be several minutes, it is imperative to take into account explicitly the percent of active virus particles, which is deduced from measurements over several hours.

Table II, which gives the rate constants and percentage of activity as a function of liposome composition, pH, and PEG content, illustrates that the largest variability is exhibited by the rate constant of fusion per se, $f$, the extreme values of which are 1 $s^{-1}$ for CL at pH 5 [this value is similar to that obtained for influenza virus (Nir et al., 1986a)] and 0.005 $s^{-1}$ for PS at pH 7.4. The result that $f$ values are most sensitive to pH supports the view that changes in the conformation of the viral proteins are most important in affecting the viral affinity for fusion with target membranes (Hoekstra et al., 1985a).

The role played by the dissociation rate constant, $D$, is complex. Ordinarily, in vesicle–vesicle fusion an increase in the value of $D$ results in a reduction in the overall fusion rate, depending on the ratio $D/f$ (Bentz et al., 1983, 1985; Nir et al., 1986a). However, in the later stages ($I \geq 15$–$30$) of the aggregation–fusion reaction in concentrated liposome suspensions, the calculated $I$ values may increase with an increase in $D$ values. A nonvanishing value of $D$ enables detachment of liposomes which are bound to inactive virus particles, so that the dissociated liposomes can encounter free and active virus particles or fusion products.

We have noted that the final fluorescence levels are best explained by assuming that inactive virus particles bind liposomes irreversibly. This finding does not contradict the use of nonvanishing $D$ values in the kinetic analysis, because our calculations indicate that the inactive virus particles bind a certain fraction of the liposomes even after long incubation times. We do not as yet have an explanation for the phenomenon of partial activity of virus particles. It can be visualized that inactivity is an expression of binding of liposomes to inactive glycoproteins of the virus. According to this point of view, the percent of fusion activity reflects a certain probability of liposome binding to inactive sites on the virus particles. The percent of inactive viral proteins is expected to be a function of pH, but it can also reflect the interaction of the viral proteins with the lipids of the target membrane. The fusion activity of Sendai virus is not merely dependent on the liposomal membrane surface charges, as is exhibited by the sequence CL > CL/DOPC > PS with activity percentages of 100, 80, and 30, respectively, at pH 5. However, as pointed out in the preceding paper (Klappe et al., 1986), an F protein independent mechanism may also contribute to the occurrence of fusion at these conditions. The mechanism likely involves a direct participation of HN in the fusion reaction, implying that physical and geometrical properties of the bilayer per se may also govern the ability of this protein to trigger membrane fusion at certain conditions. Application of the present theoretical procedure to kinetically analyze the fusion of reconstituted viral envelopes, containing one or both viral proteins, will be an important tool to further clarify the role of the viral proteins in determining the fusogenic properties of viruses with various target membranes at different conditions.

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# Absence of Detectable Ribonucleic Acid in the Purified, Untransformed Mouse Glucocorticoid Receptor†

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ABSTRACT: The glucocorticoid receptor (GC-R) isolated from the mouse AtT-20 pituitary tumor cell line exists in three forms. The untransformed (non-DNA-binding), 9.1S species (319K) can be converted into two transformed (DNA-binding) species. One of these (5.2 S, $M_r$ 132K) appears to be composed of one molecule of the hormone-binding, monomeric protein (96K) plus a small RNA, while the second transformed species is the monomeric, hormone-binding subunit (3.8 S, 96K) itself. We wished to determine whether the untransformed GC-R contains RNA or if the monomer binds to RNA subsequent to subunit dissociation (which occurs during receptor transformation). Kinetic studies using both the crude and purified untransformed GC-R show that the untransformed, 9.1S GC-R dissociates into 3.8S monomeric subunits, without forming a transient 5.2S complex. The untransformed receptor was then purified with affinity chromatography, gel filtration, and DEAE-cellulose chromatography. One major protein band, corresponding in size to the GC-R monomer (94K–96K), was observed on sodium dodecyl sulfate–polyacrylamide gels upon silver staining or fluorography of [$^3$H]dexamethasone mesylate covalently labeled receptor. In vivo $^{32}$P-labeling of AtT-20 cells, followed by purification of the untransformed GC-R, yielded two major $^{32}$P-labeled components (94K–96K and 24K). Both of these bands were protease-sensitive, contained phosphoserine, and were unaffected by ribonuclease treatment. We conclude that the untransformed mouse GC-R is wholly proteinaceous and contains no RNA. Thus, RNA binding occurs subsequent to dissociation of the oligomeric, untransformed GC-R complex into monomers.

Steroid hormone receptors are gene regulatory proteins that elicit a wide variety of physiological responses (Yamamoto & Alberts, 1976). Crucial to our understanding of the mechanism of steroid-regulated gene expression is a knowledge of the structure of these proteins. A large number of studies support the hypothesis that an oligomeric, untransformed (non-DNA-binding) receptor species dissociates into subunits upon receptor transformation, that is, conversion to a DNA-binding moiety [reviewed in Vedeckis (1985)]. Our laboratory has performed a detailed series of studies to elucidate the structure of the glucocorticoid receptor (GC-R)[1] in the mouse AtT-20 pituitary tumor cell line (Vedeckis, 1981, 1983a,b, 1985; Eastman-Reks et al., 1984; Reker et al., 1985; Kovačič-Milivojević et al., 1985; Vedeckis et al., 1985). We

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[1] Abbreviations: 94K, 24K, etc., $M_r$ of 94 000, 24 000, etc.; BSA, bovine serum albumin; DEAE, diethylaminoethyl; Dex-M, dexamethasone 21-mesylate (9α-fluoro-16β-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 21-methanesulfonate); EDTA, ethylenediaminetetraacetic acid; GC-R, glucocorticoid receptor; HPLC, high-performance liquid chromatography; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; UMP, uridine 5′-phosphate.

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