



## FUSION OF SENDAI VIRUS WITH VESICLES OF OLIGOMERIZABLE LIPIDS: A MICRO CALORIMETRIC ANALYSIS OF MEMBRANE FUSION

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Sendai virus fuses efficiently with small and large unilamellar vesicles of the lipid 1,2-di-*n*-hexadecyloxypropyl-4-(beta-nitrostyryl) phosphate (DHPBNS) at pH 7.4 and 37°C, as shown by lipid mixing assays and electron microscopy. However, fusion is strongly inhibited by oligomerization of the head groups of DHPBNS in the bilayer vesicles. The enthalpy associated with fusion of Sendai virus with DHPBNS vesicles was measured by isothermal titration microcalorimetry, comparing titrations of Sendai virus into (i) solutions of DHPBNS vesicles (which fuse with the virus) and (ii) oligomerized DHPBNS vesicles (which do not fuse with the virus), respectively. The observed heat effect of fusion of Sendai virus with DHPBNS vesicles is strongly dependent on the buffer medium, reflecting a partial charge neutralization of the Sendai F and HN proteins upon insertion into the negatively-charged vesicle membrane. No buffer effect was observed for the titration of Sendai virus into oligomerized DHPBNS vesicles, indicating that inhibition of fusion is a result of inhibition of insertion of the fusion protein into the target membrane. Fusion of Sendai virus with DHPBNS vesicles is endothermic and entropy-driven. The positive enthalpy term is dominated by heat effects resulting from merging of the protein-rich viral envelope with the lipid vesicle bilayers rather than by the fusion of the viral with the vesicle bilayers *per se*.

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**KEYWORDS:** membrane fusion; vesicles; polymerizable lipids; Sendai virus; isothermal titration microcalorimetry.

### INTRODUCTION

Sendai virus or Haemagglutinating Virus from Japan (HVJ) is a paramyxovirus that infects cells by fusion with the cell membrane at neutral pH. Pioneering studies (Shimizu *et al.*, 1974) revealed that the virus contains two different spike proteins that protrude from the viral envelope membrane: the HN glycoprotein (with two subunits of 15 kDa and 51 kDa) with haemagglutinating and neuramidase activity, and a second glycoprotein of 67 kDa, also composed of two subunits. One of the subunits of this second spike protein contains a distinct hydrophobic region at its amino terminal end

(Gething *et al.*, 1978). In later studies, the HN protein was found to be involved in receptor-mediated binding to the cell surface, whereas the second protein mediates fusion with the target membrane. Therefore, the second spike protein is called the fusion or F protein. There are many reports of fusion between Sendai virus and liposomes that indicate that the physiologically relevant mode of action of Sendai requires a temperature of 37-40°C and pH 7.4, and a target membrane of phosphatidylcholine and cholesterol that contains a sialic acid receptor (a ganglioside) to bind HN (Haywood and Boyer, 1982; Citovsky *et al.*, 1985; Klappe *et al.*, 1986). Fusion involves insertion of the hydrophobic amino terminus of one of the two subunits of the F protein into the target membrane (Hsu *et al.*, 1981; Novick and Hoekstra, 1988). Target membranes that contain

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negatively-charged lipids such as phosphatidylserine or cardiolipin fuse more efficiently than membranes composed of neutral phospholipids (Haywood and Boyer, 1984; Klappe *et al.*, 1986; Fonteijn *et al.*, 1992). It has been suggested that the negatively-charged lipids act as unspecific sialic acid substitutes (Haywood and Boyer, 1984), and/or that the decreased hydration repulsion of charged lipid bilayers favours rapid aggregation and fusion between Sendai virus and liposomes (Klappe *et al.*, 1986). In addition, it was discovered that at low pH an extremely rapid fusion process occurs between Sendai virus and liposomes containing negatively-charged lipids, as a result of unspecific electrostatic interactions between HN (not F) and the target membrane upon protonation of the HN glycoprotein below pH 6 (the isoelectric point of HN is at pH 6.5) (Haywood and Boyer, 1984; Klappe *et al.*, 1986; Chejanowsky *et al.*, 1986; Fonteijn *et al.*, 1992). This low pH, HN-mediated fusion process does not bear physiological relevance.

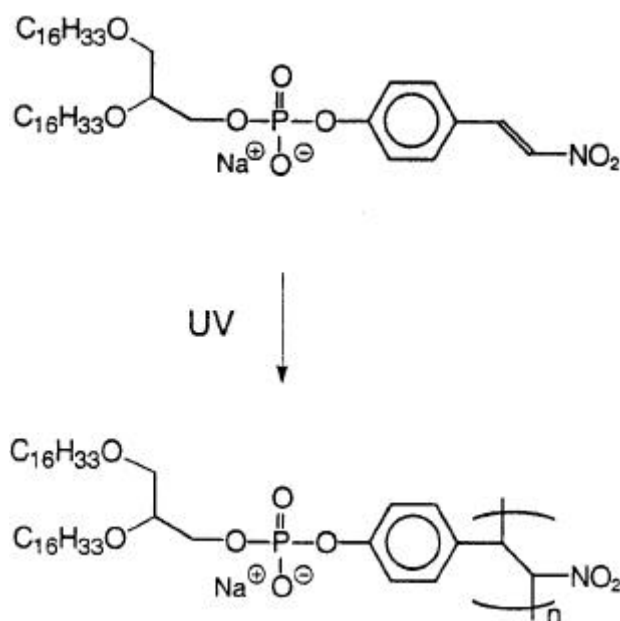
Apparently, the crucial step in Sendai virus fusion is insertion of part of the F protein into the target membrane. In view of the microcalorimetric studies on peptide-lipid interactions reported by Seelig and coworkers (Beschiaschvili and Seelig, 1992; Seelig *et al.*, 1993; Thomas and Seelig, 1993; Seelig, 1997; Wenk and Seelig, 1998), membrane insertion of the fusion peptide could have drastic influences on the thermodynamic characteristics of the fusion process. Hydrophobic membrane binding of peptides is normally endothermic and driven by an entropically favourable release of hydration water (Russell *et al.*, 1996). However, several examples of 'non-classical' hydrophobic binding are known in which binding is enthalpy-driven, and the binding entropy is zero or even negative (Smithrud *et al.*, 1991; Beschiaschvili and Seelig, 1992; Thomas and Seelig, 1993; Peterson *et al.*, 1995; Wenk and Seelig, 1998). The thermodynamics of binding are strongly dependent on the internal lateral tension of the lipid membrane (the ease with which it permits insertion of foreign molecules), which is relatively low for small vesicles, and higher for membranes with less curvature (i.e. diameters exceeding 50 nm) (Beschiaschvili and Seelig, 1992).

We have recently reported that calcium-induced fusion of small vesicles is endothermic by  $0.15 \pm 0.10$  kJ/mol lipid and driven by a gain in entropy of both lipid and water molecules as a result of bilayer merging (Ravoo *et al.*, 1998). On the other hand, fusion of influenza virus and liposomes is endothermic by 2.5-2.9 kJ/mol viral lipid (Nebel *et al.*,

1995). These authors do not elaborate on the potential entropic driving forces for membrane fusion. Fusion between influenza virus and liposomes occurs at pH 5.1 and involves insertion of a hydrophobic part of the influenza haemagglutinin protein into the target membrane. Clearly, the heat effects in protein-mediated fusion are much larger than those observed in calcium-induced fusion of pure lipid vesicles. It would be worthwhile to investigate whether the differences should be attributed to heat effects due to insertion of viral fusion proteins into the target vesicle membrane, due to lipid membrane fusion *per se*, or because of lipid-protein mixing as a result of fusion between the viral envelope and the target membrane. It has been shown that the contribution of viral and liposomal contents mixing is negligible (Nebel *et al.*, 1995).

A related issue that requires attention is the effect of buffer on the observed enthalpies of fusion, since in several calorimetric studies of peptide-lipid interactions a substantial buffer dependency has been found (Beschiaschvili and Seelig, 1992; Seelig *et al.*, 1993; Seelig, 1997; Baker and Murphy, 1996). This buffer effect has been interpreted in terms of membrane binding-induced  $pK_a$  shifts of residues in the peptide that binds to or inserts into the membrane. Such  $pK_a$  shifts can either be the result of the transfer of charged alpha-amino acid residues from a polar to a hydrophobic environment, or (in the case of negatively-charged bilayers) arise from the fact that the local pH near a negatively-charged membrane is considerably lower than the pH in bulk solution, leading to protonation of residues upon transfer (Seelig, 1997). Depending on the alpha-amino acid composition of the peptide, the membrane binding-induced  $pK_a$  shift(s) may lead to either a net uptake or a net release of protons upon binding. These protons are absorbed or provided by the buffer medium, which causes a heat effect that is directly correlated to the ionization enthalpy of the buffer. Therefore, common practice measures the heat effect of peptide-lipid interactions in several buffers of known ionization enthalpy, and determines the intrinsic heat effect by extrapolation to zero buffer ionization enthalpy. This consideration has been ignored in the calorimetric study of influenza virus-liposome fusion, possibly due to the complicated analysis of such a buffer effect in a pH-dependent fusion process (Nebel *et al.*, 1995).

This paper describes an investigation of fusion of vesicles of the negatively-charged, oligomerizable lipid 1,2-di-*n*-hexadecyloxypropyl-4-(beta-nitrostyryl) phosphate (DHPBNS, see: Ravoo *et al.*, 1996) with



**Fig. 1.** Molecular structure of DHPBNS and oligomerized DHPBNS.

Sendai virus. DHPBNS is one of the members of a family of synthetic lipids that provides a highly useful membrane mimetic system. The molecular structure of DHPBNS is presented in Figure 1. This lipid has two C16 hydrocarbon chains and a main phase transition temperature ( $T_m$ ) of 40°C (Ravoo *et al.*, 1996). The beta-nitrostyrene head groups of the lipid oligomerize upon UV irradiation (Ravoo *et al.*, 1996).  $^{31}\text{P}$ -NMR spectroscopy, permeability studies, and detergent resistance measurements indicated that the head groups have a reduced mobility and pack more closely upon oligomerization. DSC showed that the main phase transition temperature (i.e. flexibility of the alkyl chains in the bilayer interior) is unaffected, although the phase transition occurs less cooperatively in the oligomerized bilayer (Ravoo *et al.*, 1996, 1999). We have shown that vesicles of DHPBNS (and structurally related lipids) undergo efficient calcium-induced fusion (Ravoo *et al.*, 1999). After oligomerization of the lipid head groups, the vesicles aggregate upon addition of calcium ion, but calcium-induced fusion is strongly inhibited due to a combination of reduced lipid lateral diffusion and bilayer curvature effects (Ravoo *et al.*, 1998, 1999). We have exploited this membrane model system in a micro calorimetric study to determine the enthalpies associated with binding of calcium ion, vesicle aggregation, and bilayer fusion in the course of calcium-induced vesicle fusion, and we showed that fusion of small

vesicles is associated with a small positive enthalpy (Ravoo *et al.*, 1998).

The present study addresses three main questions. (1) Since fusion of Sendai virus requires insertion of (part of) the viral F protein into the target membrane, is fusion inhibited upon oligomerization of the lipids in the target membrane? (2) Is it possible to measure a heat effect that accompanies fusion of Sendai virus with DHPBNS vesicles with titration microcalorimetry? (3) Is it possible to account for the differences that are anticipated for the enthalpy of interaction of Sendai virus with vesicles of DHPBNS prior to and after lipid oligomerization? In this paper we demonstrate that Sendai virus fuses efficiently with vesicles of DHPBNS, but not with vesicles of oligomerized DHPBNS. We show that fusion of Sendai virus with DHPBNS vesicles can be analyzed using titration microcalorimetry. From the buffer medium dependency observed in titration microcalorimetry, we conclude that Sendai virus does not fuse with oligomerized DHPBNS vesicles because the Sendai F protein does not insert into the oligomerized target membrane. Comparison of titrations of Sendai virus into DHPBNS vesicles (which fuse) and oligomerized DHPBNS vesicles (which do not fuse) yields an estimate of the enthalpy associated with the fusion process. Finally, we discuss the different contributions to the observed enthalpy of fusion.

## MATERIALS AND METHODS

### Materials

DHPBNS was prepared as described (Ravoo *et al.*, 1996). R18 was generously provided by Prof. D. Hoekstra (Laboratory of Physiological Chemistry, Groningen University).  $\text{NaH}_2\text{PO}_4$  and PIPES were purchased from Merck (Darmstadt, Germany), HEPES from Calbiochem Co. (La Jolla, CA, U.S.A.), and TRIS, Aristar quality, from BHD Chemicals Ltd (Poole, UK.).

### Vesicle preparation

Vesicle solutions were prepared from a 10 mM solution of the sodium salt of DHPBNS in chloroform. Aliquots of the chloroform solution were rotary-evaporated in Pyrex tubes to yield thin lipid films, which were dried in vacuum for 30 min. Subsequently, small unilamellar vesicles (50–100 nm) were prepared by dispersion of the lipid films in the appropriate buffer (pH 7.4) at 50°C by

means of a Branson B15 sonication immersion tip (30-40% power output in 30-40% cycles, *ca.* 2 min/ml). Large unilamellar vesicles were prepared by hydration of the thin lipid film in the desired buffer solution at 50°C. The turbid lipid suspension was freeze-thawed at least five times, and large unilamellar vesicles (200 nm) were obtained by repeated extrusion (at least ten times) through a 200 nm polycarbonate membrane in a LiposoFast Basic extruder (Avestin Inc., Ottawa, Canada). The extruder was preheated in hot water. Photo-induced oligomerization of DHPBNS in vesicles was performed by UV irradiation for 10 min, as previously described (Ravoo *et al.*, 1996).

### Virus

The Z strain of Sendai virus was kindly provided by Karin Klappe (Laboratory of Physiological Chemistry, Groningen University). It was grown, isolated and stored as previously described (Klappe *et al.*, 1986). R18 labelling was performed according to Klappe *et al.* (1986). The concentration of Sendai virus was determined by protein measurement against an albumin standard, and is reported as such in the text (unless indicated otherwise). For the microcalorimetric experiments in different buffers, the virus was twice pelleted by centrifugation and resuspended in the appropriate buffer.

### R18 assay of lipid mixing

The R18 lipid mixing assay was performed as described by Klappe *et al.* (1986). Fusion assays were performed with R18-labelled Sendai virus at 5 µg protein/ml and 5-50 µM of DHPBNS in 5 mM HEPES/NaAc buffer (140 mM NaCl, pH 7.4). The extent of fusion was determined from the maximum fluorescence observed following addition of target vesicles to labelled virus at the appropriate temperature ( $F_{\text{obs}}$ ), relative to the background fluorescence of R18 labelled virus ( $F_0$ ) and the infinite dilution of R18 upon addition of 0.5% (w/w) of TX-100 ( $F_{\text{inf}}$ ), according to:  $\text{extent (\%)} = (F_{\text{obs}} - F_0) / (F_{\text{inf}} - F_0) \times 100$ . The initial rate of fusion was determined from the tangent of the increase of fluorescence in time directly upon addition of target vesicles.

### Electron microscopy

Samples of vesicle and virus solutions were brought on formvar/carbon coated 400 mesh grids made hydrophilic by glow discharge in air directly before sample preparation. After *ca.* 30 s, the sample

solution was removed by gentle blotting with filter paper, and the sample was stained with uranyl acetate (1% w/v). A drop of staining agent was applied for 3-5 s and removed by blotting. The samples were air-dried for *ca.* 15 min, and examined in a Jeol JEM EX1200 electron microscope operated at 100 kV.

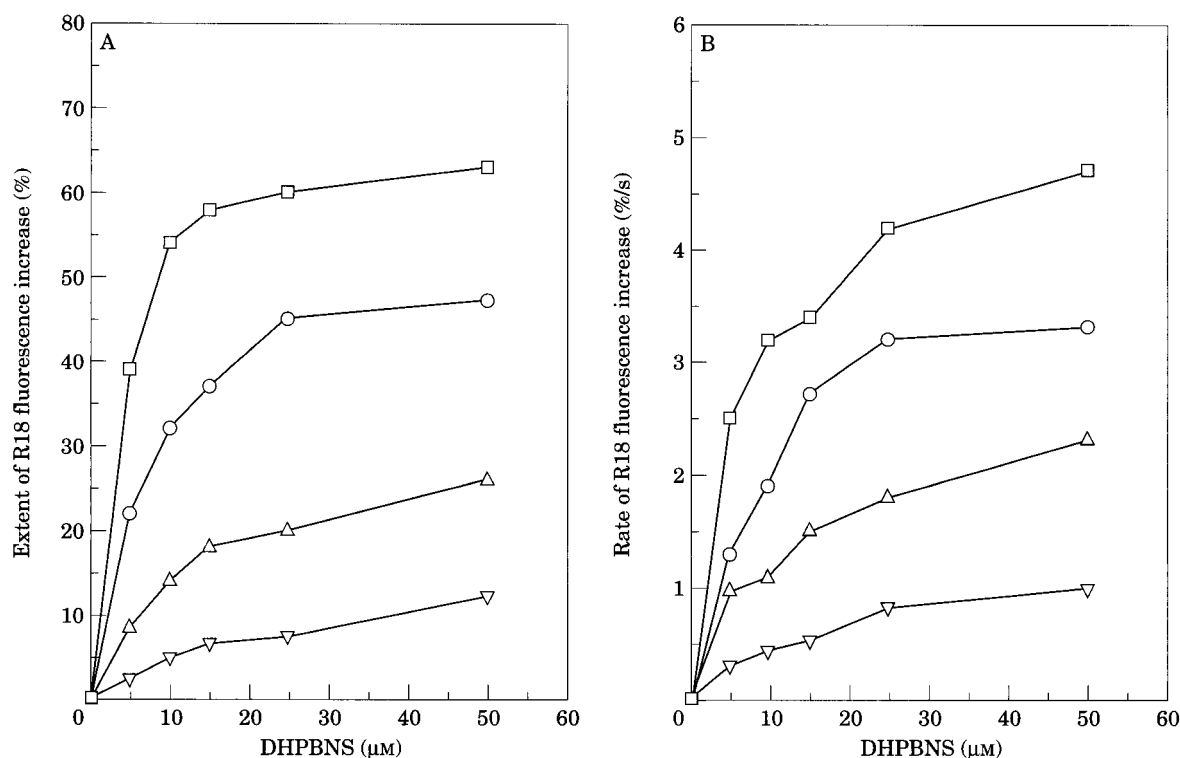
### Isothermal titration microcalorimetry

Titration microcalorimetry was performed with an Omega isothermal titration microcalorimeter (Microcal Inc., Northampton, MA, U.S.A.), operated at 37°C. 10 µl aliquots of Sendai virus suspension were injected into the sample cell containing a solution of DHPBNS vesicles (1.37 ml). 10-12 injections were carried out at 5 min intervals. The sample cell was stirred at 350 rpm. Integration of the heat flow vs time yielded the enthalpy for each injection. The concentration of Sendai virus in the syringe was 2.0 mg protein/ml and the concentration of DHPBNS in the sample cell was 0.5 mM. Both the Sendai virus and the DHPBNS vesicles were suspended/prepared in four different buffers (phosphate, PIPES, HEPES and TRIS) with [buffer]=10.0 mM, 140 mM NaCl and pH 7.40. The values of the buffer ionization enthalpies were taken from the best available literature data. Unfortunately, not all ionization enthalpies have been measured at 37°C, but for those that have been measured as a function of temperature (PIPES, HEPES and several others: Roig *et al.*, 1993) the heat capacity is rather small, and the value at 25°C can safely be used for the extrapolation. We used 5.10 kJ/mol for phosphate (Seelig, 1997), 12.4 kJ/mol for PIPES (Roig *et al.*, 1993), 22.5 kJ/mol for HEPES (Roig *et al.*, 1993) and 48.1 kJ/mol for TRIS (Seelig, 1997).

## RESULTS

### Lipid mixing assays and electron microscopy

Lipid mixing in the course of fusion between Sendai virus and vesicles of DHPBNS was monitored by the R18 assay (Hoekstra *et al.*, 1984; Klappe *et al.*, 1986). Although this assay is known to disagree with other fusion assays in some cases, it has recently been verified again that the dequenching of R18 gives an accurate measure of membrane fusion of Sendai virus, particularly in the first minutes of the fusion process (Ohki *et al.*, 1998). Unspecific probe transfer occurs at a much longer time scale.

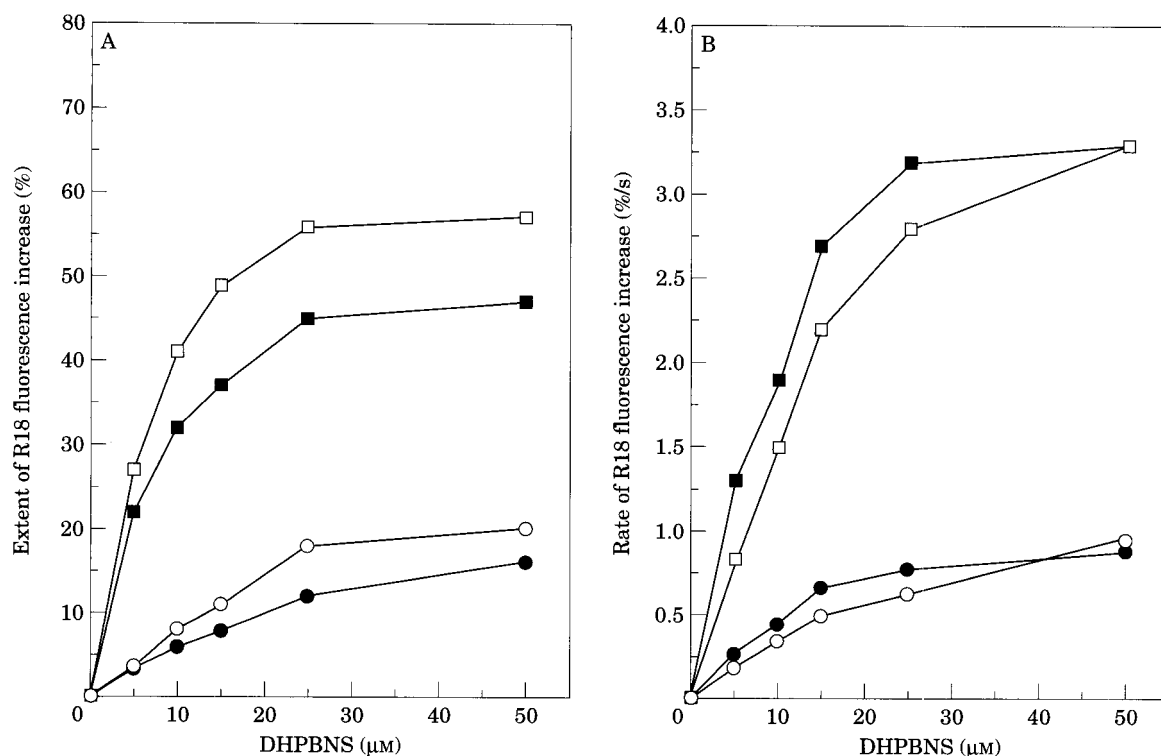


**Fig. 2.** Temperature dependence of lipid mixing during fusion of Sendai virus (5  $\mu\text{g/ml}$ ) with small vesicles of DHPBNS as a function of DHPBNS concentration. (A): Extent of fusion. (B): Initial rate of fusion. Legend: ' : 43°C; ) : 37°C; + : 30°C; , : 21°C.

The extent and initial rate of R18 fluorescence increase during fusion of Sendai virus (labelled with R18) with small (50–100 nm) vesicles of DHPBNS are shown in Figure 2. The data are reported as a function of DHPBNS concentration at four different temperatures. Clearly, fusion becomes faster and more extensive as the concentration of target membrane and/or the temperature are increased. Since the virus was added at a concentration of 5  $\mu\text{g}$  viral protein/ml, which is equivalent to 2.0  $\mu\text{M}$  viral lipid (Sendai virus contains *ca.* 400 nmol of lipid (including cholesterol) per mg of viral protein, see: Loyter and Volsky, 1982), efficient fusion requires a large excess of target membrane. Fusion is very slow and inefficient at 21°C, but occurs more readily at 30°C, and is quite efficient at 37°C and 44°C. Remarkably, Sendai virus can fuse with DHPBNS vesicles below their main phase transition temperature ( $T_m$  is 40°C: Ravoo *et al.*, 1996). We note that 100% fusion (complete mixing of all available viral envelope with the target vesicles) is not achieved in any of our fusion experiments, not even at high excess of target vesicles. This is most probably explained by the presence of a certain percentage of inactive virus, which does not participate in fusion.

Figure 3 illustrates the effect of lipid oligomerization in the DHPBNS vesicles on the extent and initial rate of fusion with Sendai virus at 37°C. Clearly, fusion is strongly inhibited and retarded. Inhibition and retardation of fusion diminish as the concentration of target membrane is increased, but even at a 25-fold excess of target membrane (50  $\mu\text{M}$  DHPBNS) oligomerization still results in a 70–75% lower extent and initial rate of fusion. As discussed in our recent report on the effect of lipid head group oligomerization on calcium-induced vesicle fusion, the reduced extent and rate of R18 dilution represent inhibition and retardation of the fusion process, and are not due to an artifact such as inhibition of dye redistribution in the oligomerized bilayer membranes (Ravoo *et al.*, 1999).

The results of a lipid mixing assay during fusion of Sendai virus with large (200 nm) vesicles of DHPBNS at 37°C are also presented in Figure 3. It is evident that large vesicles fuse with Sendai virus equally efficiently as small vesicles (Fig. 2). The initial rate of fusion tends to be somewhat smaller, but the extent of fusion is slightly higher. Fusion becomes more efficient at higher temperature (43°C), i.e. well above the  $T_m$  of DHPBNS (data not shown). Oligomerization of DHPBNS leads to a strong inhibition and retardation of fusion. As



**Fig. 3.** Lipid mixing during fusion of Sendai virus (5 µg/ml) with either small or large vesicles of DHPBNS at 37°C as a function of DHPBNS concentration before and after oligomerization of DHPBNS. (A): Extent of fusion. (B): Initial rate of fusion. Legend: □: large vesicles of DHPBNS; ■: small vesicles of DHPBNS; ○: large vesicles of oligomerized DHPBNS; ●: small vesicles of oligomerized DHPBNS.

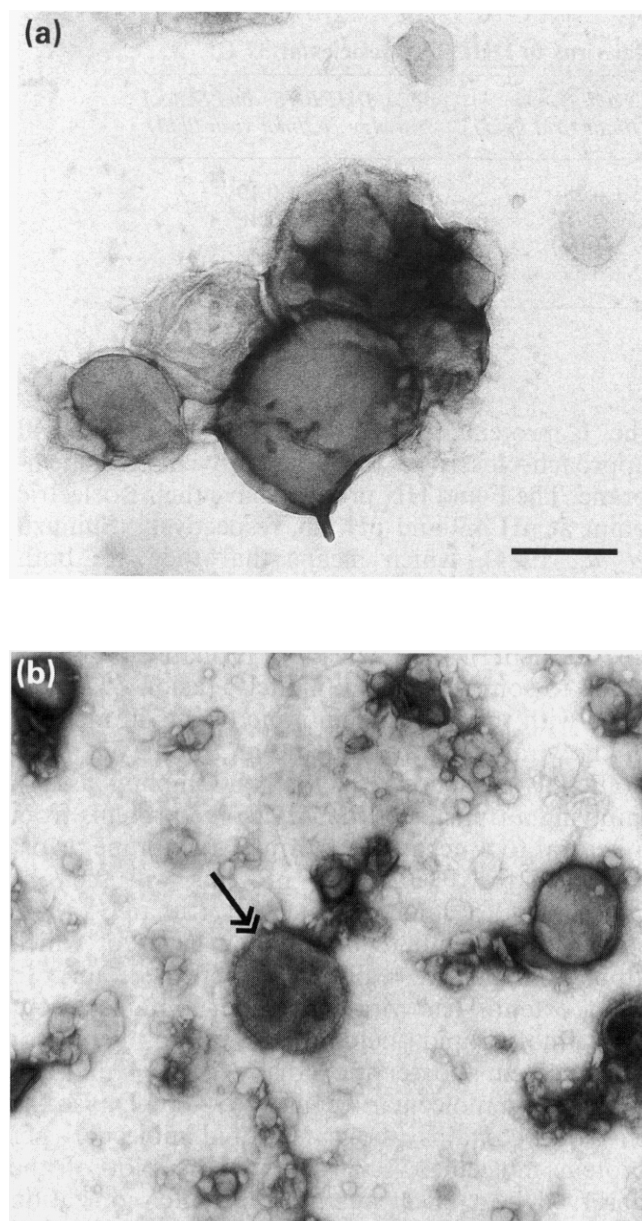
for the small vesicles, inhibition and retardation of fusion diminish as the concentration of target membrane is increased.

Electron microscopy of samples of Sendai virus and DHPBNS vesicles confirmed the conclusions from the R18 fusion assays. As illustrated in Figure 4, incubation of Sendai virus with small DHPBNS vesicles at 40°C results in the formation of large fusion products within 2 min. However, if Sendai virus is incubated at 40°C with small vesicles of oligomerized DHPBNS, hardly any fusion products are observed, and the majority of virions and vesicles persist. Moreover, the micrographs do not indicate aggregation of the virions and the oligomerized DHPBNS vesicles. Similar observations were made for Sendai virus and DHPBNS vesicles incubated at 4°C (data not shown). At this temperature, Sendai virus is not fusogenic.

#### Titration microcalorimetry

The enthalpy of the interaction between Sendai virus and small vesicles of DHPBNS was measured using isothermal titration microcalorimetry. Ten to twelve 10 µl aliquots of a suspension of Sendai virus (2.0 mg viral protein/ml, i.e. approximately

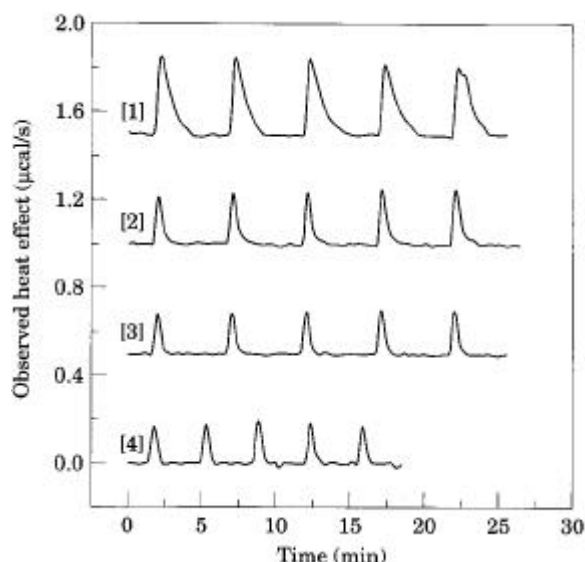
0.80 mM viral lipid) were injected into the sample cell containing 1.37 ml of a solution of small vesicles of DHPBNS (0.5 mM lipid) at 37°C. Hence *ca.* 8.0 nmol of viral lipid was added per injection and, in the course of the titration experiment, *ca.* 100 nmol of viral lipid was added to 680 nmol of DHPBNS. Upon each injection, endothermic heat effects were observed over a time scale of about 1 min. Typical plots of the heat flow *vs* time are presented in Figure 5. The experiment was repeated in four different buffers (phosphate, PIPES, HEPES and TRIS) of identical concentration and pH (7.40). The intensity of the observed heat effect was strongly dependent on the buffer medium. The heat effects observed in the titration experiments are summarized in Table 1. As the ionization enthalpy of the buffer increases, the reaction becomes more endothermic. As illustrated in Figure 6, a linear correlation ( $r=0.95$ ) relates the observed heat effect and the ionization enthalpy of the buffer. Extrapolation of the linear fit to zero buffer ionization enthalpy yields an intrinsic heat effect for the interaction of Sendai virus with small vesicles of DHPBNS of  $+4.3 \pm 1.1$  kJ/mol viral lipid. The positive slope of the plot of the observed heat effect *vs* the buffer ionization enthalpy



**Fig. 4.** Electron micrographs of mixtures of Sendai virus with small vesicles of DHPBNS. (a): virus and vesicles incubated at 40°C. (b): virus and vesicles of oligomerized DHPBNS incubated at 40°C. The arrow indicates an intact virion, easily identified because of its spike coating. Bar represents 200 nm.

indicates that buffer dissociates during the interaction of Sendai virus and the vesicles, which implies an uptake of protons in the course of the fusion process. The slope is a quantitative measure for the number of protons that are taken up, and it amounts to  $0.15 \pm 0.04$  mol protons/mol viral lipid.

Similar titration experiments of Sendai virus and small vesicles of oligomerized DHPBNS yielded much smaller endothermic heat effects (Table 1 and Fig. 6). Most interestingly, experiments in four



**Fig. 5.** Heat flow vs time during a titration of Sendai virus into small vesicles of DHPBNS at 37°C. (1) DHPBNS and virus in HEPES buffer. (2) DHPBNS and virus in phosphate buffer. (3) Oligomerized DHPBNS and virus in HEPES buffer. (4) Oligomerized DHPBNS and virus in phosphate buffer.

different buffers yielded identical heat effects of  $+2.8 \pm 0.2$  kJ/mol viral lipid. This pattern implies that no protons are taken up (or released) during the interaction of Sendai virus with small vesicles of oligomerized DHPBNS. Control experiments in which Sendai virus was titrated into phosphate and HEPES buffer solutions in the absence of vesicles revealed that the enthalpy of dilution of the concentrated virus suspension amounts to  $+2.1 \pm 0.2$  kJ/mol viral lipid, and, therefore, accounts for most of the heat effect that is observed in the titration of Sendai virus to oligomerized DHPBNS vesicles. Most probably, a small extent of fusion of the oligomerized DHPBNS vesicles (Fig. 2) is responsible for the additional heat effect, but any buffer dependence is lost in the error margins of the experiments.

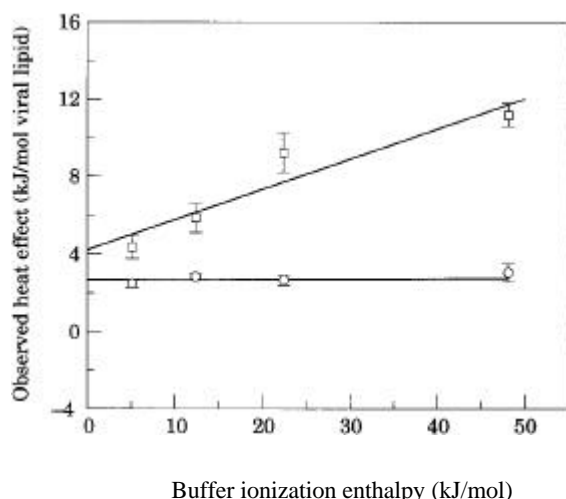
## DISCUSSION

The buffer-dependence of the heat effect observed upon fusion of Sendai virus with vesicles of DHPBNS can in part be explained by the insertion of the F protein of Sendai into the target membrane. The amino terminal part of the subunit of the F protein that is believed to insert into the target membrane contains 20 hydrophobic residues (Gething *et al.*, 1978; White *et al.*, 1983), which cannot possibly be protonated or deprotonated. In addition, the subunit contains three threonine

**Table 1.**  
**Heat effects accompanying titration of Sendai virus to DHPBNS vesicles at 37°C\***

Buffer	Buffer ionization Enthalpy (kJ/mol buffer)	DHPBNS and Sendai Enthalpy (kJ/mol viral lipid)	Oligo(DHPBNS) and Sendai Enthalpy (kJ/mol viral lipid)
Phosphate	5.10	$4.43 \pm 0.58$	$2.55 \pm 0.19$
PIPES	12.4	$5.90 \pm 0.78$	$2.88 \pm 0.18$
HEPES	22.5	$9.29 \pm 1.0$	$2.68 \pm 0.25$
TRIS	48.1	$11.2 \pm 0.59$	$3.05 \pm 0.46$

\*See Materials and Methods for experimental details.



**Fig. 6.** Plot of the observed heat effect of titration of Sendai virus to small DHPBNS vesicles in various buffers vs the enthalpy of ionization of the buffers. Legend:  $\circ$ : DHPBNS vesicles;  $\square$ : oligomerized DHPBNS vesicles.

residues which are not hydrophobic, but possess a  $pK_a$  far outside the range that could be influenced by insertion into the membrane. The only residue that contains a pH-sensitive group is the amino terminal phenylalanine, with the terminal  $NH_2$  with a  $pK_a$  of *ca.* 8. This implies that the amino group is only partially protonated at pH 7.4. The microcalorimetric data suggest that it is protonated, which is probably the result of the reduced local pH near the surface of the negatively-charged membrane (rather than deprotonated, which would imply a charge neutralization that favours membrane insertion). Similar effects have been described in a calorimetric study of binding between a model peptide and liposomes (Beschiaschvili and Seelig, 1992).

However, it would be a gross oversimplification to suggest that only the residues in the amino terminal part of the F protein are affected during fusion with the target membrane. Certainly, in the course of the fusion event, a considerable part of

the F protein as well as the HN protein will approach closely to the negatively-charged membrane. The F and HN proteins have their isoelectric point at pH 4.9 and pH 6.5, respectively (Shimizu *et al.*, 1974), which means that they are both negatively-charged at pH 7.4. Upon close association with the negatively-charged membrane (with low local pH), they will most probably be protonated to some extent. Ultimately, fusion of Sendai virus with the target membrane results in merging of the viral envelope and the target bilayer membrane (as evidenced by the lipid mixing assay), implying a transfer of the viral spike proteins from a neutral to a negatively-charged membrane. Most certainly, the F and HN proteins will then be protonated to some extent. Unfortunately, these effects are difficult to quantify in terms of which alpha-amino acid residues are effected, and to what extent. The viral envelope contains about 1600 nmol of lipid (including cholesterol) per mg of spike protein (Loyter and Volsky, 1982). F and HN both have a molecular weight of *ca.* 66 kDa, so the envelope contains about 100 lipid molecules per protein molecule. According to the plot of the observed heat effect against the buffer ionization enthalpy, about 0.15 protons are taken up per viral lipid molecule, which means about 15 protons should be taken up per protein molecule. This may appear a large number, but both F and HN are composed of *ca.* 500 alpha-amino acids, many of which are potential proton acceptors (Gething *et al.*, 1978). Assuming an equimolar ratio of HN and F, it is obvious that protonation effects specifically related to insertion of the amino terminal part of the F protein (max. 0.5 proton/protein) are modest compared to protonation of F and HN as a result of membrane merging (almost 15 protons/protein).

The R18 assays of lipid mixing and the electron microscopic observations lead to the conclusion that fusion of Sendai virus with both small and large vesicles of DHPBNS is inhibited by



oligomerization of DHPBNS. Since no buffer-dependence is observed for the heat effect of the titration of Sendai virus to vesicles of oligomerized DHPBNS, it can be concluded that the F protein does not insert into, or interact otherwise with, this target membrane. Most probably, this is a direct consequence of the covalent linking of the lipid molecules as a result of the head group oligomerization. We have previously reported a reduced head group mobility, a reduced permeability and an increased detergent resistance of the oligomerized vesicles (Ravoo *et al.*, 1999). Failure of the F protein to insert into the oligomerized vesicle membrane can explain the inhibition of fusion of Sendai virus with oligomerized DHPBNS vesicles. This conclusion is supported by the electron microscopic observation, which shows that Sendai virus and oligomerized DHPBNS vesicles do not aggregate.

Individual injections into the titration microcalorimeter can be compared to successive fusion experiments with a high ratio of target membrane to virus. Lipid and virus concentrations are lower in the R18 assays than in the microcalorimetric experiments, but the DHPBNS/viral lipid ratio is similar in both experiments. In a typical R18 fusion assay (Fig. 2A and B) at 37°C, a maximum of 45% fusion is achieved with an initial rate of 3%/s and completion (i.e. end of fluorescence increase) in about 2 min, with a DHPBNS/viral lipid ratio of at least 10. In the microcalorimetric titrations, this ratio is more than 50 at the first injection, and decreases to about seven at the last injection, and the heat effects are observed over up to 2 min following each injection (Fig. 5). These results show that the time scale of the heat effect measured in the microcalorimeter matches the rapid fusion observed in the lipid mixing assay. Therefore, the difference in the heat effect that is observed when comparing the titration of Sendai virus to DHPBNS vesicles prior to and after oligomerization can be attributed to heat effects associated with the membrane fusion process that occurs between Sendai virus and the DHPBNS vesicles, and *not* with the oligomerized DHPBNS vesicles. The enthalpy of the fusion process is  $4.3 \pm 1.1 - 2.8 \pm 0.2$  kJ/mol viral lipid =  $1.5 \pm 1.3$  kJ/mol viral lipid. Hence, fusion is an endothermic process that requires an entropic driving force.

Previously, we observed a very small positive enthalpy for calcium-induced lipid bilayer merging (0.15-0.25 kJ/mol lipid for the C12 analogue of DHPBNS (Ravoo *et al.*, 1998). Fusion of Sendai virus with DHPBNS vesicles is endothermic by about 1.5 kJ/mol viral lipid. Therefore, fusion between vesicles and Sendai is an order of magnitude

more endothermic than vesicle-vesicle fusion. Admittedly, this comparison has to be made with caution, since the thermodynamics of the fusion of vesicles of the C12 lipid could differ from fusion of vesicles of the C16 lipid, in spite of their close structural resemblance. Unfortunately, a microcalorimetric study of calcium-induced (C16) DHPBNS vesicle fusion is complicated by experimental difficulties at the required elevated temperature. On the other hand, a preliminary R18 lipid mixing assay indicated that fusion between vesicles of the C12 analogue of DHPBNS and Sendai virus is far less efficient than fusion between DHPBNS and Sendai virus. Thus, we cannot make a direct experimental comparison between the two fusion processes.

Nevertheless, it would be extremely informative if one could at least compare the enthalpy associated with these two fusion processes at a 'kJ per mol fusion events' basis. We will attempt to do so in a qualitative manner. In order to translate the enthalpy of fusion per mol lipid to the enthalpy per mol of fusion events, one needs to consider (i) the number of lipid molecules per vesicle and per virion and (ii) the number of rounds of fusion that each vesicle (or virion) participates in. Since the Sendai virions are at least twice the size of the vesicles (Fig. 4), the number of lipid molecules per virion is considerably higher than the number of lipids per vesicle. Therefore, the difference between the enthalpy of Sendai virus-vesicle fusion and the enthalpy of vesicle-vesicle fusion will be even greater when expressed per mol virion (vesicle) instead of per mol lipid. As to the number of rounds of fusion: in the calcium-induced vesicle fusion experiments, about ten small vesicles fused into one large vesicle, implying that the enthalpy reflects several rounds of fusion. Also in the course of fusion between Sendai virus and DHPBNS vesicles, each Sendai virion must participate in several rounds of fusion in order to achieve the extent of lipid mixing observed in the R18 assay, and the diameter of the fusion products shown in Figure 4. The DHPBNS vesicles are less than half the size of the Sendai virions, and an extent of fusion of 50% corresponds to two-fold membrane probe dilution, implying fusion of one virion and four vesicles. Therefore, the enthalpy of Sendai virus-vesicle fusion represents a similar if not lower number of rounds of fusion as the enthalpy of vesicle-vesicle fusion. Thus, we assume that the enthalpies reported here on a 'per mol lipid' basis indicate a very significant difference on a 'per mol fusion events' basis: the enthalpy associated with Sendai virus-vesicle fusion is an order of

magnitude larger than the enthalpy of vesicle-vesicle fusion.

This leads us to the interesting conclusion that most of the enthalpy cost of Sendai virus-vesicle fusion is a result of merging of the viral envelope and the target membrane, rather than membrane fusion *per se*. We note that merging of the protein-rich viral envelope and the DHPBNS lipid bilayer could result in curvature strain, domain formation and phase transitions of viral lipids, or DHPBNS, or both, accompanied by significant heat effects (Epand, 1998; Gil *et al.*, 1998). Most probably, DHPBNS will undergo a gel-like to liquid crystalline phase transition upon transfer from the vesicles to the viral membrane, which could contribute to the observed endothermic enthalpy of fusion. Moreover, merging of the viral envelope with the vesicle bilayers is accompanied by protonation of some of the protein residues in the viral envelope proteins, which is expected to be an exothermic reaction. The overall enthalpy of this protonation is difficult to estimate, since we do not know which residues are involved, and to what extent they are protonated. In any case, the intrinsic enthalpy of the membrane merger must be even larger than 1.5 kJ/mol viral lipid, and, consequently, a strong entropic driving force is required. A significant entropy gain could result from the liberation of hydration water upon insertion of (part of) the F protein into the target membrane, as expected for a hydrophobic binding process. In addition, the thermodynamics of the fusion process may be interpreted in terms of the model proposed by Beschiaschvili and Seelig (1992). According to this model, binding of the F protein and the target membrane is endothermic, because it requires enthalpy input to insert a protein into the target membrane. Provided the bilayer membrane is not exceedingly curved (the vesicle diameter should be 50 nm or more, which is the case for these DHPBNS vesicles), the internal tension in a lipid membrane is high, and any gain in Van der Waals energy upon inserting a foreign hydrophobic molecule is overruled by the work required to make room for it. On the other hand, merging of a protein-rich and a protein-free membrane entails a large entropy gain because of a large increase in continuous membrane surface area, which results in an increased degree of freedom of the lipid and protein molecules.

### Conclusions

Sendai virus fuses efficiently with vesicles of DHPBNS, but fusion is strongly inhibited by

oligomerization of the lipid head groups. Fusion is triggered by insertion of part of the F protein into the target membrane, which is inhibited upon oligomerization of the lipid head groups in the target membrane. Fusion is accompanied by an endothermic heat effect of at least 1.5 kJ/mol viral lipid. This heat effect is dependent on the buffer ionization enthalpy, reflecting (partial) charge neutralization of the viral spike proteins as the viral envelope merges with the negatively-charged target membrane. The enthalpy of fusion of Sendai virus with DHPBNS vesicles is ten times larger than the enthalpy of fusion of comparable pure lipid bilayers. Apparently, the loss of enthalpy during fusion is a result of insertion and incorporation of viral protein into the lipid membrane, rather than fusion of the bilayers *per se*. Fusion is driven by a gain of entropy of lipid and water molecules.

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