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INTERACTIONS OF SENDAI VIRUS WITH LIPOSOME TARGET MEMBRANE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

A model system for the study of interactions of Sendai virus with target membranes has been developed using liposomes containing viral receptor gangliosides. The fluorescence quenching dye, calcein, was entrapped into liposomal aqueous compartment to monitor the kinetics of membrane permeability changes induced by the virus. An assay method employing ultracentrifugation with airfuge was also developed to monitor the kinetics of Sendai virus-liposome and other preceding events, i.e., binding steps. In order to interpret the results at a more fundanmental level, we simplified the liposome composition to only contain two lipids, i.e., phosphotidylethanolamine and ganglioside GDla. The lipid packing of this target membrane has been characterized biophysically and was found to have a gel-liquid crystalline phase transiton between 2Ø and 25 °C. Parallel temperature dependence studies have allowed us to correlate the physical parameters of target membrane, such as lipid stucture order, fluid vs gel phase, etc., to the fusion and leakage activities of the virus. Since Sendai virus induces fusion and membrane permeability changes are intuitively related, we also have investigated the relationship between these two events.

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CHAPTER I

INTRODUCTION

Biological Significance of Membrane Fusion

Contrary to prokaryotic cells, the interior of eukaryotic cells is partitioned into a number of intracellular compartments. There are distinct advantages of having these membrane boundaries among them: (a) to constrain metabolites and enzymes of particular pathways in compartments so as that they can interect efficiently; (b) to isolate the pathways from opposing or competing reactions; (c) to integrate pathways occuring in different compartments into a coherent whole by controlling the movements of material between them. Although specific metabolites or enzymes can be transported between the cytosol and the compartments by transportors, channels, as well as cotranslational transfer (Gratzl et al., 1980), membrane fusion offers a more direct and effective way to deliver the content of one compartment into another. In addition to the aqueous components, the membrane components of one compartment can also be joined with another compartment through membrane fusion. This allows an efficient transport of both free and membrane-bound molecules to their functional sites. The communications facilitated by membrane fusion occur not only at subcellular level (such as membrane biogenesis, etc.) but also at cellular level (endocytosis, exocytosis, neurotransmitter release, etc.) as well as intercellular level (fertilization, myogenesis, etc.) (Wilschut & Hoekstra, 1984 ;

Blumenthal, 1985). Some viruses also take advantage of their membrane-fusing activities to deliver their genomes into the host cells (White et al., 1983).

Sendai Virus Induced Membrane Fusion and Leakage

Sendai virus is the best studied member of the paramyxoviruses, which are enveloped negative strand RNA viruses able to deliver their genomes into host cell via fusion with the plasma membrane. The virus contains two surface glycoproteins (HN and F), a nonglycosylated membrane protein (M), a structual nucleocapsid protein (NP), and two viral transcriptases (L and P) (Choppin & Compans, 1975). HN is responsible for binding of the virus to the cell surface (on sialic acid-bearing glycoproteins and glycolipids). F is responsible for the fusion between viral membrane and plasma membranes (Choppin & Scheid, 1980). The viral genome can then be delivered into the cytoplasm of the host cell for virus replication. When the virus particles are added at high multiplicity, or when the viral progenies are budding out of the host cell plasma membrane, cell-cell fusion may occur (White et al., 1983; Ishida & Homma, 1978). There is a large number of studies focusing on the morphology, physiological stimuli and consequences of membrane fusion. (Poste & Nicholson, 1978). One of the consequences is the leakage of cellular metabolites (Impraim et al., 1980). It is believed to be caused by the addition of leaky viral membrane to the cell membrane as a result of virus-cell fusion (Shimizu et al., 1976 ; Wyke et al., 1980). It is also believed to be the cause of both hemolysis and the swelling of cells, which leads to

polykaryon formation (Wyke et al., 1980). However, the correlation between Sendai virus induced fusion and leakage has not been rigorously established. Mechanisitic studies at the molecular level thus has been carried out to complement the morphological studies. One of the major focuses of the mechanisitic studies was on the function of HN and F protein.

Both HN (67,000 dalton) and F (66,000 dalton) are glycoproteins containing fucose, mannose, galactose, and glucosamine but no galactosamine and sialic acid (Yoshima et al., 1981). In detergents they form dumbbell-shaped objects which are similar to the spikes seen on the surface of the virion (Shimizu et al., 1974). Judging from negative-stain electron microscopic images F-spike has a similar size and shape compared to the spike formed by hemagglutinin trimers in the influenza viral envelope (Wilson et al., 1981), and has a total length of 21 nm, knob mean diameter of 7 nm, shaft length of 7 nm, and shaft width of 2.5-3.0 nm. While, HN-spike has a smaller knob and a more slender shaft than the F-spike (Shimizu et al., 1974). The density of HN and F in the viral envelope is about one membrane protein molecule per 100 molecules of lipid (Hsu et al., 1979). HN has both hemagglutinating and neuraminidase activities which reside on different portions of the molecule (Portner, 1981 ; Sugawara et al., 1982). It is generally accepted that the former is responsible for the attachment of virus to host cell receptors before infection while the latter is responsible for newly synthesized virus to elute from the surface of the host cell (Portner, 1981). HN has been suggested to

participate in the fusion activity of F protein with a mechanism which has not yet been revealed (Ozawa et al., 1979 ; Miura et al., 1982 ; Nakanishi et al., 1982). Neuraminidase activity may be essential for virus induced fusion (Huang et al., 198Ø). However, it was not consistent with the observation that (a) reconstituted liposomes containing F alone could express hemolytic activity in the presence of lectins (Hsu et al., 1979); (b) fusion activity was enhanced when the neuraminidase activity was specifically blocked (Neurath et al., 1972); and (c) non-hemolytic L Sendai virus could become hemolytic by trypsin-treatment without changing its neuraminidase activity (Ishida & Homma, 1978). It is well established that F protein is responsible for the fusion activity of the virus. However, the F protein of newly synthesized and assembled virus is in a precusor form which has no fusion activity until it is proteolytically cleaved by extracellular trypsin-like protease into fusion active F1 (51,ØØØ dalton) and F2 (15,ØØØ dalton) polypeptides, which are linked by a disulfite bond (Scheid & Choppin, 1977). This mechanism can prevent the virion to fuse back with the original host cell and offer enough opportunity for neuraminidase to elute the virus from the cell surface. The N-terminus of F1 protein is only exposed after proteolytic cleavage and has been found to be unusually hydrophobic and quite homologous to the fusion proteins of other paramyxoviruses. This terminal peptide is believed, from indications of a number of studies, to destabilize the target membrane and bring about fusion (White et al., 1983). This is consistent with the observation that trypsin treatment of the virus, which removes the N-terminal portion of F1 protein, obliterates the

fusion activity of the virus. The nature of F protein-target membrane interaction is not known. It is therefore important to study the effects of chemical composition and physical property of the target membrane on the viral fusion activity. However, the complexity of natural target membranes and the lack of suitable assay methods to monitor the membrane fusion activity have prevented indepth mechanistical studies. Thus, a simple model membrane system is needed for studies of mechanism of fusion between the virus and the target membranes.

The Mechanism of Membrane Fusion

Although biological membrane fusion occurs extensively as described above, it is a very specific and highly regulated process. Natural membranes themselves do not tend to fuse with each other while the intended fusion event can be completed within a very short period of time, such as few milliseconds. It is assumed that fusion between two natural membranes is usually hampered by the energy barriers between them until the latter are removed by the fusion triggering mechanism (Blumenthal, 1985). The formation of specific macromolecular bridges is to compete against and overcome the repulsive forces arising from electrostatic forces and steric hindrances between two opposing membranes (Bell et al., 1984). However, there must be some other energy barriers between the two opposing membranes because binding alone does not necessarily lead to the fusion of the membranes. Although many cellular polypeptides and viral proteins are

thought to directly participate in fusion (Lucy, 1984 ; White et al., 1983), some membrane proteins may actually prevent fusion according to the observation from freeze-fracture studies that membrane proteins were depleted and "bare lipid patches" were formed in the contact areas of the two opposing membranes before fusion (Ahkong et al., 1975). Recent studies using ultrafast freezing technique, however, indicated that membrane fusion may start within a small localized region without any large-scale rearrangement of membrane proteins (Plattner, 1981). Therefore, there must be energy barriers other than proteins to prevent fusion. Some lipid structures and packing patterns are also thought to be the prerequisites of membrane fusion. The first candidate is the generation of phase boundaries between fluid and gel-phase lipid domains by isothermal phase transitions or lateral lipid phase separations (Papahadjopoulos et al., 1977). However, it was recently found to be the result rather than the cause of divalent cation induced fusion of negatively charged liposomes (Wilschut et al., 1985). The second candidate is the formation of hexagonal-like structures according to the observation of "lipidic particles" on the fusing membrane by freeze-fracture electron microscopy (de Kruijff et al., 1980). Although it is the case for many membrane systems (Cullis et al., 1983), some fusion incidences can take place under the conditions that no hexagonal phase could be formed (Wilschut & Hoekstra, 1984). The third candidate is the hydrophobicity of the membrane surface (Ohki, 1984). An increase in the hydrophobic interactions between the lipids of the opposing membranes can render membrane-membrane fusion, especially when the formation of point

defects are facilitated by high membrane sulface tension (due to the effects of curvature, temperature, osmotic pressure, etc.) as well as the formation of hexagonal phase (Isrelachvilli & Pashley, 1982). The hydrophobicity of a membrane surface is related to the head group as well as the fluidity of the membrane lipids (Parsegian & Rand, 1983), the binding of calcium ions to negatively charged membrane lipids (Hui et al., 1981), and the exposure of the membranes to a relatively hydrophobic substance, such as polyethylene glycol, hydrophobic portion of a membrane protein, etc.. The fourth candidate is the fluidity of the membranes. This was recently shown to be involved in divalent cation-induced fusion of negatively charged liposomes (Wilschut et al., 1985). The fifth candidate is the dehydration of lipid headgroup which may lead to hexagonal phase formation of some lipids (Kirk et al., 1984). Dehydration is known to greatly increase the interactions between lipid bilayers (Gruen et al., 1984; LeNeveu et al., 1976) and is well correlated to the fusion activity of the membranes (Blumenthal, 1985; Parsegian and Rand, 1983). The above conclusions concerning fuison prerequisite of lipid structure were largely drawn from model studies in which, unlike the biological membrane fusion, no protein was involved. It is interesting to see whether these prerequisites are required for biological membrane fusion.

Studies of biological membrane fusion are much more difficult, and often result in controversies. The controversies came partly from the failure of the investigators to directly and quantatively measure

the physical parameters of the membranes and the fusion activity. They also partly come from the potential "side effects" of manipulating the biological membranes. For example, it was concluded from some studies that fusion required a fluid membrane because it could be facilitated by raising the temperature and adding cis-unsaturated fatty acids or arachadonic acid (Ahkong et al, 1973; Creutz, 1981). Contrarily, it was concluded from other studies that fusion required a less fluid membrane because it could be enhanced by increasing the cholesterol content of the membrane (Lucy, 1978; Horwitz et al., 1978; Blumenthal, 1985). Cholesterol in general decreases the fluidity of the natural occuring membranes (Houslay & Stanley, 1982), however, it is known that cholesterol can also affect molecular exchange (Houslay & Stanley, 1982), formation of nonbilayer structures (Cullis et al., 1978; Jensen & Schutzbach, 1984), and interaction with proteins (Dahl et al., 1981). Therefore, no solid conclusion can be drawn from this kind of studies unless a better defined system and more precise techniques are employed.

Approach Chosen in this Study and its Significance

We chose to study the interactions between Sendai virus (which contains a biological membrane) and liposomes (contain a model target membrane). Liposomes can be manipulated to contain a desired receptor species, chemical composition, physical state, and appropriate probes to monitor fusion, thus allowing us to study the fusion mechanism without manipulating the more complicated Sendai viral membrane. In order to monitor the kinetics of membrane fusion, we have developed a

liposome leakage assay. Based upon the well defined physical parameters of the model membrane, the dependence of the fusion activity on the fluidity and the receptor density of the target membranes was revealed. The correlation between the virus induced leakage and fusion has also been established. Since the overall fusion event can be dissected into several kinetically distinguishable steps, e.g., binding and fusion, we have also developed the assays for these individual steps. Taking advantage of simple kinetics of observed virus-liposome fusion, we have successfully measured the rate constant for each step. This is the first time that kinetic analyses of the fusion event has been carried out with any details. The kinetic data were analyzed using a model for the structure of the liposomes. Therefore, this approach not only provides a new insight into the kinetic intermediates of biological membrane fusion, but also sheds light on the prerequisites of the target membrane structure.

CHAPTER II

SENDAI VIRUS INDUCED LEAKAGE OF LIPOSOMES CONTAINING GANGLIOSIDES

Sendai virus induced liposome leakage has been studied using liposomes containing a self-quenching dye, calcein. The liposomes used in this study were prepared by a freeze and thaw method and were composed of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine (1:2.60:1.48 molar ratio) as well as various amounts of gangliosides and cholesterol. The leakage rate was calculated from the fluorescence increment as the entrapped calcein leaked out of the liposomal compartment and was diluted into the media. It was shown that the target liposome leakage was virus dose dependent. Trypsin treated Sendai virus in which the F protein had been quantitatively removed did not induce liposome leakage, indicating that the leakage was a direct result of F protein interaction with the target bilayer membrane. The activation energy of this process was approximately 12 Kcal/mole below 17 °C and approximately 25 Kcal/mole above 17 °C. Gangliosides GM1, GD1a, and GT1b could serve as viral receptor under appropriate conditions. Liposome leakage showed a bell-shaped curve dependence on the concentration of ganglioside in the liposomes. No leakage was observed if the ganglioside content was too low or too high. Inclusion of cholesterol in the liposome bilayer suppressed the leakage rate of liposomes containing GD1a. It is proposed that liposome leakage is a consequence of fusion between Sendai virus and liposomes.

Introduction

Sendai virus and other paramyxoviruses can interact with a wide spectrum of cells resulting in the following consequences: (a) virus-cell fusion; (b) cell membrane changes which lead to leakage of small intracellular constituents such as ions; (c) swelling of cells; (d) lysis of cells, e.g., hemolysis; (e) cell-cell fusion and/or polykaryon formation. The interrelationships among these processes are of great interest. It has been suggested that cellular permeability change is the consequence of the virus-cell fusion and the cause of both hemolysis and the swelling of cells which leads to polykaryon formation (Wyke et al., 1980; Impraim et al., 1980; Knutton & Bachi, 1980). Osmotic stress induced by the permeability changes may be the driving force which leads either to cell lysis or to polykaryon formation depending on the ability of the cell to retain or restore the membrane integrity after the stress (Peretz et al., 1974; Maeda et al., 1977; Knutton & Bachi, 1980).

Although this causal relationship seems reasonable, the true mechanism remains unclear. There exists a strong need for mechanistic studies at the molecular level to supplement the morphological studies. The use of a model target membrane seems particularly suitable for this purpose due to its simple composition. Using this approach it has been shown that membrane fusion induced by Sendai virus is not dependent on metabolic energy, divalent cations, cytoskeletons, and is not dependent on any protein in the target membrane other than the Sendai virus receptor, glycophorin. (Umeda et

al., 1983; Oku et al., 1982). Furthermore, gangliosides can replace glycophorin as Sendai virus receptor and mediate the fusion process (Haywood and Boyer, 1981; 1982). However, because the lack of a sensitive assay, systematic studies were not easy to carry out.

Calcein, (2',7'-((bis(carboxymethyl)amino)methyl)fluorescein, is a self-quenching dye. At concentrations greater than 5 uM, progressive fluorescence quenching takes place. It has been widely used for the liposome leakage assay because it is not necessary to remove liposomes for leakage measurements (Allen & Cleland, 1980; Kundrot et al., 1983). We have encapsulated calcein in the aqueous compartment of the ganglioside containing liposomes. When the liposomes interact with the Sendai virus, the fluorescence increment can be continuously measured, kinetic studies can easily be done.

In this study we have characterized the optimal conditions for liposome leakage induced by Sendai virus. We have also shown the relevance of this process to the activities of the F protein of Sendai virus. The dependence of the liposome leakage process on temperature, osmotic pressure, receptor concentration, and the presence of cholesterol in the target membranes have also been studied.

Experimental procedures

Materials

Egg phosphatidylcholine, and egg phosphatidylethanolamine were purchased from Sigma. Bovine brain phosphatidylserine, TPCK-treated bovine pancreas trypsin, turkey egg white trypisn inhibitor, and

calcein were purchased from Sigma. Bovine brain gangliosides GDla, GTlb, and GMl were purchased from Supelco. 3Ø ug each of gangliosides were applied to a TLC plate and developed with chloroform: methanol: 2.5 N ammonium hydroxide (6Ø:4Ø:9) to check the purity (Skipski, 1975; Kundu, 1981). Only one spot was found for each kind of gangliosides. Ficoll 4ØØ was purchased from Pharmacia.

Methods

Liposome preparation. Mixture of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine (1:2.6:1.48 molar ratio) and various amounts of gangliosides and cholesterol were evaporated free of the solvant under a stream of nitrogen, desiccated under reduced pressure overnight and suspended by vortexing in HEPES-buffered saline (15Ø mM NaCl, 5 mM KCl, 5 mM HEPES), and Ø.Ø2% sodium azide, pH 7.4 to a final concentration of 16 mg/ml. Lipid suspensions were sonicated and mixed with the same volume of either 14Ø or 8Ø mM calcein in HEPES-buffered saline. The mixture was sonicated thoroughly in a bath sonicator (Laboratory Spplies, Hicksville, NY) and then carried through 9 cycles of freeze-and-thaw in an acentone-dry ice bath. The final lipid concentration was 8 mg/ml and the final calcein concentration was either 7Ø mM for experiments concerning temperature, F protein, and osmotic dependence or 4Ø mM for experiments concerning the membrane composition. Column chromatography on Biogel AØ.5M (1 x 12 cm) was used to separate free calcein from calcein-loaded liposomes. The liposomes were oligolamellar vesicles and had an average diameter of 7ØØ nm as estimated by negative stain electron

microscopy. Lipid concentration was determined by phosphate assay (Ames & Dubin, 1960).

Virus preparation. Sendai virus was grown in 10 day old embryonated chicken eggs. The allantoic fluid was harvested and centrifuged at 3,000 g for 20 min to remove particulate material. The virus in the supernatant was pelleted by centrifugation at 13,300 g for 4 h. It was further purified by discontinuous sucrose density gradient centrifugation at interface of 20-50% sucrose after centrifugation at 52,000 g for 2 h. The virus was washed by dilution with standard sodium citrate solution (150 mM NaCl, 30 mM sodium citrate, pH 7,4) and repelleting at 52,200 g for 70 min.

Trypsin treatment of Sendai virus. 400 uL TPCK-trypsin (1 mg/mL) was added to 200 uL Sendai virus (10 mg protein/mL) and incubated at 37 °C for 1.5 h. 400 uL trypsin inhibitor (1 mg/mL) was added to the latter solution and Sendai virus was separated from reagents by using a Biogel A0.5M column. A hemagglutination assay was used to check the binding activity of HN protein (see below), while a hemolysis assay was used to check the activity of F protein (see below). SDS-PAGE (7.5% acrylamide) was used to check the integrity of both HN and F protein.

Hemagglutination assay and hemolysis assay. 50 uL of 0.5% human red blood cells was added to each of 50 uL two-fold serially diluted Sendai virus solutions in V-bottomed Costar 96-well serocluster and incubated at room temperature for 5 h. The titers of hemagglutination

and hemolysis can be easily determined by visual inspection of the formation of red blood cell network and the clearness of the suspension, respectively. The highest dilution at which hemagglutination and hemolysis occured were recorded. One hemagglutination unit is defined as the amount of Sendai virus to hemagglutinate 1 mL of 1% red blood cells. The hemolysis unit is defined similarly. The results of hemagglutination assay was found identical to the conventional hemagglutination assay, which was performed in the cold over 2 h.

Liposome leakage assay. 2 mL of HEPES-buffered saline containing 750 uM EDTA was preequilibrated to a desired temperature. 5 uL liposomes was then added to a final concentration of 1.75 ug/mL. After 1 min incubation with constant stirring, 5 uL Sendai virus preequilibrated to the same temperature was added to the mixture to a final concentration of 12.5 ug viral protein/mL. To monitor the calcein leakage, a Perkin-Elmer LS-5 spectrofluorometer equipped with a thermostated cuvette holder connected to a Neslab RTE-8 constant temperature circulator was used. Excitation was at 490 nm, emission was measured at 520 nm. The excitation slit was 5 nm, the emission slit was 3 nm. At the end of the experiment concentrated deoxycholate solution was added to a final concentration of 0.06% to obtain complete dye release. The reading of total fluorescence was about 100-130 at fixed scale of 0.2 depending on the temperature used for the liposomes containing 70 mM calcein. Fraction total fluorescence is defined as:

$$F(t) = \frac{I(t) - Io}{If - Io}$$

where Io = initial fluorescence, If = total fluorescence measured in the presence of deoxycholate, and I(t) = fluorescence at time t.

Viscosity and osmolarity measurements. Relative viscosity was measured as follows: A 9 inch Pasteur pipette was filled with the test solution up to the constriction ring. The pipette was held verticallly and the solution was allowed to flow downward by gravity. The time in seconds it took the solution to flow from the constriction ring to 8 cm downward toward the nose was used as a measure of viscosity. Osmolarity of solutions was measured with a Wescor 5130A vapor pressure osmometer. To measure the effect of osmolarity on liposome leakage, liposomes and buffer were preequilibrated in the medium with the desired osmolarity at 37 °C. Then 5 uL Sendai virus was added to start the reaction. The measurement was the same as described in the last section.

Results

Temperature Dependence of the Leakage Rate

Fluorescence intensity changes as the result of Sendai virus induced leakage of liposmes containing 6.5% GDla showed a dependence on both time and temperature (Figure 1, top). To avoid the inaccurate initial rate determination from the curvlinear profiles (Figure 1, top), we have determined the first order leakage rate. The latter was obtained from the slope of the plots, ln (1 - F(t)) vs t (Figure 1,

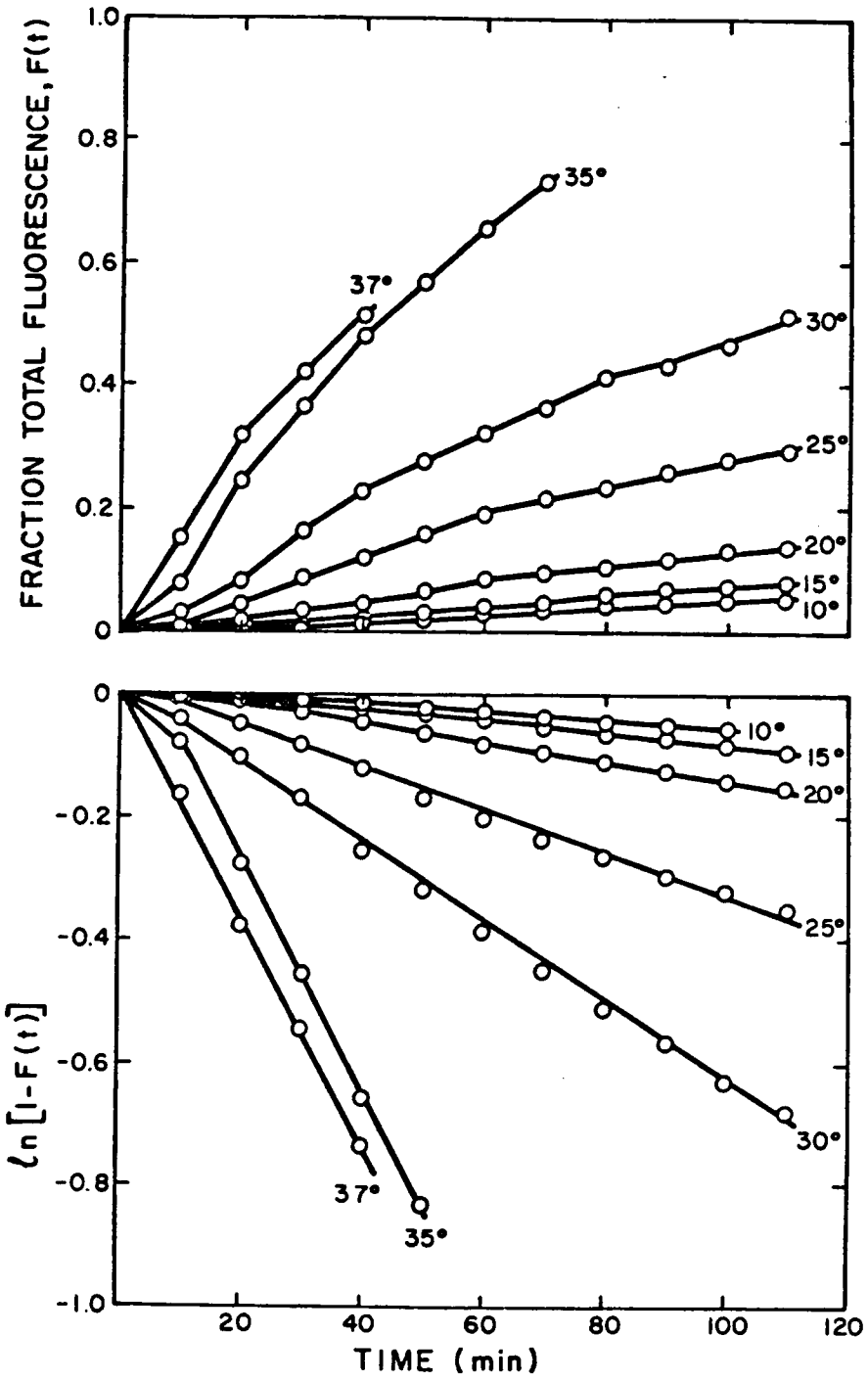


Figure 1: Time and temperature dependence of the calcein leakage process. Top Panel: Time-dependent increases as result of calcein leakage induced by Sendai virus at different temperatures. Bottom Panel: First order plot of the above data.

bottom). It showed that the leakage had a brief lag period of less than ten minutes at 10-35 °C and then followed first order kinetics at all temperatures tested. When the first order rate of liposome leakage was plotted in an Arrhenius plot, two broken straight lines were obtained. The activation energy for the leakage process was obtained from the slopes. It was approximately 25 Kcal/mole for leakage at higher temperatures and approvimately 12 Kcal/mole at lower temperature. The transition temperature was at 17 °C (data not shown). Trypsin-treated Sendai virus, from which part of F protein was cleaved, did not induce the leakage of liposomes at any temperature tested.

For all other experiments we recorded the fluorescence changes as a result of liposome leakage at 37 °C for 2 h and then plotted ln (1 - F(t)) vs t, ignoring the lag perioid, to obtain leakage rates from the slopes.

F Protein Dependence of the Leakage Rate

Trypsinized virus was used to show that liposome leakage was not due to nonspecific interactions between the virus and the liposome. Sendai virus has two major membrane proteins: (a) HN protein, which is responsible for the binding (hemagglutination) activity, and (b) F protein, which is responsible for the fusion and membrane leakage activities (Ishida & Homma, 1978; Wyke et al., 1980). TPCK-trypsin was used to gradually and specifically cleave the F protein but leave HN protein intact (Neurath et al., 1973; Shimizu & Ishida, 1975). After digestion as short as 10 min, the hemolytic activity of the virus was

lost (greater than 99%) and only residual levels (~60%) of the liposme lytic activity of the virus remained (Figure 2). SDS-PAGE analysis of the trypsin treated virus indicated a gradual loss of the F protein but not the HN protein upon the protease digestion. By 30 minutes of digestion, no intact F protein could be detected. These results indicate that both the lipoosme leakage and the hemolysis activity of the virus are closely dependent on the presence of the intact F protein of the virus and not the HN protein. It is not clear whether the apparent higher sensitivity of the hemolysis activity than the liposome leakage activity to the trypsin treatment is due to the difference in the two target membranes or due to the size difference of the two marker molecules, i.e., hemoglobin vs calcein.

All trypsin-treated Sendai viruses used in the following experiments were prepared as described in the Methods section.

Sendai Virus Dose Dependence of the Leakage Rate

For liposomes containing 6.5% GDla (1.75 ug lipid/mL) the leakage rate linearly increased with increasing amount of intact Sendai virus up to about 12.5 ug viral protein/mL and reached a plateau at about 25.0 ug viral protein/mL. The second order rate constant was $1.18 \pm 0.12 \times 10^{-3}$ mL g^{-2} min^{-1}. The trypsin-treated Sendai virus could cause minute amount of liposome leakage only at very high concentration (50 ug viral protein/mL).

For all other experiments both intact and trypsin-treated Sendai virus were used at the concentration of 12.5 ug viral protein/mL, at

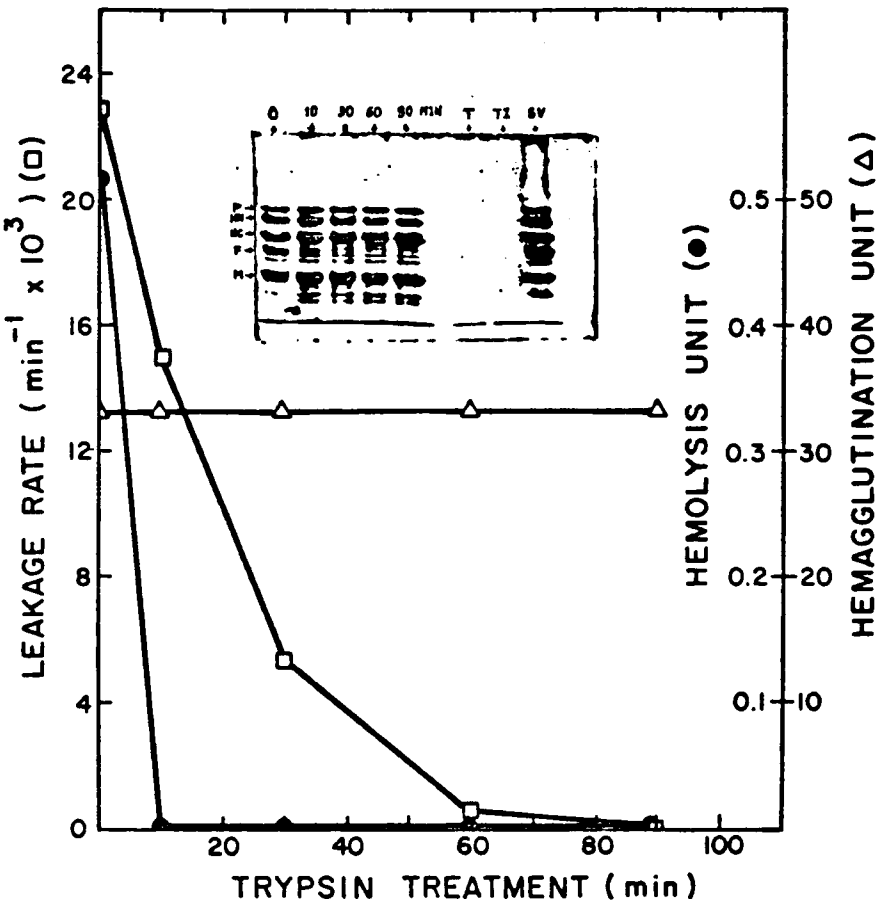


Figure 2: Effect of trypsin digestion on the Sendai virus induced calcein leakage activity. The calcein leakage (□) from liposomes containing 6.5% GD1a was monitored at 37 C. Hemolysis (●) and hemagglutination (△) activities of the treated virus were also measured. Inset: SDS-PAGE analysis of the viral proteins during the course of trypsin treatment.

which non-specific interactions between virus and liposomes were negligible.

Osmotic Pressure and Viscosity Dependence of the Leakage Rate

70 mM calcein in HEPES-buffered saline, which had an osmolarity of 592 mOs/L, was entrapped inside the liposomes. The osmolarity of the medium was increased starting from that of the HEPES-buffered saline, which had an osmolarity of 242 mOs/L, by adding sodium chloride, sucrose or Ficoll into the medium. Osmolarity of the medium less than, equal to, or greater than 592 mOs/L is defined as hypoosmotic, isoosmotic, or hyperosmotic, respectively.

As shown in Figure 3 the leakage rate decreased as the osmolarity of the medium increased. Slight hyperosmolarity of the medium drastically decreased the rate. Sucrose suppressed the leakage rate much more effectively than did sodium chloride at the same osmolarity. This discrepancy can be attributed to a viscosity effect as shown by the data in Table 1. 18% Ficoll as opposed to 1.86 Ficoll was only slightly higher in osmolarity (305 vs 276 mOs/L) but had much higher viscosity (49 s vs 5 s in relative viscosity units). This viscosity change greatly decreased the leakage rate. On the other hand, 18 % sucrose had the same viscosity as 1.86 % Ficoll (5 s) but had much higher osmolarity (980 vs 278 mOs/L). This osmolarity change also caused a large decrease in the leakage rate. Thus, adding sucrose to medium had both an osmotic effect and a viscosity effect. It is reasonable to expect that the leakage rate is reduced in a high

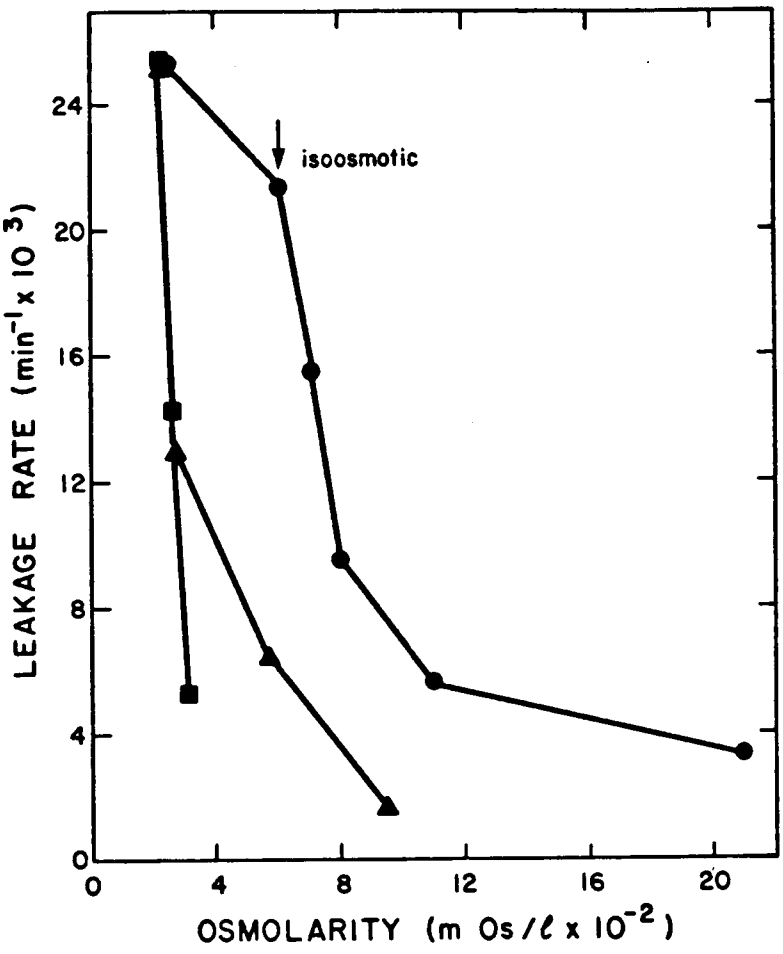


Figure 3: Osmotic pressure dependence of calcein leakage from liposomes containing 6.5% GDla at 37 C. The osmolarity of the medium was varied by adding sodium chloride (●), sucrose (▲), or Ficoll (■). The isoosmotic point to liposomes was at 592 mOs/L.

viscosity medium since the frequency of collision between the virus and liposomes would be reduced.

Table 1. Osmotic vs Viscosity Effect on the Liposome Leakage Rate.

Medium	Relative Viscosity (sec)	Osmolarity (mOs/L)	Leakage Rate (min x 10)
1.86% sucrose	3.5	360	11.0
18.00% sucrose	5.0	980	1.5
1.86% Ficoll	5.0	278	14.2
18.00% Ficoll	49.0	305	5.3

For all other experiments HEPES-buffered saline (242 mOs/L) was used as medium. For the experiments concerning temperature, F protein, and osmotic dependence, 70 mM calcein was entrapped inside the liposomes. For experiments concerning the membrane composition only 40 mM calcein was entrapped inside liposomes so as to reduce the chance of spontaneous leakage. Some liposomes with certain compositions were not as stable as others.

Receptor Species and Concentration Dependence of the Leakage Rate

There were three types of liposome leakage in these experiments: (a) spontaneous leakage, (b) leakage induced by the binding of the trypsin-treated virus to the liposomes, (c) leakage induced by the binding of the intact virus. In Figures 4-6 we show five profiles comparing the different types of leakage (a) liposome leakage induced by intact virus; (b) liposome leakage induced by trypsin-treated virus; (c) spontaneous leakage; (d) F protein dependent leakage which

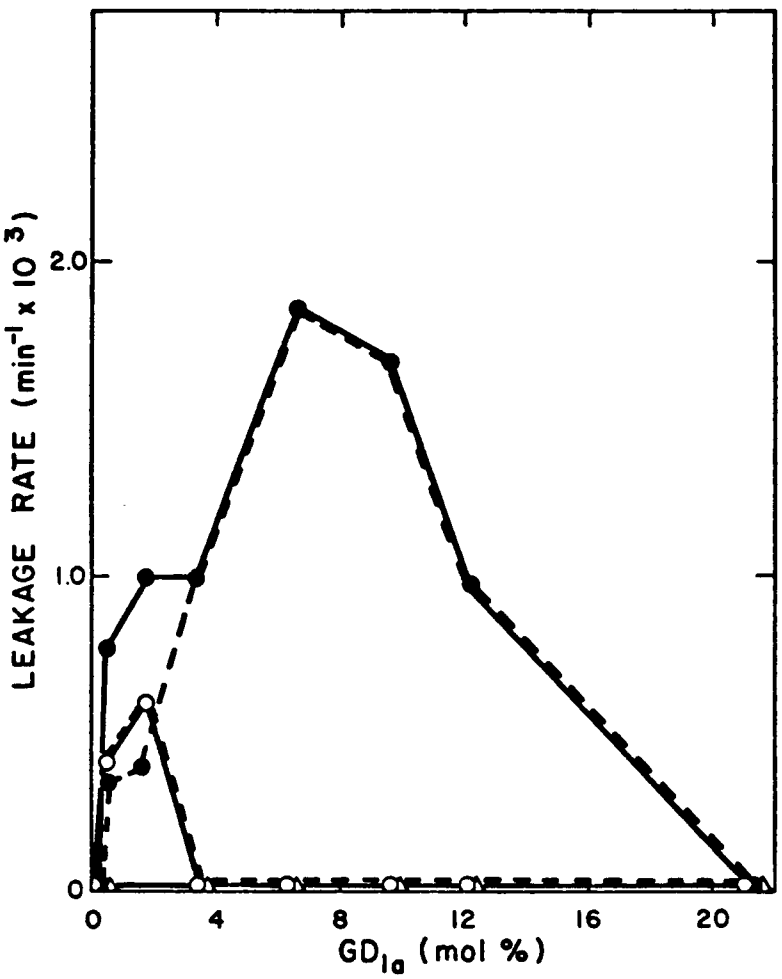


Figure 4. The rate of Sendai virus induced calcein leakage as functions of GDla concentration in liposome membrane. Leakage induced by intact virus (●—●), by trypsin-treated virus (○—○), spontaneous leakage (△—△), F protein dependent leakage (●--●), and F protein independent leakage (○--○).

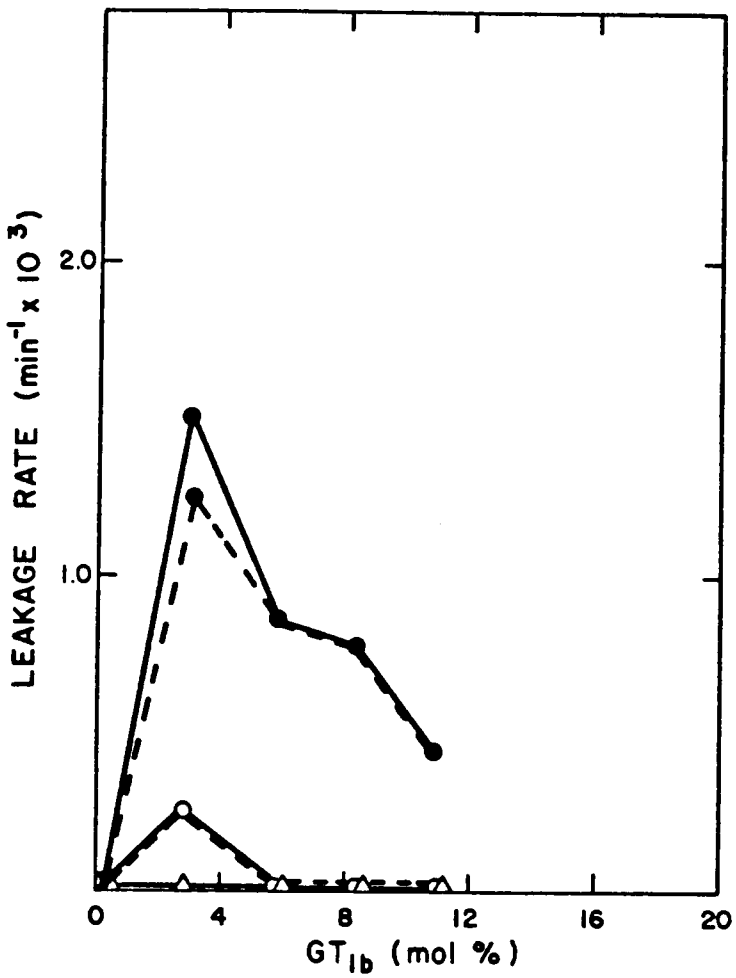


Figure 5. The rate of Sendai virus induced calcein leakage as functions of GT1b concentration in liposome membrane. Symbols same as those in Figure 4.

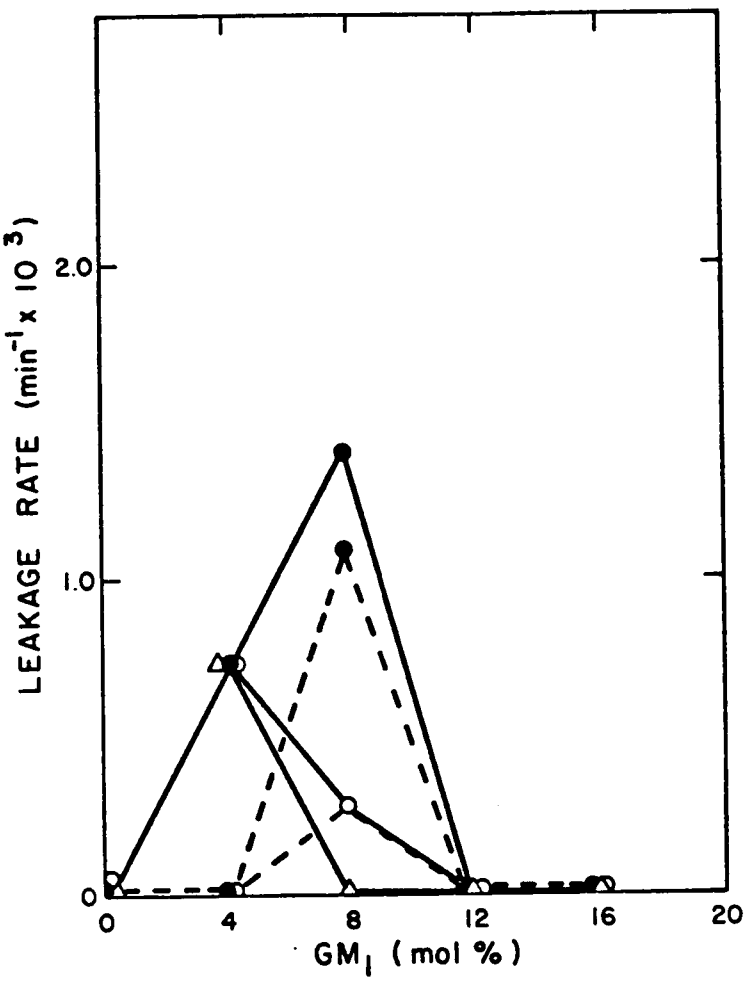


Figure 6. The rate of Sendai virus induced calcein leakage as functions of GM1 concentration in liposome membrane. Symbols same as those in Figure 4.

resulted from subtracting (b) from (a); (e) F protein independent leakage which resulted from subtracting (c) from (b). IN doing so we have assumed that F protein dependent and independent leakage were independent of each other. Figure 4 shows that both F protein dependent leakage and F protein independent leakage have a bell-shape dependence on GDla concentration which peaked at 6.5 mol% and 1.6 mol%, respectively. The shoulder of the intact virus profile at 1.6 mol% could be due to the F protein indenpent leakage. The leakage occuring at the GDla greater than 4 mol% was only F protein dependent. We routinely used liposomes containing 6.5 mol% GDla for all the experiments in other sections to obtain optimized and purely F protein dependent leakage.

F protein dependent and F protein independent leakage also showed a bell-shaped dependence on GTlb and GMl concentrations (Figures 5, 6). However, both leakage rates peaked at about 3 mol% of GTlb and at about 8 mol% of GMl, which were approximately the same concentrations at which F protein independent leakage occured. The F protein dependent leakage was the only or the predominant process at all GTlb or GMl concentrations except at 4 mol% of GMl, at which the spontaneous leakage was predominant.

Effect of Cholesterol on the Leakage Rate.

Inclusion of cholesterol into liposomal membrane suppressed the leakage rate induced by intact virus (Figure 7). At 33 mol% cholesterol F protein dependent leakage rate was only about 23% of the of control liposome without choleserol. On the other hand, 13%

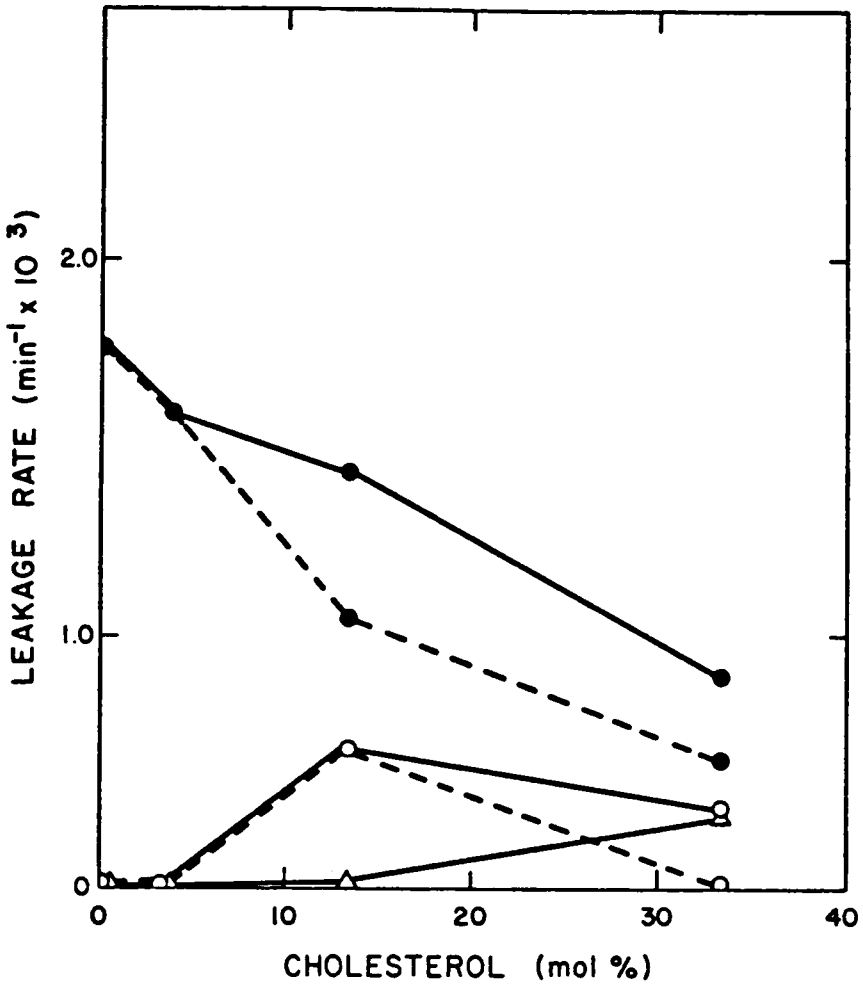


Figure 7. The rate of Sendai virus induced calcein leakage as functions of cholesterol concentration in liposome membrane containing 6.5% GDla. Symbols same as those in Figure 4.

cholesterol increased the F protein independent leakage rate. At 33 mol% cholesterol, the spontaneous leakage also became significant. For other experiments we did not incorporate cholesterol into liposomes to eliminate the complications induced by cholesterol.

Divalent Cation and the Leakage Rate

Divalent cations are not required in Sendai virus-induced liposome leakage. All the experiments were done without divalent cations. In addition, EDTA was presented in all experiments to chelate the possible contaminating divalent cations from Sendai virus preparations.

Discussion

By using ganglioside containing liposomes as well defined, easily manipulated target membranes, we have studied in detail the Sendai virus induced lytic process. Although the major receptor on the cell membranes for Sendai virus was considered to be sialic acid-bearing glycoproteins, e.g., glycophorin in erythrocyte membrane (Kundrot et al., 1983; Wu et al., 1980), gangliosides can also be receptors. The ability of GDla and mixed ganglisides as receptors for Sendai virus was supported by the virus-liposome fusion studies (Haywood & Boyer, 1981; 1982). However, (Markswell et al., 1981) postulated that only GDla and GTlb, but not GM1, could function as natural receptors for Sendai virus in host cells. The postulation was based on the ability of these gangliosides to render the sialidase-treated cells susceptible to Sendai virus. Contradictorily, we found that GM1 could

also serve as a receptor in liposomes. Failure to detect the receptor activity of GM1 in the cell system (Markwell et al., 1981) may be due to the narrow effective range of GM1 mole fraction (Figure 6). Ganglioside concentrations which allow the maximal leakage rate are 7.7 mol% for GM1, 6.5 mol% for GD1a, and 3 mol% for GT1b. Thus the receptor activity of ganglioside follows the order of GT1b GD1a GM1. It is interesting to note that GT1b, GD1a, and GM1 contain respectively three, two and one sialic acid residues, which is the virus binding moiety of gangliosides. This correlation suggests that the number of the sialic acid residues determines the receptor activty of the ganglioside.

F protein independent leakage process occurs over a very narrow range of ganglioiside content for all the gangliosides tested, i.e., GM1, GD1a, and GT1b. When Sendai virus binds to liposomes via the HN protein-ganglioside linkage, ganglisides would be clustered in the contact area and phase-separated from other lipids in the liposomal membrane. Ganglioside clusters could form hydrophobic pores and be susceptible to the permeation of calcein as proposed by Delmelle et al. (1980). Alternatively, the phase junctions between ganglioside clusters and the rest of the membrane could also allow the permeation of calcein, as suggested by Papahadjopoloulos et al. (1973) who postulated that sodium ion could permeate through the lipid phase boundary region of membranes.

It is generally accepted that the cellular leakage induced by Sendai virus is the consequence of virus-cell fusion (Homma et al., 1976; Impraim et al., 1980; Lyles & Landsberger, 1979). This is

because the addition of leaky viral membrane to the cell membrane as a result of fusion makes the cell leaky. F protein dependent liposome leakage may also be the consequence of liposome-virus fusion (Kundrot et al., 1983). In this case the rate-limiting step measured in our experiments may be the fusion step. It is interesting to note that the F protein dependent liposome leakage has an activation energy of 25 Kcal/mole, which is similar to 26 Kcal/mole found for the erythrocyte-Sendai virus fusion (Lyles & Landsberger, 1979). Nonbilayer structure formation has been suggested to cause leakage of cellular or liposomal membranes (Cullis & de Kruijff, 1978; Janoff et al., 1983) and may be an intermediate step in the fusion process (Hope & Cullis, 1981; Nir et al., 1983; Verkleii et al., de Kruijff et al., 1980; Siegel, 1984). Nonbilayer structure formation, which might be enhanced by the presence of F protein in the contact region, could also be important in the F protein dependent liposome leakage because there are two good candidates of nonbilayer-forming lipids in our liposomes, i.e., phosphatidylethanolamine (Cullis & de Kruijff, 1978) and gangliosides (Tomasi et al., 1982). Alternatively, bilayer perturbation by the hydrophobic segment near the N-terminal region of the F protein could also play an important role in the induction of membrane fusion (Hsu et al., 1981; Richardson et al., 1980). In fact, recent studies by Richardson and Choppin (1983) have shown that peptides of sequence analogous to the N-terminus of the F protein bind to the target cell membrane in a specific and saturable manner. Although it is not clear

about the molecular identity of the F protein receptor, our results suggest that it may be a lipid(s).

The present model study is different from other model studies. It is known that cholesterol is required for the lytic response of glycophorin-incorporated lipoosmes induced by Sendai virus (Kundrot et al., 1983). In the present study we did not observe any cholesterol requirement in the F protein dependent leakage of the liposomes. In fact the presence of cholesterol suppressed the leakage response. The discrepancy is probably due to the difference in the viral receptor used. Glycoporin could require cholesterol for the proper expression of the viral binding sites, whereas the gangliosides, having a much simpler oligosaccharide structure than glycophorin, do not. It has also been shown that the presence of cholesterol enhances the fusion between Sendai virus and liposomes containing phophatidylcholine and mixed gangliosides (Hsu et al., 1983). Thus the effect of cholesterol on the action of Sendai virus depends on the lipid composition of the liposomes. One possible involvement of cholesterol is to regulate the formation of the nonbilayer structure. Cholesterol enhances the nonbilayer formation in some cases and suppresses it in others depending on the phospholipid composition (Cullis et al., 1978; Jensen & Schutzbach, 1984). Thus the presence of cholesterol in the target membrane may positively or negatively modulate the membrane leakage depending on the lipid composition. The detailed mechanism of Sendai virus induced liposome leakage will be discussed in the following chapters.

CHAPTER III

A SIMPLIFIED TARGET MEMBRANE FOR SENDAI VIRUS: THE STRUCTURE AND PHASE BEHAVIOR OF LIPOSOMES CONTAINING GANGLIOSIDE GD1A AND PHOSPHATIDYLETHANOLAMINE

The Sendai virus-induced leakage of large liposomes composed of egg phosphatidylethanolamine (PE) and different mole fractions of ganglioside GD1a have been investigated by fluorescence spectroscopy, using the self-quenching fluorescent dye calcein. The kinetics of leakage were obtained by following the enhancement of fluorescence intensity at 520 nm (excitation wavelength 490 nm) as a function of time. Leakage of the entrapped dye was only observed when F-protein was present in the Sendai virus. Removal of the F-protein by trypsin digestion resulted in negligible leakage rates. The leakage rate was also dependent on the mole fraction of ganglioside GD1a and was maximal at 6.3 mole percent from 20 to 35 °C. Arrehnius plots of the leakage rates showed breaks in the 20-25 °C temperature range. Calorimetric, DPH steady-state anisotropy and pyrenedecanoic acid excimer formation experiments reveal that the breaks were associated with the gel-liquid crystalline phase transition of the liposomes. High sensitivity differential scanning calorimetry measurements indicated that mixed PE-GD1a liposomes undergo a broad phase transition centered between 17 and 21 °C. The enthalpy change of the transition was linearly dependent on the ganglioside concentration up to 10.0 mole percent and had a maximal plateau at 11.4-16.2 mole percent. It must be noted that under the conditions of the

experiments, neither PE or GDla are able to form bilayer structures by themselves and that liposome formation is the result of interactions between PE and ganglioside. It is proposed that semi-fluid domains containing six PE and one ganglioside molecules are present in the PE-GDla membranes at temperatures above gel-liquid crystalline phase transition.

Introduction

Membrane fusion is common in biological systems, such as in viral infection, intracellular traffic (endocytosis, exocytosis, etc.), fertilization and myogenesis. Among these systems virus-induced membrane fusion has great advantage for fusion studies because many viral membrane structures have already been well characterized. Even so, virus-induced membrane fusion is still a complex event; the mechanism of which remains unclear. Generally, membrane fusion involves at least three well defined steps: (a) recognition between the two membrane, which may be mediated by membrane proteins, glycolipids, soluble peptides or ions, (b) changes in membrane lipid packing, which may include lipid transfer, "flip-flop", lipid phase separation, or hexagonal phase formation, and (c) mechanical forces, such as osmotic force, etc., which bring about complete fusion between the two membranes. Thus mechanistic studies of membrane fusion often requires an in-depth understanding of the microscopic membrane structures and their changes during fusion.

Membrane fluidity is one of the structural parameters that may correlate with membrane fusion because it is a reflection of lipid

packing in membranes. However, the role of membrane fluidity in membrane fusion is still not defined despite intensive investigation. The general approach has been to change the overall fluidity of cellular or model membranes by changing membrane lipid composition (Blumenthal, 1985), especially the content of cholesterol (Lucy, 1978; Horwitz et al, 1978; Hsu et al, 1983) and cis-unsaturated fatty acids (Ahkong et al, 1973; Creutz, 1981; Kleinfeld, 1984). One of the major problems in these approaches is the lack of a membrane system with a well defined gel-liquid crystalline phase transition temperature. Thus, the fluidity dependence of membrane fusion remains unsubstantiated.

We have previously developed a sensitive and quantitative assay to study the Sendai virus-induced target membrane permeability change (Chapter II; Tsao & Huang, 1985). We have also demonstrated that the permeability change is due to the activity of the F glycoprotein of the Sendai virus and is intuitively related to membrane fusion. We have simplified the composition of the model target membrane to contain only two lipids, i.e., phosphatidylethanolamine (PE) and ganglioside GD1a, such that the lipid phase transition can be readily defined. This greatly simplified model system has allowed us to rigorously examine the relationship between the membrane lipid fluidity and the ability of the membrane to serve as a target for Sendai virus. In the present paper we look at the leakage event, while in the accompany paper fusuion and other preceding events are also investigated.

Gangliosides belong to a class of lipids with many important functions in cellular recognition and other physiological processes (Brunngraber, 1979). They mediate the binding and internalization of hormones, interferons, lectins, toxins and viruses into cells. They may also be involved in the regulation of the activities of membrane bound enzymes, such as adenylate cyclase, phosphodiesterase, Na^+-K^+ ATPase and acetylcholinesterase. Interestingly, both gangliosides and PE are enriched in the synaptosomal membranes and chromaffin granules. In this study we have also revealed a unique PE-GDla packing property which may be important for the biological functions of these lipids in natural membranes.

Experimental Procedures

Materials

PE which was transesterified from egg phosphatidylcholine was purchased from Avanti (Birmingham, AL). TPCK-trypsin was purchased from Sigma. Ganglioside GDla was purchased from Supelco. The purity of GDla was confirmed with TLC using chloroform : methanol : 2.5 N ammonium hydroxide (60 : 40 : 9) as a developing solvent system (Skipski, 1975; Kundu, 1981). Hexadecyl [^{3}H] cholestanyl ether was synthesized and purified as described (Baumann & Mangold, 1964; Pool et al., 1982). DPH was purchaed from Eastman. Pyrenedecanoic acid was purchased from Molecular Probes.

Methods

Liposome preparations. Liposomes were prepared by sonication followed by freeze and thaw according to the methods in Chapter II (Tsao & Huang; 1985) with some modifications . 20 mM of sonicated lipid mixture including PE, GDla and a tracer amount of lipid marker hexadecyl [^{3}H] cholestanyl ether was mixed with same volume of 140 mM calcein in HEPES-buffered saline (for leakage experiments only) or with same volume of HEPES-buffered saline (for all other experiments). Nine cycles of freeze and thaw of the liposomes allowed calcein be entrapped into the aqueous compartment of the liposomes. Column chromatography on Biogel A 0.5 M (1 x 12 cm) was used to separate liposomes from untrapped calcein.

Virus preparations and hemagglutination, hemolysis assays. The preparation of intact and trypsin-treated Sendai virus and assays for their protein integrity by SDS-PAGE, functions (hemagglutination, hemolysis) were done according to the methods in Chapter II (Tsao and Huang, 1985).

Liposome leakage assay. Liposome leakage assay has been described in Chapter II (Tsao and Huang, 1985). 5 ul of Sendai virus (8 mg viral protein/ml) preicubated to the desired temperature was added to 2 ml of reaction mixture (1.8 ug/ml of lipid and 750 uM of EDTA in HEPES-buffered saline) at the same temperature to initiate the reaction. To monitor the calcein leakage, a Perkin-Elmer LS-5 spectrofluorometer was used. Excitation was at 490 nm, while emission was measured at 520 nm. At the end of the experiment concentrated deoxycholate solution

was added to a final concentration of 0.06% to completely release calcein. The leakage rate was obtained from the slope of the plots, ln (1 - F(t)) vs t,

where $F(t) = \frac{I(t) - Io}{If - Io}$ and

Io = initial fluorescence, If = total fluorescence measured in the presence of deoxycholate, and I(t) = fluorescence at time t.

High sensitivity differential scanning calorimetry. Calorimetric measurements were performed using a Microcal MC1 differential calorimeter equipped with two separate Keithley amplifiers connected to the heat capacity and temperature output, respectively. The calorimeter data were automatically collected and processed by an interfaced IBM PC mirocomputer and digitized by a Data Translation DT-805 A/D converter. The measurements of PE-GD1a vesicles were performed at a scanning rate of 15 °C/hr at a concentration of 10 mM lipid with total sample volume of 0.7 ml.

Steady-state fluorescence spectroscopy. Steady-state fluorescence anisotropy measurements were performed using a Perkin-Elmer LS-5 spectrofluorometer equipped with a red sensitive photomultiplier tube, a polarization accessory and a thermostated cuvette holder connected to a Neslab RTE-8 bath circulator. DPH was dissolved in acetonitrile and added to the vesicle suspensions at a final ratio of 1 probe molecule per 1000 lipid molecules. Lipid concentration was 0.3 mM. Probe incorporation was allowed to continue for 5 hours at 35 °C prior

to fluorescence measurements. Fluorescence was measured by excitation of DPH at 360 nm while emission was monitored at 430 nm. Anisotropy r(t) was calculated according to Barenholz et al, (1976) :

$$r(t) = \frac{I_{\parallel} - I_{\perp} \times G}{I_{\parallel} + 2\ I_{\perp} \times G}$$

where G is the correction factor obtained using horizontally polarized light ($G = I_{\parallel} / I_{\perp}$).

Excimer formation. Excimer formation studies were performed with a Perkin-Elmer LS-5 spectrofluorometer. Pyrenedecanoic acid was dissolved in acetonitrile and added to the vesicle suspensions at a final ratio of 1 probe molecule per 20 lipid molecules. Lipid concentration was 15 uM. Probe incorporation was allowed to continue for 2 hours at 35 °C prior to fluorescence measurements. Fluorescence was measured by excitation of pyrenedecanoic acid at 320 nm while emission for monomers and excimers were monitored at 395 nm and 480 nm, respectively. Excimer to monomer ratio (I'/I) was obtained from the fluorescence peak height ratio at 480 nm to 395 nm, according to Galla and Hartman, (1980).

Results

Temperature Dependence of the Leakage Rate

The fluorescence intensity increase as the result of Sendai virus-induced calcein leakage from liposomes was monitored as a function of time. The leakage was found to follow first order kinetics by fitting the data into linear ln (1 - F(t)) plots (data not shown).

The temperature dependence of the first order leakage rate is shown in the Arrhenius plots in Figure 8. As shown by the break point of two broken straight lines for each profile, a phase transition is apparent between 20 and 25 °C for every PE-GDla liposome sample tested with the GDla concentration varied from 3.2 to 20.5% (although it is not as obvious as others for 20.5% GDla sample). The activation energies, obtained from the slopes of the Arrhenius plots, were between 32 and 37 Kcal/mole (except 47.8 Kcal/mole for 6.3% GDla sample) at temperatures below the transition and between 15 and 21 Kcal/mole (except 34.5 Kcal/mole for 20.5% GDla sample) at temperatures above the transition. The leakage rate was not only dependent on temperature but also on the mole fraction of GDla. It decreased as GDla mole fraction increased. This is particularly apparent for GDla mole fractions between 6.3 to 20.5 %. The leakage rate was also F-protein dependent since trypsin-treated Sendai virus, from which part of F protein was cleaved, did not induce the leakage of any liposomes at any temperatures tested (data not shown).

Steady State Fluorescence Anisotropy

The membrane fluidity of PE-GDla liposomes was investigated by DPH steady state anisotropy as a function of temperature and GDla mole fraction. All the Arrhenius plots, ln anisotropy vs. 1/Temp. (Figure 9), showed two broken straight lines consistent with the existence of a gel-liquid crystalline phase transition between 20 and 25 °C for each PE-GDla liposome tested. The activation energies were between -7.0 and -8.8 Kcal/mole at temperatures below the transition and

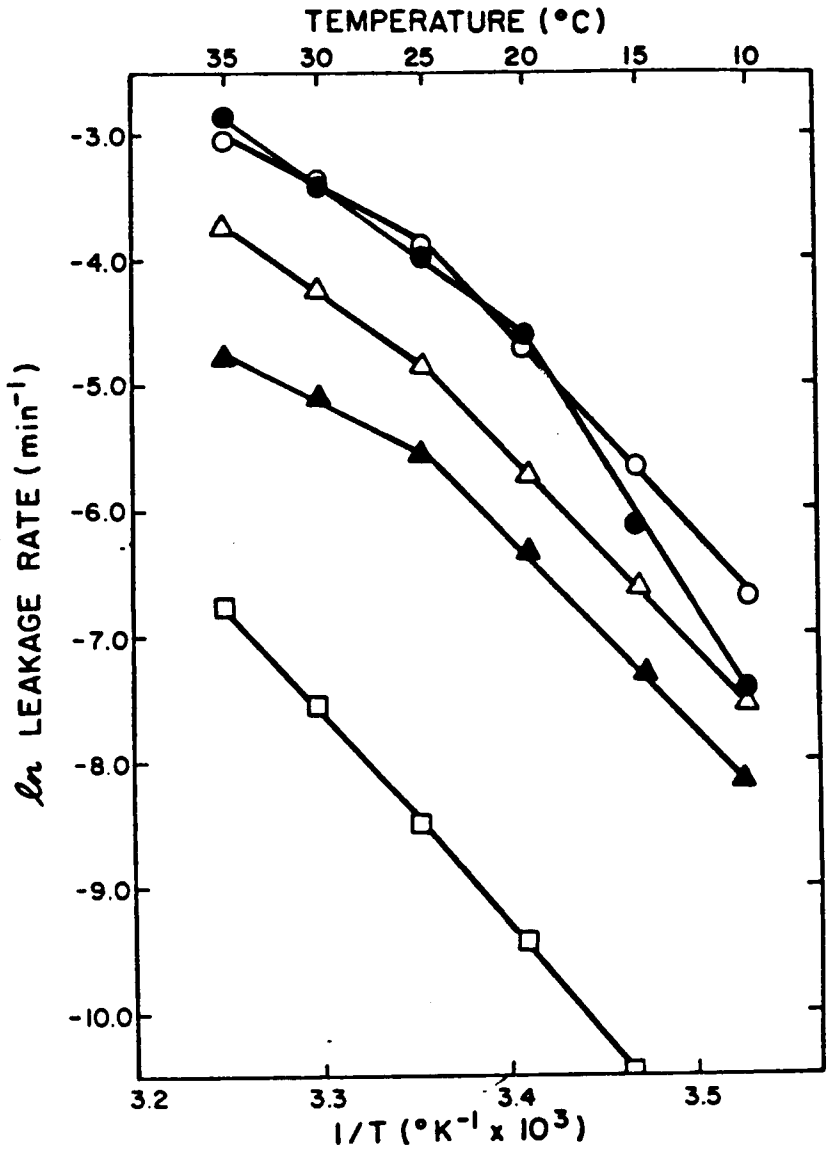


Figure 8: Temperature dependence of calcein leakage from liposomes containing different mole percents of GDla. Samples are 3.2% (o), 6.3% (●), 11.4% (Δ), 16.2% (▲) and 2Ø.5% (□) GDla containing liposomes. The activation energies were different at higher and lower temperatures. Transitions occured between 2Ø and 25 C.

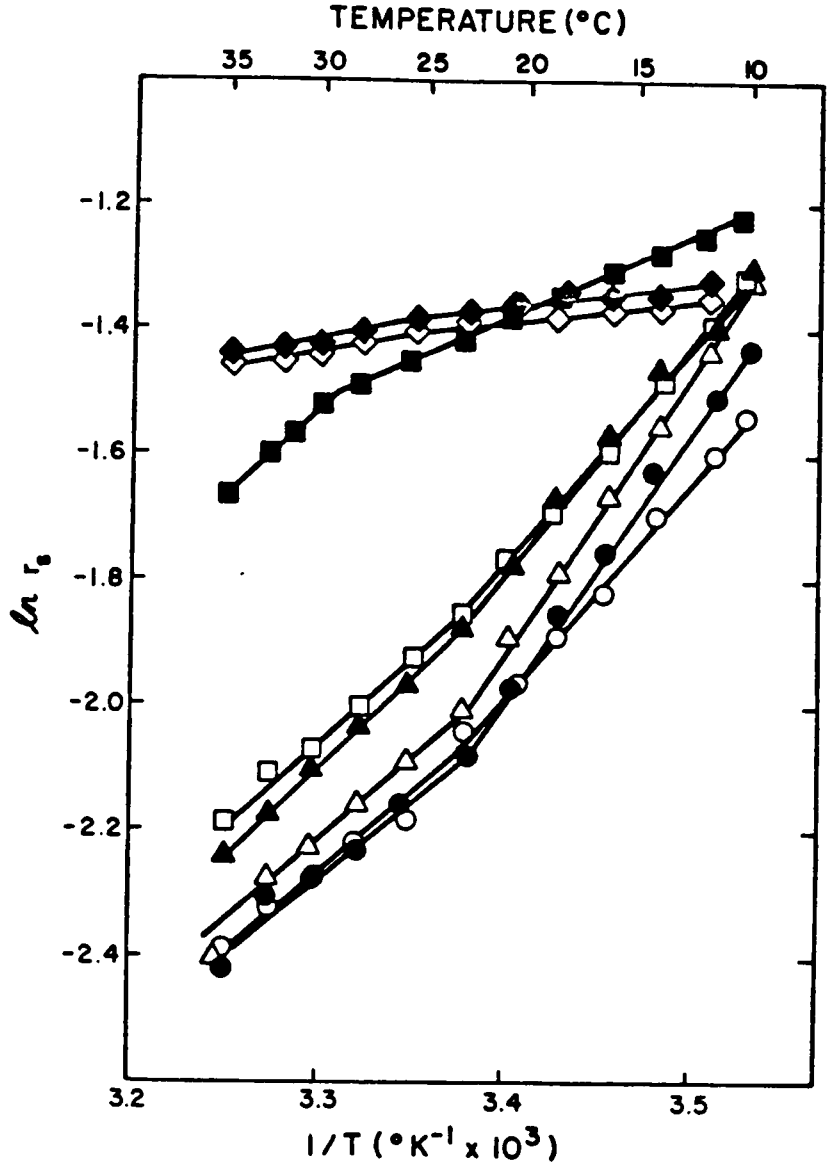


Figure 9: Temperature dependence of fluorescence anisotropy of DPH in different lipid structures. Samples are liposomes containing 3.2% (O), 6.3% (●), 11.4% (△), 16.2% (▲) and 20.5% (□) of GDla as well as GDla micelles (■), Sendai viral membranes (◆) and trypsin-treated Sendai viral membranes (◇). Transitions occured between 20 and 25 C for liposomal membranes alone.

between -5.3 and -6.1 Kcal/mole at the temperatures above transition. The anisotropy increased as temperature decreased or as GDla mole fraction increased, except for 3.2% GDla sample that had an anisotropy comparable to or slightly higher than the 6.3% GDla sample depending on the temperature. Sendai virus, trypsin-treated Sendai virus and GDla micelles had much higher anisotropy values than PE-GDla liposomes and had no phase transition between 2Ø and 25 °C. Thus, the DPH fluorescence anisotropy measurement revealed a similar phase transition temperature as the measurements of the liposome leakage rate (Figure 8). However, their dependence on the GDla mole fraction showed the opposite relationship. This is more clearly shown in Figure 1Ø in which the leakage rates and the anisotropy of the liposomes at 35 °C are plotted against the GDla mole fraction. These data indicate that the leakage induced by Sendai virus requires a fluid target membrane.

Excimer Formation

The physical state of the PE-GDla liposomes were further explored with pyrenedecanoic acid excimer formation. Since excimer formation depends on the concentration as well as the lateral diffusion coefficient of the probe, a high excimer to monomer ratio indicates either a more fluid membrane or the existence of pyrenedecanoic acid-enriched domains within the membrane. Arrhenius plots, ln (excimer to monomer ratio) vs. 1/Temp., of each PE-GDla samples are shown in Figure 11. From 3Ø to 35 °C all liposomes had activation energies between 4.5 and 5.Ø Kcal/mole. Below 3Ø °C liposomes with 3.2, 6.3,

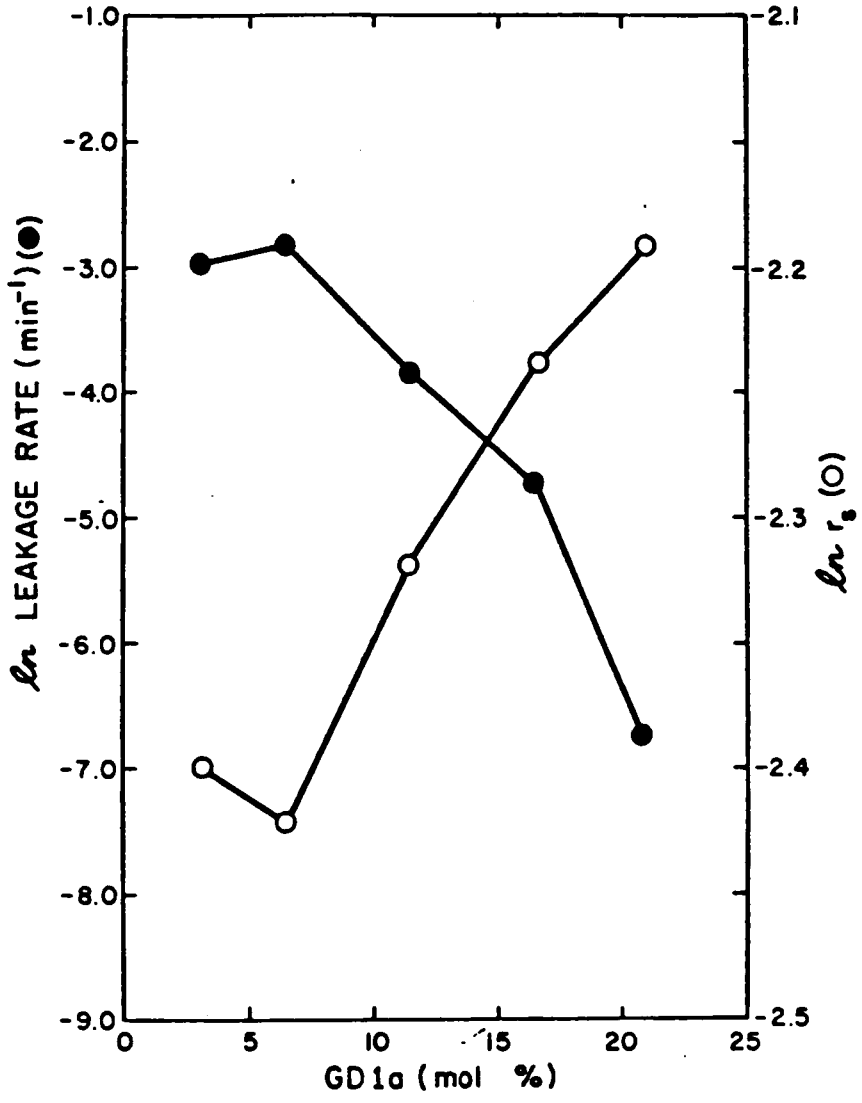


Figure 1Ø: Comparisons of the GD1a concentration dependence of the calcein leakage and fluorescence anisotropy of liposomal membranes at 35 C. The calcein leakage (●) was inversely proportional to the fluorescence anisotropy (O).

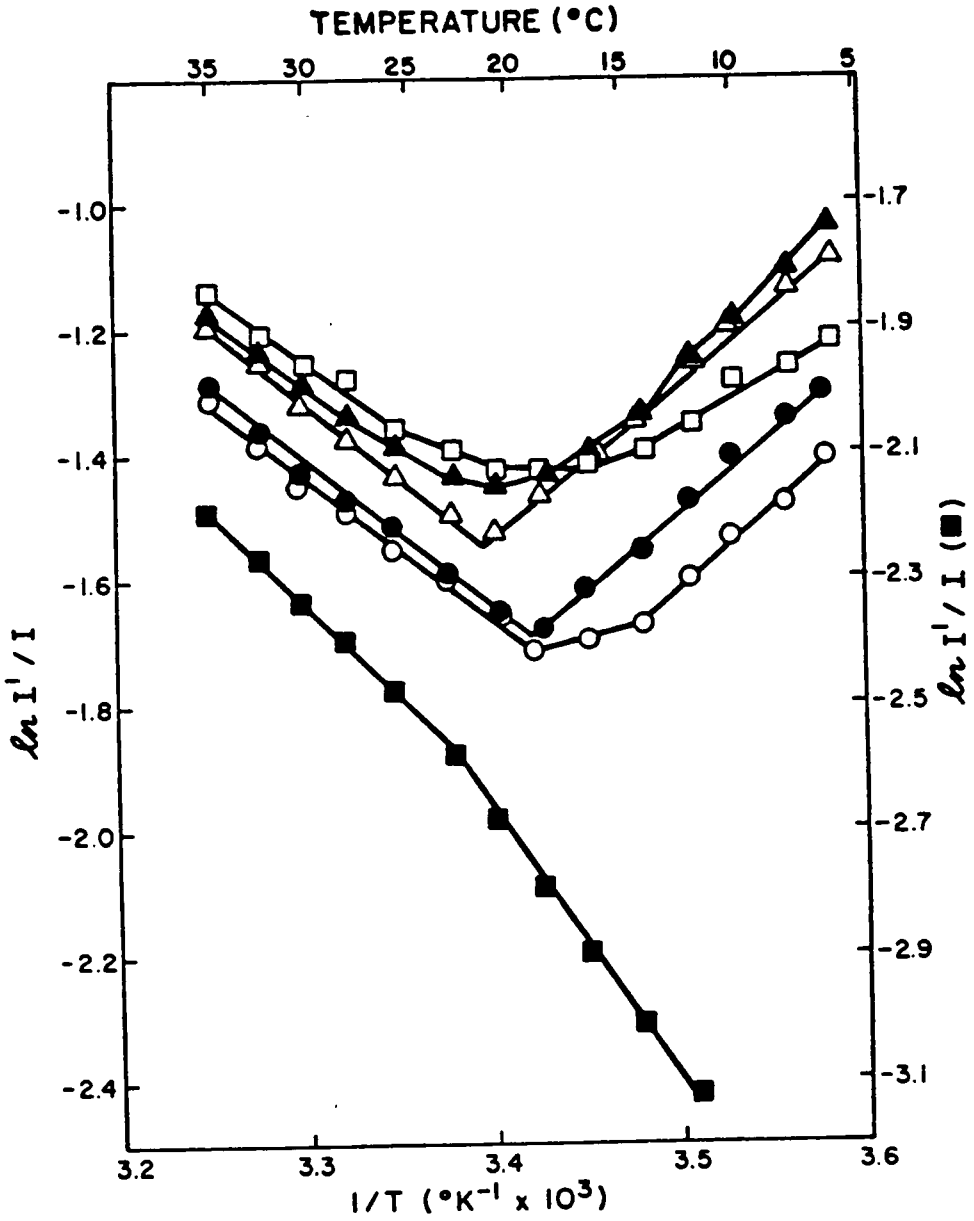


Figure 11: Temperature dependence of pyrenedecanoic acid excimer formations in liposomal membranes containing different mole percents of GDla as well as in GDla micelles. Symbols are the same as in the legend of Figure 9. Most of the transitions occured in between 15 and 21 C for liposome samples.

and 11.4% GDla still kept the same activation energies until an abrupt phase transition occured at 19, 19, and 21 °C, respectively; while liposomes with 16.2 and 20.5% liposomes had a gradual change in activation energies with a range of transition temperature between 5 and 35 °C. Below the midpoint of the transitions all the liposomes had an increased excimer to monomer ratio and from 7.5 to 14 °C they had activation energies between 4.9 and 5.6 Kcal/mole, except 3.4 Kcal/mole for 20.5% GDla liposomes. Sendai virus and GDla micelles did not show phase transition between 10 and 25 °C. They also had very low excimer to monomer ratio at all temperatures tested, especially for GDla micelles.

High Sensitivity Scanning Calorimetry

The thermodynamic properties of the PE-GDla liposomes were investigated by high sensitivity differential scanning calorimetry. All the heat capacity vs. temp. plots with different GDla mole fractions had broad phase transitions peaked between 17.3 and 21.0 °C depending on the GDla mole fraction (Figure 12). The phase transitions share the same temperature range, while the peak temperature shifted up with GDla mole fraction until 16.2% . The enthalpy change associated with the phase transition initially increased linearly with GDla mole fraction and leveled off between 10% and 16% GDla and dropped drastically at higher mole fractions (Figure 13). This behavior is consistent with the fact that pure PE as well as pure gangliosides are unable to form bilayer structures and that these structures only exist for a certain range of mole fractions. The

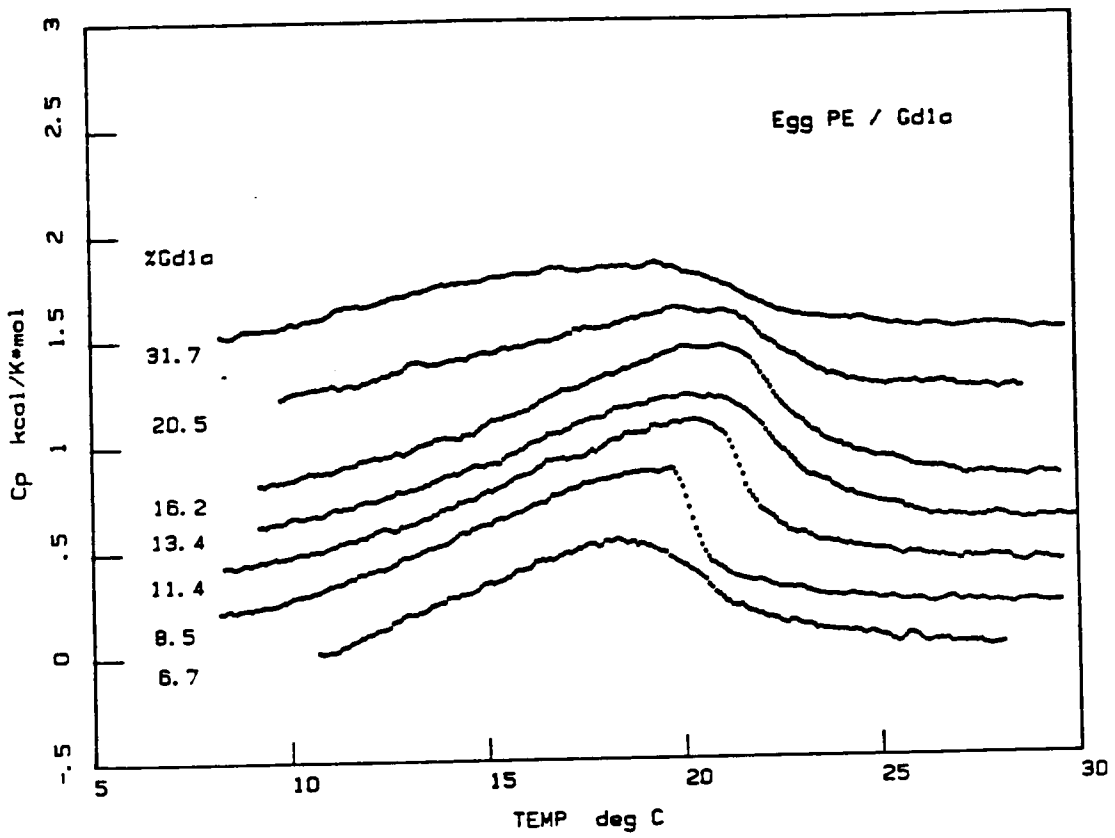


Figure 12: Temperature dependence of heat capacity of the gel-liquid crystalline phase transitions for liposomes containing different mole percents of GD1a.

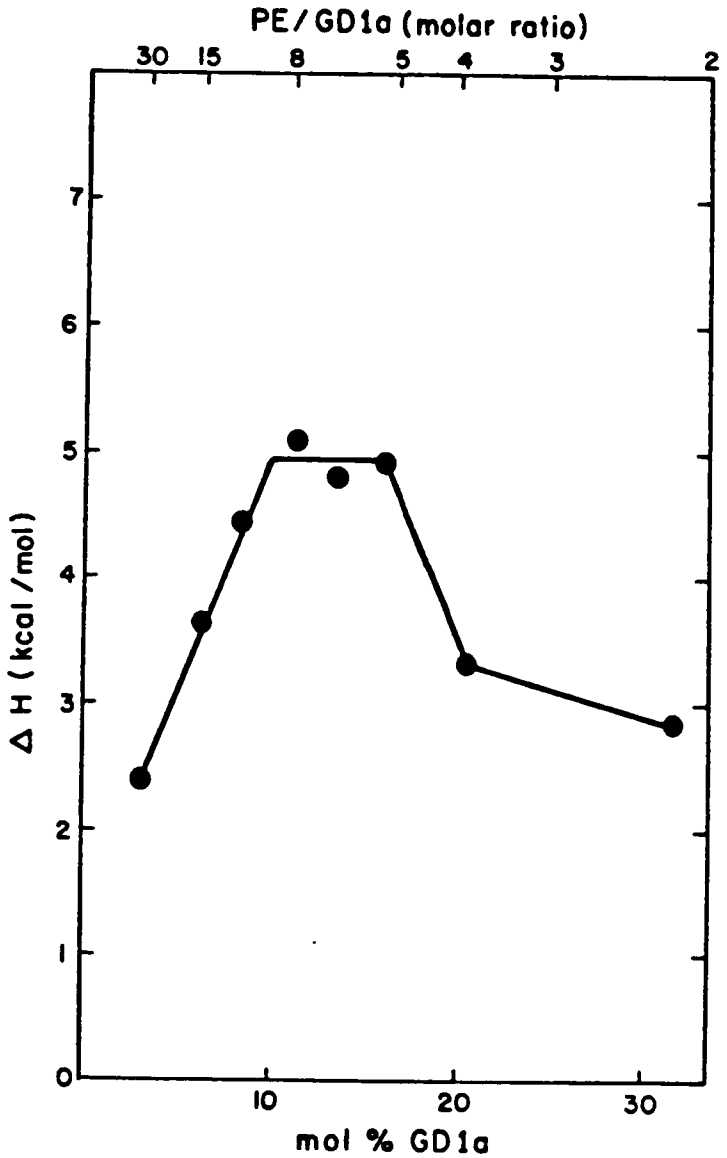


Figure 13: GD1a concentration dependence of heat capacity change of the gel-liquid crystalline phase transitions for liposomes containing different mole percents of GD1a.

initial slope of the enthalpy dependence on the ganglioside mole fraction was 37 Kcal/mole. This number should be proportional to the number of PE molecules packed in a bilayer configuration under the influence of a ganglioside molecule.

Discussion

We have previously shown that calcein leakage from liposomes induced by Sendai virus is a result of the Sendai viral F glycoprotein activity on target membranes in Chapter II (Tsao & Huang, 1985). In order to investigate the correlations between the leakage and the structure of the target membranes, we have further simplified the liposomal composition to contain only PE and ganglioside GDla. The Sendai virus induced leakage of these liposomes all show an abrupt change in the activation energy of the leakage rate between 2Ø and 25 °C (Figure 8, p. 41). That this is due to a gel-liquid crystalline phase transition of target liposome membranes instead of the viral membrane stems from the following observations: (a) DPH fluorescence anisotropy studies indicate that liposomal membranes, but not viral membranes, show a phase transition in lipid packing (Van Blitterswijk et al., 1981 Pottel et al., 1983; Shinitzky and Yuli, 1982; Kleinfeld et al., 1981) between 15 and 25 °C (Figure 9, p. 42); (b) Pyrenedecanoic acid excimer formation of the liposomes also shows a distinct phase transition in the same temperature range (Figure 1Ø, p. 44). (c) High sensitivity scanning calorimetry experiments further reveal that the enthalpy change during phase transition under optimal liposome compositions, i.e. 1Ø-16% of GDla, is about 5 Kcal/mole,

which is too high for a phase transition of a bilayer-to-hexagonal or cluster formation (McElhaney, 1982 ; Lee, 1977). With this well-defined model system we have shown that the Sendai virus induced leakage rate is much higher at temperatures above the gel-liquid crystalline phase transition (Figure 8, p. 50). Because the avoidance of the use of cholesterol as a membrane-fluidizing mean, we have explicitly demonstrated that Sendai virus induced leakage is much more efficient with a fluid target membrane. The small discrepancy (within $4\ ^{o}C$) in phase transition temperatures among fluorescence anisotropy, excimer formation and differential scanning calorimetry studies may be because they are sensitive to the different events during the phase transition. DPH steady-state fluorescence anisotropy is sensitive to the structural order of lipids, i.e., the degree to which the probe rotations are resricted by the molecular packing of lipids (Van Blitterswijk, 1981; Pottel, 1983). Excimer formation, on the other hand, is sensitive to the fluidity in the vicinity of the probe (Georghiou, 1981). In addition, the strong increase in excimer formation with decreasing temperature during phase transition could be due to the formation of pyrenedecanoic acid-enriched domains which are squeezed out of crystalline phase of lipid matrix into the remaining fluid part of the membrane (Galla & Hartmann, 1980). Differential scanning calorimetry provides a direct estimate of the overall enthalpy change, which is sensitive to the close van der Waal's contact of aliphatic side chains in a gel-liquid crystalline phase transition (Biltonen & Freire, 1978). The phase transition temperature of Sendai virus induced leakage seems to agree most closely with the

DPH fluorescence anisotropy data (compare Figures 8 and 9, pp. 41, 42). This may mean that the rate-limiting step of the leakage is also sensitive to the changes in structural order of the lipid bilayer. The inverse relationship of the leakage rate with structural order of the lipid bilayer is also seen when the latter is varied by changing the concentration of GDla in the target membranes (Figure 1Ø, p. 44).

PE and gangliosides do not form bilayers by themselves under physiological conditions or under the conditions of these experiments. Below 37 °C PE alone exists in hexagonal phase instead of lamellar phase (Cullis & de Kruijff, 1978). However, many biological membranes containing a high concentration of PE, e.g. outer membranes of E. coli where 85% of the membrane lipids are PE (Trauble & Overath, 1973) still exist as the lamellar phase. This suggests that proteins and other lipids in the membranes such as cholesterol, phosphatidylserine and sphingomyelin, may express a stabilization effect on PE to form bilayers (Cullis & de Kruijff, 1978; Cullis & Kruijff, 1979; Cullis & Verkleij, 1979). We found that ganglioside GDla could also stabilize PE to form bilayers and also liposomes. These liposomes are very stable in that they could retain 7Ø mM calcein in a hypoosmotic condition (592 mOs/l in the aqueous interior vs. 242 mOs/l in the external medium) without any spontaneous leakage for several days. Gangliosides, including GDla, by themselves form only micelles at concentrations above critical micellar concentration (Corti & Degiorgio, 198Ø; Ulrich-Bott & Wiegandt, 1984). Together with phosphatidylcholine or sphingomyelin, however, stable liposomes can be prepared (Hinz et al., 1981; Cestaro et al.). According to the "Shape

Concept" (Cullis et al., 1983) phospatidylcholine and sphingomyelin may have functional roles related to maintenance of lamellar structure because of their cylindrically shaped structures, in contrast to the inverted cone shape of micelle-forming molecules and the cone shape of hexagonal phase-forming lipids. It is known that the mixtures of some of the inverted cone molecules (such as lyso-phosphatidylcholine, detergents, and anethetics) with some of the cone molecules (such as cholesterol, fatty acids, and PE) exibit bilayer structures at appropriate ratios (Madden & Cullis, 1981; Rand et al., 1975; van Echteld et al., 1981; Jain et al., 1980; Hornby & Cullis, 1978). Therefore, it is likely that cone-shaped PE and inverted cone-shaped GDla molecules form complemetary structures in the lipid matrix.

The existence of these PE-GDla "complexes" in the membranes is supported by the fact that 1 mole of GDla could add 37 Kcal/mole of enthalpy change under the phase transitions (Figure 13, p. 48). It is unlikely that 37 Kcal enthalpy change can be attributed to 1 mole of a single lipid undergoing a gel-liquid crystalline phase transition (McElhaney, 1982; Boggs, 1980). Furthermore, gangliosides alone do not have a cooperative phase transition between 5 and 50 ^{o}C. (Hinz et al., 1981; Sillerud et al., 1979) Apparently, PE is influenced by GDla such that a phase transition between 15 and 25 ^{o}C is observed. The enthalpy change of the phase transition is maximal at PE/GDla ratio of six to eight (11 to 14% GDla) (Figure 13, p. 48). It drops abruptly when the ratio is below six. This indicates the regular PE-GDla "complex" may be difficult to form and to undergo the phase transition between 15 and 25 ^{o}C when the PE/GDla ratio is below 6. Thus, it is reasonable to

assume that the PE/GDla ratio within the "complex" is six. According to Sela & Bach (1984) the enthalpy change of egg PE alone undergoing phase transition is about 5.5 Kcal/mole . The enthalpy change for six PE in the "complex" to undergo phase transition would be about 33 Kcal/mole, which is reasonably close to the observed value, 37.Ø Kcal/mole, assuming that there is no contribution from the interaction between PE and GDla or GDla alone.

Interestingly, both DPH fluorescence anisotropy and pyrene-decanoic acid excimer formation show a GDla mole fraction dependence even at temperatures above the phase transition (Figures 9 & 1Ø, pp. 42, 44). This may indicate the existence of semi-fluid domains in the membranes. The abundance of these putative domains is apparently proportional to the GDla content. These domains not only cause the membrane to be more ordered (and thus exibit greater DPH fluorescence anisotropy) but also partially separate pyrenedecanoic acid (and thus have greater excimer formation). These observations are consistent with the following model (Figure 14) for PE-GDla membrane packing: (a) "complexes" that contain one GDla molecule and 6 surrounding "boundary PEs" with a structure analogous to the association between membrane proteins and their boundary-lipid counterparts are uniformly distributed in the "free PE" matrix of the bilayer membranes; (b) the boundary PE may be in a semi-fluid state at the temperatures above the transition, whereas free PE stays in fluid state; (c) only boundary PE or 6:1 "complexes", but not free PE or GDla alone, can undergo a gel-liquid crystalline phase transition between 15 and 25 °C. For differential scanning calorimetry studies (Figure 13, p. 48) this

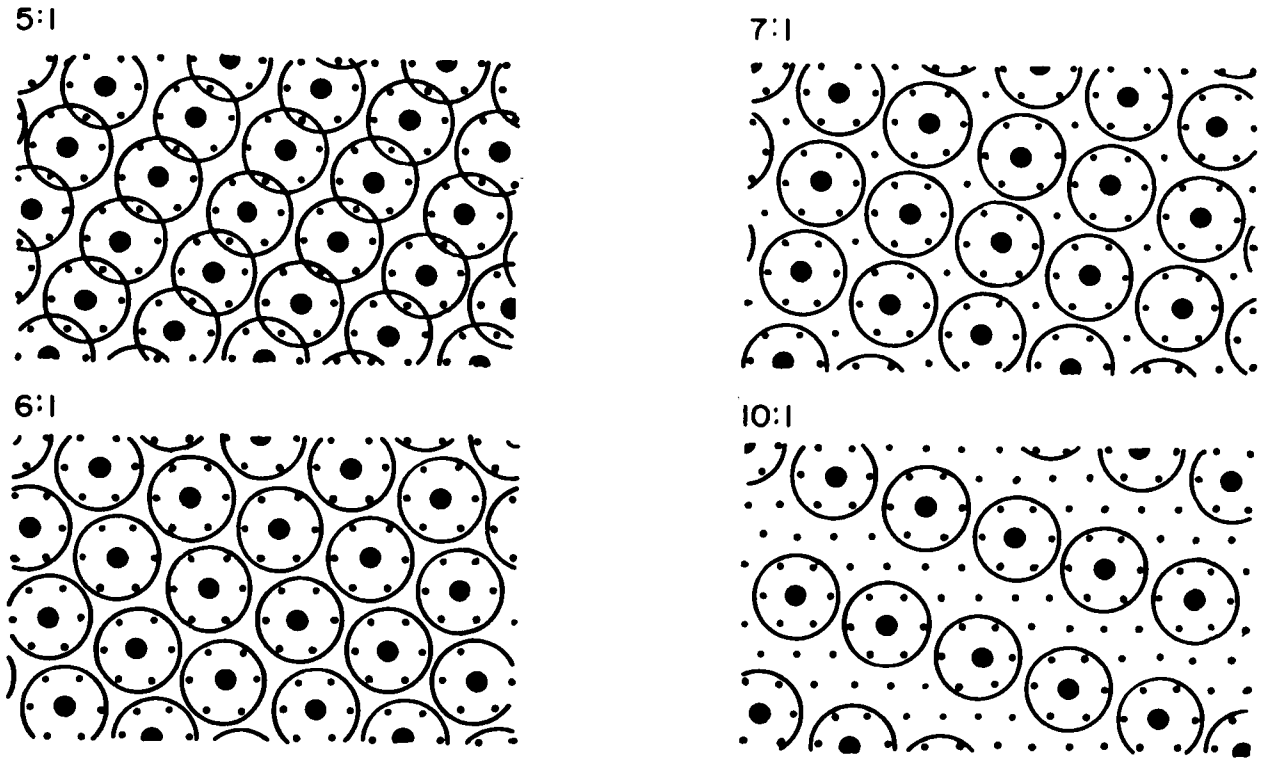


Figure 14: A proposed model for the lipid packing of the liposomes containing different PE to GDla ratios. The PE to GDla ratio is shown at the upper left corner of each panel. Each GDla molecule (●) is surrounded by 6 boundary PE molecules (•) to form a semi-fluid "complex" (defined by a large circle). The areas outside the large cycle are fluid PE domains.

model predicts an initial linear increase in the enthalpy change of phase transition with increased GDla content of the membrane due to the increasing amount of the 6:1 "complex" formation. The enthalpy change reaches a maximun at higher GDla content (1Ø to 16 %) due to the interactions between "complexes" as a result of close contact among "complexes". At even higher GDla content (PE/GDla ratio equals to five or smaller) the enthalpy change decreases due to the overlapping boundary PE among the neighboring "complexes". For fluorescence anisotropy and excimer formation studies (Figures 9 & 1Ø, pp. 42, 44) this model predicts a phase transition between 15 and 25 °C for all liposome compositions, and a higher anisotropy and excimer formation at higher GDla mole fractions even at temperatures above the phase transition. Thus, this model is consistant with most of our data.

The gel-liquid crystalline phase transition of pure PE occurs around 1Ø °C (Sela & Bach, 1984). The mechanism of the ability of GDla to influence PE to undergo phase transition between 15 and 25 °C is mostly due to the known modifying effect of gangliosides in lipid bilayers (Sheron & Grant, 1977). Intermolecular hydrogen bonding is known to shift up gel-liquid crystalline phase transition temperature for many lipids (Eibl et al., 1977; Boggs, 198Ø). There are many possible functional groups in PE and GDla which are capable of forming such intermolecular hydrogen bondings, e.g., negatively charged phosphate and protonated amine groups of PE, and hydroxyl and amide groups of gangliosides (Boggs, 198Ø). Therefore, intermolecular hydrogen bonding between PE and GDla is a possible candidate to

influence PE to undergo a gel-liquid crystalline phase transition between 15 and 25 °C.

CHAPTER IV

MODEL STUDIES OF SENDAI VIRUS-TARGET MEMBRANE FUSION WHICH ALLOW INDEPENDENT ANALYSIS OF BINDING AND FUSION

Fusion between Sendai virus and liposomes containing phosphatidylethanolamine (PE) and different mole fractions of ganglioside GD1a have been investigated. At different times after mixing the virus and liposome the mixture was diluted with a sucrose solution and centrifuged in an airfuge to separate the free and virus-associated liposomes. Since the HN protein of the virus was sensitive to reducing reagent, inclusion of dithiothreitol in the sucrose solution dissociated the bound but not the fused liposomes. Thus the kinetics of liposome-virus binding and fusion could be independently measured. The validity of the assay was confirmed by electron microscopic obsevation of the virus-liposome mixtures. Using trypsin treated Sendai virus, in which the F glycoprotein of the virus had been selectively removed, only virus-liposome binding but not fusion was observed. The kinetic experiments were done under the condition of virus in large excess. Following a very fast initial binding phase, which was completed at the "zero time" of the measurement, the virus-liposome binding followed a pseudo-first order kinetics. The subsequent fusion step was zeroth order. Both the rate constants for binding and fusion showed a temperature dependence such that a phase transition was apparent between 20 and 25 °C. The binding rate constants had activation energies between 16 and 23 Kcal/mole at temperatures above the transition. They were not sensitive to

temperature change at temperatures below the transition. On the other hand, the fusion rate constants were not sensitive to temperature change above the transition, except for 6.3% GDla liposomes. The activation energies were between 20 and 44 Kcal/mole at the temperatures below the transition. The binding step, but not fusion step, was found to be the most probable rate-limiting step in the overall process leading to fusion and leakage at temperatures above the gel-liquid crystalline phase transition. This observation is opposite to the popular belief that the kinetics of the overall process which results in fusion reflects the kinetics of the fusion step itself.

Introduction

Membrane fusion is an effective process to deliver both aqueous and membrane-bound contents from one compartment to another. Cells and viruses take advantage of this important characteristic of membranes to control the synthesis of materials and distribute them to their functional sites efficiently. Although membranes are composed of a heterogeneous collection of different molecules, membrane fusion is spatially and temporally controlled in a precise manner. The mechanism of fusion has become one of the most challenging studies in membrane biology. Although there are some sophisticated mechanistic studies using pure model membrane systems, studies of biological membrane fusion remains largely morphological and descriptive. The main reason is that proper techniques and suitable experimental systems for studying this complex phenomenon are not developed. Sendai virus-

liposome interaction is a promising compromise because of the relative ease of manipulating the chemical and physical properties of the liposomes without a major perturbation of the biological function activity of the virus, while the biological fusion activity of the virus can be studied without major perturbation.

In Chapter III (Tsao et al., 1985) we have demonstrated the correlations between target membrane phase behavior and Sendai virus induced leakage, which is intuitively related to Sendai virus induced membrane fusion. In this chapter we have developed kinetic assays for both Sendai virus-liposome binding and fusion. The kinetic relationship between Sendai virus induced fusion and leakage can thus be investigated. Furthermore, because the overall process which results in virus-liposome fusion can be dissected into binding steps and a subsequent fusion step by independently determining the binding and fusion rate constants. Our studies have shed some light onto the prerequisites of target membrane for fusion with Sendai virus.

Experimental Procedures

Material

PE, TPCK-trypsin, ganglioside GDla, and hexadecyl [^{3}H] cholestanyl ether were obtained as described in the previous paper. Chloramine T, dithiothreitol and soybean trypsin inhibitor were perchased from Sigma. Sucrose, potassium phosphotungstate, and sodium metabisulfite were purchased from Fisher. Polyallomer centrifuge tubes were purchase from Beckmann.

Methods

Preparation of virus, liposomes, and ganglioside micelles.

Liposomes and virus were prepared as described in Chapter III (Tsao et al., 1985) Ganglioside micelles were prepared same way as liposomes except omitting the freeze-thaw step.

Radioiodination and trypsin treatment of Sendai virus.

^{125}I-radioiodinated virus was prepared by using the chloramine T method (Greenwood et al., 1963). Thirty uL of chloramine T (10 mg/mL) was added to 3 mL of the mixture containing Sendai virus (40 mg of viral protein) and 25 mCi of $Na^{125}I$ to start the reaction. After 10 min the reaction was stopped by adding 60 uL of sodium metabisulfite (10 mg/mL). The residual reagents were removed by dialyzing against a large quantity of HEPES-buffered saline (150 mM NaCl, 5 mM KCl, 5 mM HEPES, and 0.02% sodium azide, pH 7.4). Trypsin treatment of Sendai virus was done according to a method modified from Tsao and Huang (1985). Three hundred uL of TPCK-trypsin (1mg/mL) was added to 300 uL of Sendai virus suspension (15 mg/mL of viral protein). The reaction was stopped by adding 1.5 mg of trypsin inhibitor (10 mg/mL).

Sendai virus-liposome binding and fusion assays. Five microliters of liposomes (1 mM lipid, unless otherwise stated), 10 uL of Sendai virus (10 mg/mL), and 35 uL of HEPES-buffered saline were placed as separate droplets at the bottom of a Wheaton omni-vial and preequlibrated to the desired temperature. Reaction was started by vortex mixing the omni-vial for two seconds and the tube placed into a

thermostated Neslab RTE-8 bath. Total virus-liposome association (binding and fusion) was stopped by adding 200 uL of 20% (w/w) sucrose solution into the tube and immediately mixed by vortex. In a separate tube, identical incubation was stopped by using 200 uL of 20% (w/w) sucrose solution containing 50 mM DTT to dissociate those liposomes bound to, but not yet fused with, virus. Two hundred microliters of virus-liposome suspension was then loaded in a 5 x 20 mm polyallomer tube and spun at 165,000 g (generated at 30 psi air pressure) for 25 min in a 30° fixed angle rotor by using a Backman Airfuge. Following centrifugation the sample in the polyallomer tubes was frozen at -70° C for 30 min or longer and then sliced with a razor blade into a top (170 uL, containing free liposomes), middle (160 uL), and bottom (170 uL, containing free virus and virus-liposome pellet) portion. Thereafter,these three portions were placed into separate omni-vials and 500 uL of 10% sodium dodecyl sulfate solution was added to dissolve the pellet by incubating at 37° C for 3 h or longer. ^{3}H-cpm (lipid) of these three portions was then counted in a Beckman LS 7500 Scintilation Counter.

Determination of rate constants. The association between Sendai virus and liposomes was found to follow pseudo-first order, consective-irreversible, three-step kinetics under the condition of virus in large excess. The reaction may be described by the following equation:

$$a \xrightarrow{k1} b \xrightarrow{k2} c \xrightarrow{k3} d$$

where the first step (with a rate constant k1) converted free liposomes (a) into virus-associated species (b) which was DTT-dissociable and completed at the "zero time" (after few seconds) of the measurement; the second step (with a rate constant k2) converted b into virus-associated species (c) that was also DTT-dissociable but its kinetics was slow enough for measurement; the third step (with a rate constant k3) converted c into DTT-insensitive species (d). According to Capellos & Bielski (1972) the time dependent concentration of a (denoted as A), b (denoted as B), c (denoted as C), d (denoted as D) can be calculated from the following equations:

$$A = Ao \exp(-k1\ t) \tag{1}$$

$$B = (k1\ Ao/k2 - k1)\ [\exp(-k1\ t) - \exp(-k2\ t)] \tag{2}$$

$$C = Ao\ k1\ k2\ \{\ [1/(k2 - k1)(k3 - k1)]\exp(-k1\ t) + [1/(k1 - k2)(k3 - k2)]\exp(-k2\ t) + [1/(k1 - k3)(k2 - k3)]\exp(-k3\ t)\ \} \tag{3}$$

$$D = Ao\ \{\ 1 - [k2\ k3/(k2 - k1)(k3 - k1)]\exp(-k1\ t) - [k1\ k3/(k1 - k2)(k3 - k2)]\exp(-k2\ t) - [k1\ k2/(k1 - k3)(k2 - k3)]\exp(-k3\ t)\ \} \tag{4}$$

where Ao = A at zero time of the measurement.

Since k1 is much greater than k2 and k3, equations (1), (2), (3), (4) can be simplified into:

$$A = Ao = Bo \tag{5}$$

$$B = Bo \exp(-k2\ t) \tag{6}$$

$$C = (\ Bo\ k2/k3 - k2\ )\ [\exp(-k2\ t) - \exp(-k3\ t)] \tag{7}$$

$$D = Bo\ \{\ 1 + (k3/k2 - k3)\exp(-k2\ t) - (k2/k2 - k3)\exp(-k3\ t)\ \} \tag{8}$$

Since Bo = Ao, where Bo = B at t = Ø (obtained from equation (6)), the

virus-liposome association can then be described as a pseudo-first, consective-irreversible, two-step process:

$$b \xrightarrow{k2} c \xrightarrow{k3} d$$

The rate constants k2 and k3 can then be determined according to Benson (1960) and Szabo (1969). The kinetics of total association (refering to binding and fusion) and fusion of virus and liposomes was followed by counting the percentage of liposomes (^3H-cpm) associated with the pellet at different time intervals with and without DTT, respectively. The data of total association were plotted as ln (100 - F (t)) vs. t , where

$$F(t) = \frac{I(t) - Io}{Ioo - Io} \times 100$$

Io = background ^3H-cpm in the pellet, determined by replacing virus with DTT-pretreated virus in the assay, Ioo = total ^3H-cpm in the pellet after overnight incubation, and I(t) = ^3H-cpm in the pellet at time t. The pseudo-first order rate constant k2 was calculated from the slope of the linear line best fitting data points.

The fusion rate constant k3 was determined by the equation

$$k2\ B = k3\ C \quad \text{at time } t_{max}$$

where t_{max} = the time when B reaches maximum, which can be determined graphically (see results). The rate constants k3 calculated from the above method were reconfirmed by the equation

t_{max} = [1/(k3 - k2)] ln(k3/k2) when the initial values of C and D are zero.

The activation energy Ea of the process could be determined from the slope of the plot, ln rate constant vs. 1/Temp plot. The entropy of activation of the process could then be calculated from the following equation:

entropy of activation

= R [(Ea/RT) + ln rate constant - ln (kT/h)]

where R = the gas constant; k = the Boltzmann constant; h = the Planck constant; T = temperature.

Electron microscopy. Mixtures containing Sendai virus (125-250 ug/mL viral protein) and liposomes (50-100 uM lipids) in HEPES-buffered saline was incubated at 35 °C for 30 min. Three uL of the mixture was placed on carbon-stabilized, formvar-coated, and glow-discharged copper grids for 6 seconds. Samples were then negatively stained with 1% potassium phosphotungstate, pH 7.5, for 3 min. Samples were examined with a Hitachi H-600 electron microscope operating at 75 KV.

Results

Sendai Virus-Liposome Binding and Fusion Measured by a Centrifugation Assay

We have assumed that the association of Sendai virus and liposomes had three consective steps: fast binding, stabilization of binding, and fusion. Experimentally, we could only measure the extent of fusion (centrifugation after DTT treatment of virus-liposome complex) and the extent of total association (centrifugation without DTT treatment). The latter included both binding and fusion. The extent of fusion and total association of virus with liposomes containing 6.3% of GDla as a function of time at 35 °C are shown in Figure 15. The time course of total association contained a fast binding phase, which completed at "zero time" and had an extent of 11%, and a subsequent slower phase, which followed first order kinetics. The time course of fusion showed no fast phase and had an extent less than total association. The amount of bound but not yet fused liposomes, obtained from the difference between total association and DTT insensitive portion, showed a maximum at about 1 min. To check the effectiveness of the addition of sucrose solution to prevent further association (in the absence of DTT) and fusion (in the presence of DTT), we did the following experiments. The association and fusion of virus and liposomes were allowed to proceed for 1Ø s. and 2Ø s., respectively. The reactions were then stopped as described above except that the solutions after sucrose addition were allowed to stand at room temperature for a varing period of time

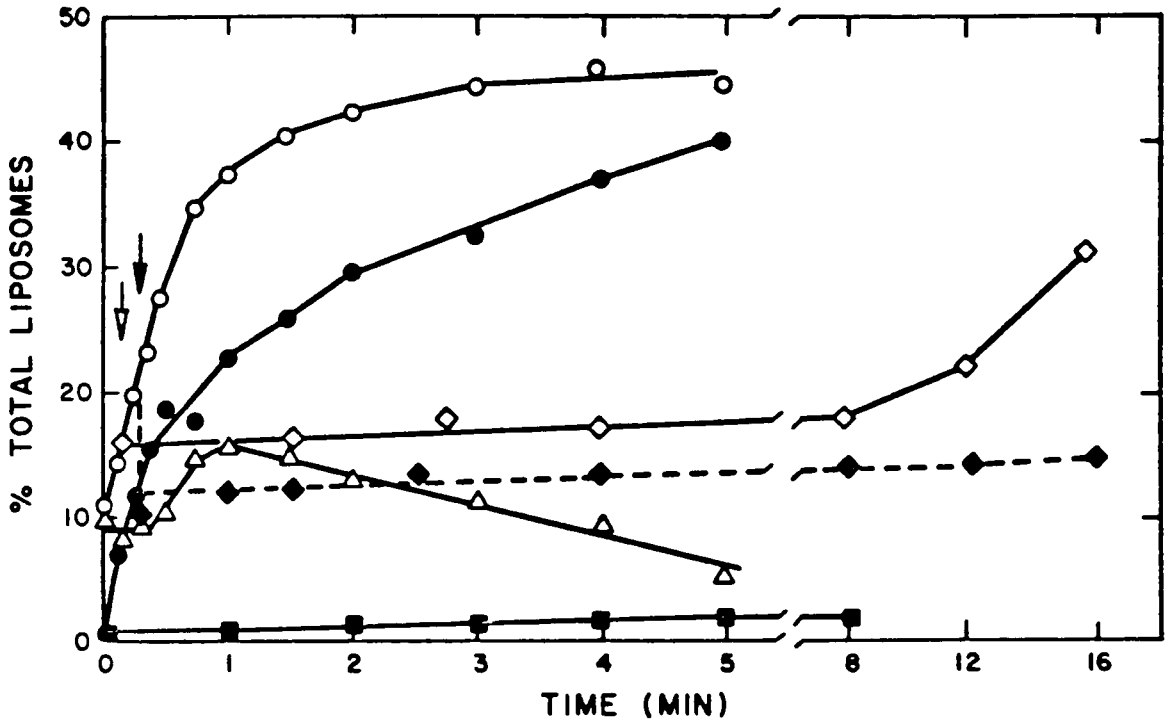


Figure 15: Kinetic measurements of the associations between Sendai virus and liposomes containing 6.3% GDla at 35 C. Binding (O) and fusion (●) were measured after the addition of sucrose solutions without and with DTT, respectively, at the time indicated. The effectiveness of the sucrose solutions to stop the reactions was tested by the addition of sucrose without DTT (◆) at 1Ø s. (as indicated by ↓), or with DTT (◇) at 2Ø s. (as indicated by ↓) and allowed further incubation at room temperature for the time indicated before centrifugation. DTT-pretreated virus (■) was used to show the effect of DTT on binding activity of the virus.

before centrifugation. After the addition of sucrose solution (without DTT) the amount of liposomes associated with viruses stayed almost constant for the first 8 min and then increased. This indicated that unreacted liposomes and virus during the reaction were physically separated by diluting with sucrose solution and thus further virus-liposome association was hampered until they encountered each other again. The addition of sucrose solution containing DTT, on the other hand, caused an instant drop in the amount of liposomes associated with virus from the total association level to the DTT insensitive level and then stayed nearly constant. DTT could immediately reverse and eliminate the binding between virus and liposome so as no furhter virus-liposome association could proceed. This was supported by the fact that DTT-pretreated virus completely lost its ability to associate with liposomes. Trypsinized virus was used to show that the DTT-resistant association of liposomes with virus was related to fusion between virus and liposomes. Sendai virus is known to lose its fusion activity after trypsin treatment, which preferentially digests the viral F glycoprotein (Neurath et al., 1973; Shimizu and Ishida, 1975; Tsao & Huang, 1985). We measured the kinetics of both DTT sensitive and insensitive associations of liposomes with either intact virus or trypsin-treated virus. Consistently, trypsinized virus also lost its activity to associate liposomes in a DTT-resistant manner (fusion) while the total association (binding) activity was only partially impaired (Figure 16). It should be noted that the fast binding component was not affected by trypsin treatment; only the rate of subsequent slow binding phase was greatly reduced.

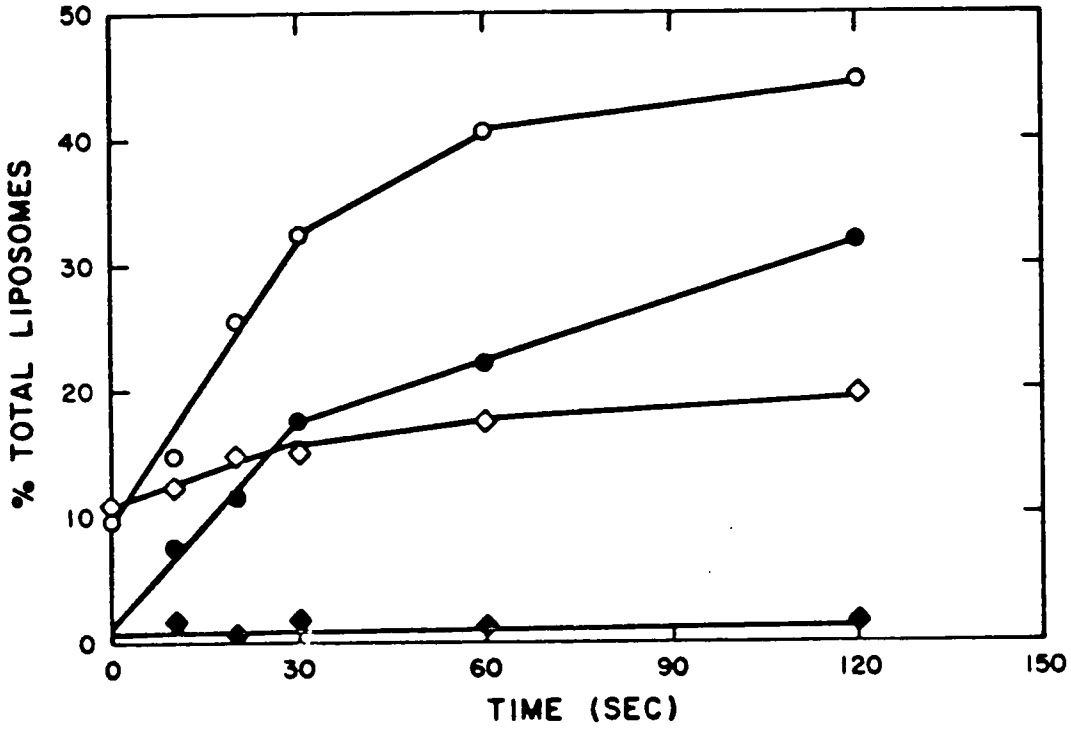


Figure 16: Effect of trypsin digestion of the Sendai virus on the binding stabilization and fusion. Binding (open symbols) and fusion (closed symbols) of liposomes to intact virus (○, ◇), and to trypsin-treated virus (●, ◆) as a function of time was measured with the centrifugation assay.

Fusion between Sendai virus and liposomes was further substantiated by electron microscopic observations. Fusion and binding could be clearly distinguished using this technique because viral membrane proteins (spikes) and viral ribonucleoproteins can serve as excellent markers for identifying fusion species, i.e., to show the presence of viral spikes on liposomal membrane and the delivery of viral internal components into the liposomal aqueous compartment (Figure 17). Thus, a statistical investigation was carried out. The results are compared with those obtained from the centrifugation assay (Table II). Both electron microscopic investigation and centrifugation assay were done after allowing virus and liposomes to react under initial condition; i.e., a condition far from the steady state. Although the total amount of virus associated liposomes was different in the two assays, approximately 65% of which had been fused with virus. This level of fusion was independent of the virus to liposome ratio over a wide range. This result is expected because fusion takes place only when virus and liposome have already physically associated and thus should be completely independent of the concentrations of free liposome and virus. In other words, the fusion step is a zeroth order process. This result indicates that DTT resistant virus-liposome association observed in centrifugation assay indeed corresponds to the fusion hybrid of liposomes and virus observed with electron microscopy. Conversely, DTT sensitive portion of the virus-liposome association corresponds to the binding between virus and liposomes.

Fusion rate constant k3 and the rate constant k2 for the binding stabilization could be determined from the kinetics of total

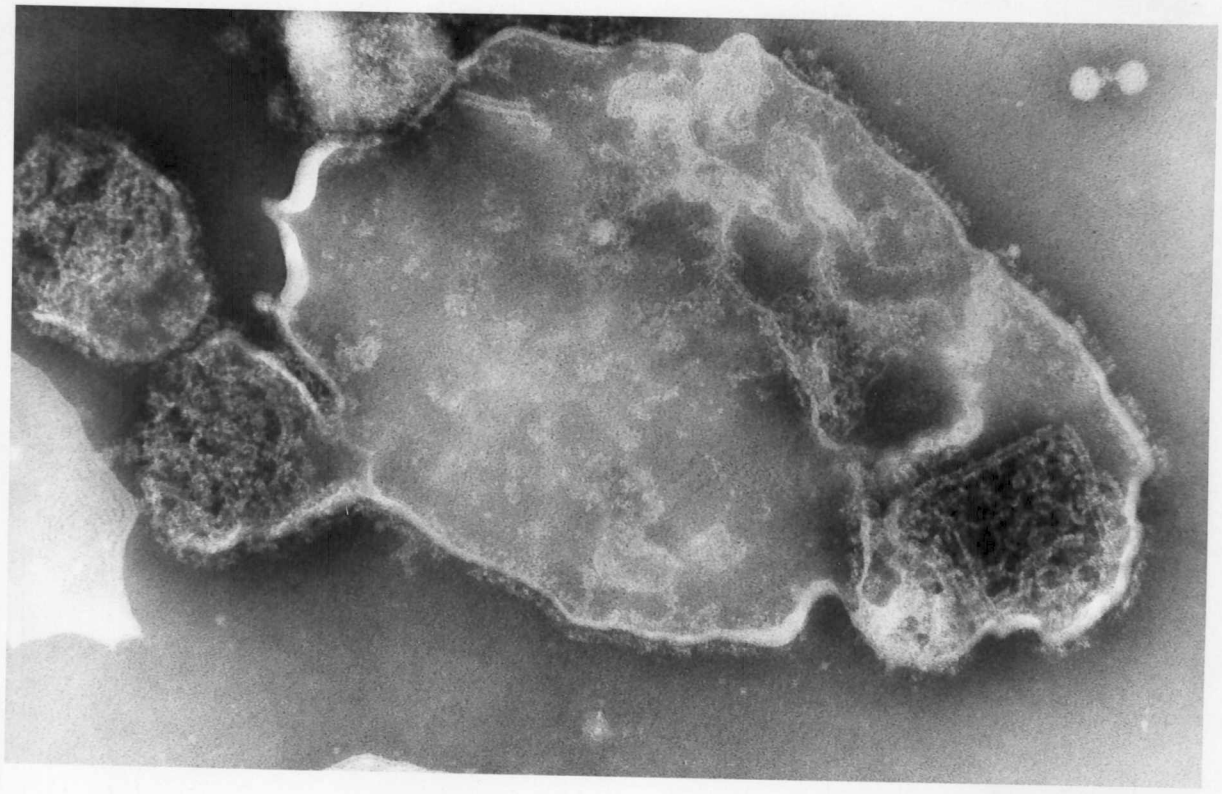


Figure 17: A typical electron micrograph showing liposome interaction with Sendai virus. There are 4 viral particles surrounding the liposome; three on the left, and one on the right. The viral particle on the right has already fused with the liposome. Note that the nuclear capsid (noodle-shaped material) of the virus is inside the aqueous compartment of the liposome, while the membrane proteins (the spikes) are evenly distributed over the surface of the liposome. The virus on the extreme left is a free virus with spikes surround the surface of the particle itself. The virus at upper left is bound to or in close contact with the liposome (slightly out of focus). And the virus at the bottom is probably fusing with the liposome. Note that there is a constriction area between the viral and the liposomal compartment in this last case. Bar is Ø.1 um.

Table II. Interactions between sendai virus and liposomes investigated by electron microscopy and by airfuge centrifugation[a]

Investigation method	Virus to liposome ratio	% of total liposomes			% virus associated liposomes fused
		free	bound	fused	
Electron Microscopy	7.6	Ø	31	69	69
	1.8	35	12	2Ø	62
Centrifugation Assay	1.Ø	76	9	15	63
	Ø.5	7Ø	1Ø	2Ø	67
	Ø.25	66	11	23	65

[a] Centrifugation assays were performed after incubation of a mixture containing Sendai virus (2 mg viral protein/ml) and 6.5% GDla liposomes (5Ø-2ØØ uM) at 35 C for 3Ø s. Due to technical limitations electron microscopy investigation was performed using a mixture with lower concentration: Sendai virus (125-25Ø ug viral protein/ml) and 6.5% GDla liposomes (5Ø-1ØØ uM). The mixture was incubated at 35 C for 3Ø min. The conditions for both investigations were initial state.

association and fusion as described in Methods section. On the other hand, only the extent but not the rate constant of fast binding phase could be estimated because it was completed at "zero time" of (or few seconds after) the reaction. Fast binding could be due to the formation of weakly associated transient complexes between virus and liposome and only a small fraction appeared as virus -associated after centrifugation. Thus, the extent of fast binding was probably related to the condition of centrifugation. We did not further characterize this component.

The Temperature Dependence of Rate Constants

The temperature dependence of the rate constant k2 is shown as an Arrhenius plot in Figure 18. As evidenced by the discontinuity of each curve, a phase transition is apparent between 20 and 25 ^{o}C for every PE-GDla liposome sample with the GDla content varied from 3.2 to 20.5%. The activation energies, obtained from the slopes of the Arrhenius plots, were between 16 and 23 Kcal/mole at temperatures above the transition. Below the transition the activation energy was small. The rate constant k2 was not only dependent on temperature but also on the molar fraction of GDla. Both 3.2% and 6.3% GDla liposomes were fast binders, especially for 6.3% sample. The entropy of activation was slightly dependent on the GDla molar fraction at temperatures above gel-liquid crystalline phase transition, i.e., ranging approximately from -3 (6.3% GDla sample) to -19 cal/mol/deg (20.5% GDla sample) at 35 ^{o}C. It had a large negative value at at the temperatures above the gel-liquid crystalline phase transition

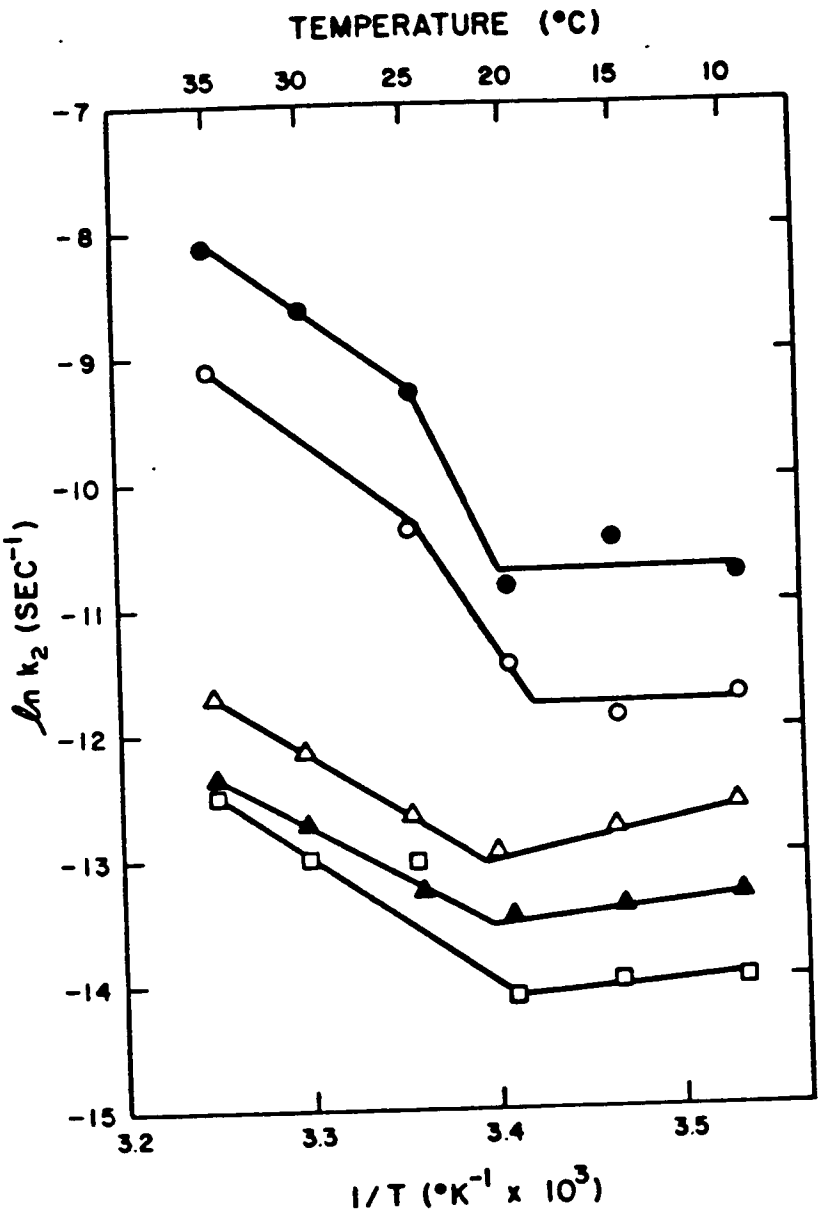


Figure 18: Temperature dependence of the binding rate constant k2 for liposomes containing different mole percents of GDla. Samples are 3.2% (○), 6.3% (●), 11.4% (△), 16.2% (▲), and 20.5% (□) of GDla. The standard deviation of k2 measurement does not exceed 7% and has an average of 3.4%.

temperatures below the transition, i.e., ranging from -80 (6.3% GDla sample) to -87 cal/mol/deg (20.5% GDla sample) at 10 °C.

The temperature dependence of fusion rate constant k3, which was independently measured, is shown in the Arrhenius plots in Figure 19. A phase transition is apparent between 20 and 25 °C for every PE-GDla liposome sample with the GDla content varied from 3.2 to 20.5%. The activation energies were between 20 and 44 Kcal/mole at temperatures below the transition. Above the transition it was negligibly small for 3.2%, 16.2%, and 20.5% GDla samples, while it was 11 Kcal/mole for 6.3% GDla sample. The rate of fusion for 11.4% sample (not shown in Figure 19) was extremely fast above the transition; practically all liposomes associated with virus were fused. Thus the fusion rate constant k3 could not be accurately measured, however, it should not be smaller than k2. The activation energy was not determined in this case. Since the activation energy of the process was small, the activation of the fusion step was entropy-controlled, i.e., when the entropy of activation ranged approximately from -70 to -80 cal/mol/deg at 35 °C for fluid-state liposomes (except 40 cal/mol/deg for 6.3% GDla sample) and from 12 (20.5% GDla sample) to 74 cal/mol/deg (3.2% GDla sample) at 10 °C for gel-state liposomes.

Comparison of the Rate Constants of Binding Stabilization and Fusion.

By comparing the value of rate constants from Figures 18 & 19 (with the exception of 6.3% GDla liposomes) the rate constant of stabilization binding was always lower than that of fusion, especially

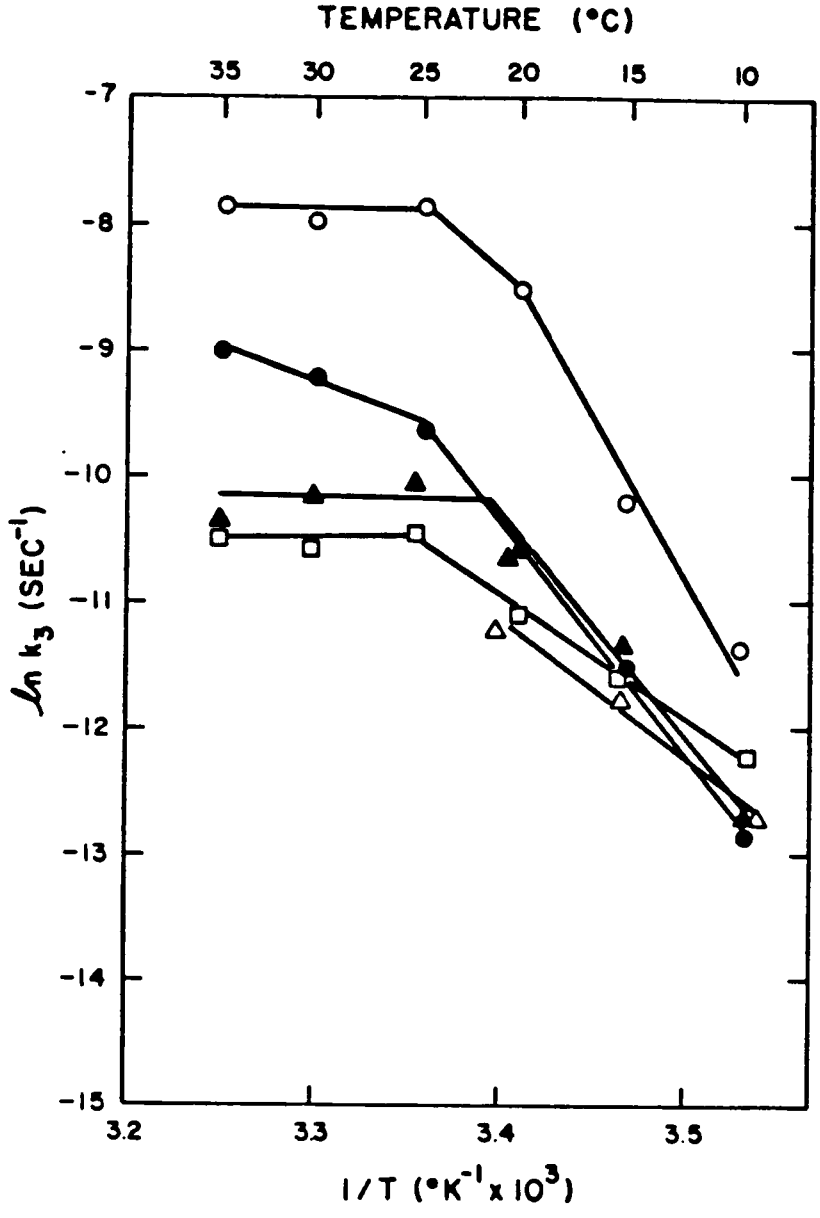


Figure 19: Temperature dependence of the fusion rate constant k3 for liposomes containing different GDla mole percents. Symbols are the same as described in the legend of Figure 18. The standard deviation of k3 measurement does not exceed 1Ø% and has an average of 5.2%.

Therefore, the rate-limiting step should be the stabilization binding with fluid membranes. Below the transition (with the exception of 6.3% GDla liposomes) although the stabilization rate constant was still lower, but it approached the fusion rate constant. Thus binding may no longer be a rate-limiting step below the transition. The exception of 6.3% GDla liposomes could be attributed to its exceptionally high binding rate constant and its fusion rate constant was not low comparing to other liposome samples.

Discussion

Dithiothreitol (DTT) is known to disrupt disulfite linkage(s) that are essential for the hemagglutination (binding), and neuraminidase activities of the Sendai HN glycoprotein (Ozawa et al., 1979). It has also been observated that DTT can remove 90-95% of virus particles that only bound to but not fused with erythrocyte membranes (Chejanovsky et al., 1984). Based on the above observations, we have developed a new assay for studying Sendai virus-liposome binding and fusion with a rapid ultracentrifugation method using an airfuge. The separation of free liposomes and virus-associated liposomes was very efficient in this assay. Over 95% of virus (free and liposome-associated) and free liposomes appeared at the bottom and the top, respectively, of a 16% sucrose solution after centrifugation (data not shown). Thus, the kinetics of liposome association with virus can be monitored conveniently. As shown in the Result section DTT-resistent and DTT-sensitive portion of the virus-liposome association correspond to fusion and binding, respectively. This conclusion was based on the

following observations: (1) DTT-resistent association followed the zeroth order kinetics, which indicated that this step occured after the binding step; (2) it was in quantitative agreement with the kinetics of fusion observed by electron microscopy; and (3) it was abolished when the viral F glycoprotein was selectively removed by trypsin. This convenient and accurate analytical method has allowed us to determine the rate constants of binding and fusion independently.

From the centrifugation assay Sendai virus-liposome association appeared to be composed of three consecutive steps: fast binding, stabilization of binding, and fusion. This model is postulated partly based on a kinetic model of cell-cell adhesion (Bell, 1978). The fast binding step likely represents the formation of weakly associated virus-liposome transient complexes. The latter is due to the immediate matching, which is independent of diffusion along the membranes, of opposing receptors and ligands upon the encounter of virus and liposome. Unfortunately, this mechanism has not been examined because the kinetics are too fast to measure with the present method. The stabilization of binding, described by rate constant k2, is the step of stabilizing the transient complex due to the lateral movement of receptors and ligands to match each other in the contact area. We examine the mechanism of this step and the subsequent fusion step, described by rate constant k3, with the kinetic data obtained. For fluid-state liposomes the stabilization of binding has an activation energy of 16-23 Kcal/mole. It is interesting to note that the activation energy of this step was not sensitive to GDla mole fraction, as was the case for the entropy of activation. It might

indicate that there were at least two separate processes involved, one was entropy-controlled and the other enthalpy-controlled, in the activation of the stabilization step. The negative values of the entropy of activation indicate the formation of a transition complex that had more restricted molecular motions in the entropy-controlled process. Although it could be due to the effects of changes in solvation state of membrane surface (Gutfreund, 1975), the physical binding between GDla and HN glycoproteins, which restrict the molecular motion of themselves, was also consistent with the results. The dependence of the entropy of activation on both density of GDla and fluidity of liposomal membranes was consistent with this hypothesis. A GDla content of 6.3% in the liposome membrane may provide the best GDla density to match HN proteins on the opposing viral membrane when the two particles initially encounter. Initial binding (i.e., fast binding) with a low density of GDla (3.2%) might be so weak that dissociation occurs during centrifugation. Such weak binding may be strengthened by the subsequent lateral movement of GDla molecules into a proper position for binding (i.e., stabilization of binding). On the other hand, it is also known that many multiple binding processes involve negatively cooperative interactions, from steric hinderance and charge repulsion among receptor sites and ligands which result in low binding rates (Wank et al., 1983; Pincus et al., 1981). The oligosaccharide chains of gangliosides have been thought to limit the access of ligands to the receptors (Wank et al., 1983; Pecoud et al., 1981). Thus, excess GDla molecules might hamper the stabilization of binding until they moved away from the contact

area. This idea is consistent with the fact that large nagative values for the entropy of activation were only observed either when the liposomes had high GDla density or when the lateral motion of GDla was restricted (in the gel state). This is also consistent with the observations that the haptenated liposomes bound to cell surface much more efficiently at the fluid state than at the solid state (Lewis et al., 1980). The difference was attributed to the difference in the lateral diffusion coefficients of the haptens on the liposomal surface. Alternatively, high GDla density and low reaction temperature might cause a change in membrane packing, which is known to influence the orientation of the oligosaccharides chain of the gangliosides (Maggio et al., 1981). The latter might determine the rate of stabilization. The activation energy of stabilization, on the other hand, was high and only slightly variable for all fluid-state liposomes and was negligibly low for all gel-state liposomes. This indicates that there is a common endothermic activation process which is only sensitive to the fluidity but not to the density of GDla of the liposome membranes. The high positive enthalpy change might be due to bond breakage, charge repulsion, steric hinderance, etc.. While the precise molecular nature of these processes is unknown, it is clear that F glycoprotein is involved in this step. Removal of F glycoprotein decreased both the rate and the extent of the binding stabilization (Figure 16, p. 68). This result indicates that F glycoprotein actually assists the binding stabilization. The exact mechamism of this observation also remains unclear. There are several possibilities. Because of the negative charge content and the

bulkiness of the F glycoprotein (Shimizu et al., 1974), they could simply exclude GDla molecules to an area in which HN glycoproteins are easily accessible, thus increasing the possibility of binding stabilization. Alternatively, the exposure and insertion of hydrophobic N-terminus of F glycoprotein into liposomal membrane might occur in this step (Hsu et al., 1982; Helenius & Simons, 1975). This would explain why the activation energy was high and only sensitive to the fluidity but not to the GDla density of the liposome membranes. The transient exposure of hydrophobic N-terminal polypeptide to the aqueous medium during the process of insertion (from the hydrophobic interior of F protein to the hydrophobic interior of liposome membrane) might also introduce a large negative entropy of activation to the stabilization process. The small activation energy for gel-state liposomes might reflect that the slow stabilization step became rate-limiting in the gel state, and the insertion of the N-terminus of F glycoprotein was no longer obvious in the activation process. However, the latter process still occured because the fusion proceed even at gel state (Figure 19, p. 75). Part of activation energy and the entropy of activation might also be contributed by the removal of membrane-associated water from the contact area (Cevc & Marsh, 1985). The latter could be facilitated by the exposure of the hydrophobic N-terminus of F glycoprotein in the contact area. These possibilities need to be examined in the future experiments.

By comparing Figures 18 & 19 (pp. 73, 75) it is found that rate constants k2 are always greater than k3 above the transition

temperature except 6.3% GDla sample. For example, ln k2 and ln k3 have values of -9.1 and -7.9, respectively, for 3.2% GDla sample, and of -12.4 and -1Ø.3, respectively, for 16.2% GDla sample. The stabilization of binding is thus the most probable rate-limiting step of the overall process which results in fusion with fluid target membranes. This observation is opposite to the popular belief that the kinetics of the overall process which results in fusion reflects the kinetics of the fusion step itself. The exception of 6.3% GDla liposome that the fusion rate was comparable to the binding rate should be attributed to its exceptional high binding rate, because its fusion rate constant is not low comparing to other liposomes. According to our model 6.3% GDla is the optimum GDla density which allows effective binding between virus and liposomes. Since the stabilization of binding step is the rate-limiting step, one would expect those crucial events preceding fusion, such as the insertion of N-terminus of F protein, the removal of water be involved in this step. This prediction is consistant with our model.

The fusion rate constant k3 showed a sharp dependence on the phase transition, and it was much smaller at temperatures below the transition (Figure 19, p. 75). It clearly shows that a fluid target membrane facilitates fusion. This conclusion agrees well with those drawn from the studies on divalent cation induced fusion of phosphatidylserine vesicles (Wilschut et al., 1985). Moreover, the fusion rate constant had a similar temperature dependence with membrane fluidity, i.e., very sensitive to temperature change only below but not above the gel-liquid crystalline phase transition of the

target membrane (Figure 9 of Chapter III, p. 42; Tsao et al., 1985). The large values in activation energy and the entropy of activation might reflect the involvement of a large scale rearrangement of membrane lipids during membrane fusion. The liposomes with 3.2% GDla had a high fusion rate constant at all temperatures tested, compared to most of the other target membranes (Figure 19, p. 75). This may be due to its high content of free PE, a nonbilayer structure-forming lipid which is known to facilitate fusion (Cullis & de Kruijff, 1978; de Kruijff et al., 1980). Liposomes containing 11.4% GDla, however, had the highest fusion rate at the temperatures above gel-liquid crystalline phase transition. According to our model of target membrane structure, which is described in Chapter III (Tsao et al., 1985), there are six boundary PEs for each GDla molecule to form semi-fluid domains which are uniformly distributed in the fluid phase PE matrix of the bilayer membrane. The calorimetry data showed that the amount of boundary PEs was proportional to GDla mole fraction until the latter reached about 10%. This indicates that at around 10% of GDla the membrane has the maximal boundary circumference between domains of PE-GDla "complex" and free PE. It may explain why the target membrane with 11.4% of GDla has the highest fusion rate constant. Phase boundary is thought to facilitate the formation of "membrane defect", which may be the localized point for fusion (Gibson & Strauss, 1984; Blumenthal, 1985). This result suggests that the step of fusion take place at the boundary of the PE-GDla "complex" and free PE.

It is generally accepted that the cellular leakage induced by Sendai virus is the consequence of adding a leaky viral membrane to the cell membrane as the result of virus-cell membrane fusion (Homma et al., 1976; Impraim et al., 1980; Lyles and Landsberger, 1979). In order to compare the temperature dependence of binding, fusion and leakage processes, the values of activation energy and the temperatures of liquid crystalline phase transition of the processes are compared (Table III). The data for leakage was obtained from Figure 8 of Chapter III (Tsao et al., 1985). The phase transition temperature of the three processes are all correlated to the gel-liquid crystalline phase transition of the liposomes, which had been carefully measured with DPH fluorescence anisotropy, pyrenedecanoic acid excimer formation and high sensitivity differential scanning calorimetry (Tsao et al., 1985). As can be seen from data in Table III, the activation energy of the leakage is better correlated to that of binding than fusion above the transition. It indicates that for the fluid membranes the rate-limiting step of leakage may be the stabilization of binding, the same rate-limiting step for the overall process leading to fusion. On the other hand, at temperatures below transition the activation energy of leakage process is better correlated, although not identical, to the fusion step than the binding step. This indicates that binding step may be no longer rate-limiting with a gel-state membrane. The contribution from low fusion rate to the rate-limiting step may explain the discrepancy between the activation energy of fusion rate constant and leakage rate below the transition. Alternatively, fusion and leakage might have different

Table III. A comparison of the activation energy of liposome binding, fusion and leakage [a].

GDla mole percent	Activation energy (Kcal/mol) above Tc			below Tc			Phase transition temperature (Tc) (C)		
	k2	k3	Leakage	k2	k3	Leakage	k2	k3	Leakage
3.2	23	s[b]	18	s	44	34	22	23	23
6.3	22	11	20	s	37	58	23	25	21
11.4	19	nd[b]	21	s	22	32	21	nd	24
16.2	16	s	14	s	35	32	21	21	24
20.5	20	s	31	s	20	36	20	25	24

[a] Data obtained from Figures 17 & 18 of Chapter III (Tsao et al., 1985.)

[b] s: activation energy was negligibly small; nd: not determined.

mechanisms with a gel-state membrane. It is interesting to note that the major discrepancy was only observed either when the temperature was below the transition or it was with the liposomes containing 20.5% GDla. The latter, comparing to other liposomes, had a less fluid membrane and a different phase behavior, i.e., there were less PE which could undergo the phase transition under the influence of GDla (Figure 13 of Chapter III, p. 48; Tsao et al., 1985).

In summary, we have developed a model system which is highly amenable to kinetic analyses of Sendai virus induced fusion and the accompany membrane permeability changes. These kinetic studies have revealed some mechanistic insight of a complex fusion event which should be helpful in our understanding of biological fusion processe.

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