# CHEMICAL STUDIES OF VIRAL ENTRY MECHANISMS:
# I. HYDROPHOBIC PROTEIN-LIPID INTERACTIONS DURING SENDAI VIRUS MEMBRANE FUSION
# II. KINETICS OF BACTERIOPHAGE λ DNA INJECTION

Thesis by

Steven Louis Novick

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1990

(Submitted July 24, 1989)

**CALVIN AND HOBBES** By Bill Watterson


**CALVIN AND HOBBES** By Bill Watterson


*Here are a few small bones...*

**ACKNOWLEDGMENTS**

Many people at Caltech have made my time here very challenging and enjoyable. It seems like I had the chance to grow a lot while I was here, personally as well as scientifically, and I think it will end up being one of the major developmental stages in my life.

My first thanks go to my advisor, John Baldeschwieler, who has been wonderful in many regards. His constant enthusiasm, encouragement, and support were always reassuring and inspiring. I am especially grateful for his policy of letting graduate students go into the lab on their own and be creative. This was the biggest part of my scientific growth, because I knew that I was solely responsible for directing my research. Sometimes it means making mistakes, but I think it was the best way to learn to do research. John was also a role model for me in learning how to budget my time, and he showed me that there is a healthy balance of lab work and outside life.

Dick Hoekstra has also been like a second advisor to me, with many helpful suggestions about science and life in general. Dick taught me that scientific writing can be colorful. I feel very fortunate to have him as a collaborator and as a friend. Our many excursions through the countryside in the Netherlands were among the highlights of my summer there.

Several people at Caltech have been especially valuable to me as sources of advice and as friends I could talk to about problems. I would like to thank Wilton Vannier, Tom Amatruda, and Mary Roberts for their interest, patience, and friendship.

One of the most important factors in having a positive work environment at Caltech was being in the JDB group. The graduate students, post-docs, and associates always provided a lively atmosphere. Much of what I learned I owe to them, both about science and people. I am happy to have shared my excitement about science with them, and I count them as lifelong friends.

My thesis committee members have also been very willing to take time from their busy schedules to sit and talk with me about scientific issues or counsel me on career directions. In addition, I would like to express my gratitude to the other students, faculty, and staff who made my stay here enjoyable and productive.

It is also necessary to acknowledge the sources of funding for this work. I am grateful to the National Science Foundation and California Institute of Technology for fellowship support. Parts of this work were also supported by grants from the Army Research Organization, the National Institutes of Health, Amersham and Monsanto.

Finally, and most importantly, I want to acknowledge the love and patience of my wife, Teresa. Being the wife of a professional student might not be the most fulfilling role in life, and her willingness to let me indulge my scientific curiosity before I start a career is greatly appreciated. Her encouragement and support have also been instrumental in my getting this far. Our family has also been a great source of support and encouragement. I owe special thanks to them for always helping me to pursue my dreams, for helping me believe I could do it, and for being there when I really needed them. They are the ones responsible for shaping me into someone capable of earning a Ph.D. and doing something positive.

## ABSTRACT

Viruses possess very specific methods of targeting and entering cells. These methods would be extremely useful if they could also be applied to drug delivery, but little is known about the molecular mechanisms of the viral entry process. In order to gain further insight into mechanisms of viral entry, chemical and spectroscopic studies in two systems were conducted, examining hydrophobic protein-lipid interactions during Sendai virus membrane fusion, and the kinetics of bacteriophage λ DNA injection.

Sendai virus glycoprotein interactions with target membranes during the early stages of fusion were examined using time-resolved hydrophobic photoaffinity labeling with the lipid-soluble carbene generator 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID). The probe was incorporated in target membranes prior to virus addition and photolysis. During Sendai virus fusion with liposomes composed of cardiolipin (CL) or phosphatidylserine (PS), the viral fusion (F) protein is preferentially labeled at early time points, supporting the hypothesis that hydrophobic interaction of the fusion peptide at the N-terminus of the $F_1$ subunit with the target membrane is an initiating event in fusion. Correlation of the hydrophobic interactions with independently monitored fusion kinetics further supports this conclusion. Separation of proteins after labeling shows that the $F_1$ subunit, containing the putative hydrophobic fusion sequence, is exclusively labeled, and that the $F_2$ subunit does not participate in fusion. Labeling shows temperature and pH dependence consistent with a need for protein conformational mobility and fusion at neutral pH. Higher amounts of labeling during fusion with CL vesicles than during virus-PS vesicle fusion reflects membrane packing regulation of peptide insertion into target membranes. Labeling of the viral hemagglutinin/neuraminidase (HN) at low pH indicates that HN-mediated fusion is triggered by hydrophobic interactions, after titration of acidic amino acids.

HN labeling under nonfusogenic conditions reveals that viral binding may involve hydrophobic as well as electrostatic interactions. Controls for diffusional labeling exclude a major contribution from this source. Labeling during reconstituted Sendai virus envelope-liposome fusion shows that functional reconstitution involves protein retention of the ability to undergo hydrophobic interactions.

Examination of Sendai virus fusion with erythrocyte membranes indicates that hydrophobic interactions also trigger fusion between biological membranes, and that HN binding may involve hydrophobic interactions as well. Labeling of the erythrocyte membranes revealed close membrane association of spectrin, which may play a role in regulating membrane fusion. The data show that hydrophobic fusion protein interaction with both artificial and biological membranes is a triggering event in fusion. Correlation of these results with earlier studies of membrane hydration and fusion kinetics provides a more detailed view of the mechanism of fusion.

The kinetics of DNA injection by bacteriophage λ into liposomes bearing reconstituted receptors were measured using fluorescence spectroscopy. LamB, the bacteriophage receptor, was extracted from bacteria and reconstituted into liposomes by detergent removal dialysis. The DNA binding fluorophore ethidium bromide was encapsulated in the liposomes during dialysis. Enhanced fluorescence of ethidium bromide upon binding to injected DNA was monitored, and showed that injection is a rapid, one-step process. The bimolecular rate law, determined by the method of initial rates, revealed that injection occurs several times faster than indicated by earlier studies employing indirect assays.

It is hoped that these studies will increase the understanding of the mechanisms of virus entry into cells, and to facilitate the development of virus-mimetic drug delivery strategies.

# TABLE OF CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| ACAC | acetylacetone |
| BHA | bromelain-solubilized influenza hemagglutinin ectodomain |
| CF | 5(6)-carboxyfluorescein |
| CL | cardiolipin |
| DDAB | didodecyldimethylammonium bromide |
| DDP | didodecylphosphate |
| DOPC | dioleoylphosphatidylcholine |
| $E_1$ | Semliki Forest virus fusion glycoprotein |
| EDTA | ethylenediaminetetraacetic acid |
| F | Sendai virus fusion glycoprotein |
| G | vesicular stomatitis virus glycoprotein |
| HA | influenza virus hemagglutinin |
| Hepes | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HN | Sendai virus hemagglutinin/neuraminidase |
| kbp | kilobasepair |
| KNP | 120 mM KCl/30 mM NaCl/10 mM $Na_2HPO_4$, pH 7.4 |
| N-NBD-PE | N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoylphosphatidylethanolamine |
| N-Rh-PE | N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine |
| NTA | nitrilotriacetic acid |
| PAC | perturbed angular correlation spectroscopy |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | 150 mM NaCl/5 mM $Na_2HPO_4$, pH 7.4 |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PEG | poly(ethylene glycol) |
| pfu | plaque-forming units |
| PS | phosphatidylserine |
| $R_{18}$ | octadecyl Rhodamine B chloride |
| RET | resonance energy transfer |
| RSVE | reconstituted Sendai virus envelope |
| SDS | sodium dodecyl sulfate |
| SFV | Semliki Forest virus |

| | |
|---|---|
| TID | 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine |
| Tris | tris(hydroxymethyl)aminomethane |
| VSV | vesicular stomatitis virus |

# Part 1:
# Hydrophobic Protein-Lipid Interactions During Sendai Virus Membrane Fusion

# *Chapter 1*
# *Introduction*

## MOTIVATION AND GENERAL APPROACH

In spite of many technological advances which have occurred recently, many diseases continue to afflict mankind, causing great suffering and limiting human potential. Whether they are diseases of internal defect or external agents, members of all societies are hampered by illness. Much of the modern medical treatment of these illnesses is nonspecific and causes unintended toxicity and harm. For example, a cytotoxic drug used for cancer treatment may cause significant damage to healthy parts of the body. This is due to the inability to target the drug to the disease site. For a drug to be useful, its therapeutic efficacy must outweigh its toxicity.

Many infectious agents are quite specific, however, and infect only one site or type of cell. By developing drug delivery vehicles which mimic these agents, it might be possible to minimize the toxicity caused by treating diseases of specific tissues with a drug. One model of an infectious agent which very efficiently targets and enters specific cell types is a virus.

The goal of these studies is to probe the biochemical mechanisms which occur during the initial interactions between a virus and a cell, with the application in mind of disease treatment using virus-mimetic targeted drug delivery. The understanding gained of viral entry mechanisms may also help to develop strategies for treating viral disease. Some success in drug targeting has already been attained using phospholipid vesicles ("liposomes") as targeted drug delivery vehicles (1-5).

Liposomes are spherical phospholipid bilayer vesicles which enclose an aqueous volume, and can be made readily in the laboratory (6,7). Water soluble drugs can be encapsulated in the aqueous compartment, and hydrophobic molecules can be solubilized within the bilayer core. Targeting molecules, e.g., antibodies or ligands for specific cellular receptors, can be attached to the liposome surface (8-10). This assembly resembles the membrane of an enveloped virus, and may be useful for

targeting drugs or genes to specific sites in the body, in the same way that a virus targets the cell types it infects.

## PROPERTIES OF VIRUSES

Many animal viruses are composed of a core of nucleic acid surrounded by a protein coat, enveloped within a phospholipid membrane (11). Embedded in the membrane are glycoproteins which allow the virus to recognize specific receptors on cells, and to efficiently enter the cell (Figure 1). Sendai virus, a paramyxovirus, has both a binding protein, the hemagglutinin/neuraminidase (HN), and a fusion protein (F; 13,14). The HN protein is responsible for viral binding to cellular sialic acid containing receptors, and the F protein mediates fusion of the viral and cellular membranes. After the viral nucleic acids are inside the cell, the virus uses the cell's replicative machinery to reproduce.

The viral membrane glycoproteins confer a great deal of specificity to the ability of a virus to enter and infect cells. The viral binding protein attaches to molecules on the cell surface. The fusion protein allows the virus to enter the cell by fusion of the viral membrane with either the plasma membrane or endosomal membrane of the cell (Figure 2). This membrane fusion event allows efficient delivery of the viral genome into the cell.

Although the involvement of proteins in viral entry has been apparent for some time (15,16), the mechanism by which they function is poorly understood. Many fusion proteins from a wide range of viral families contain homologous hydrophobic stretches of amino acids in their primary sequences (13,14,17,18; Table 1). This high degree of homology led to the hypothesis that the hydrophobic segment of a fusion protein might induce membrane fusion by insertion into the hydrophobic core of a cell membrane (Figure 3). This hypothesis was based on observations that other proteins

**Figure 1**

Diagram of Sendai virus structure, showing fusion (F) and hemagglutinin/neuraminidase (HN) glycoproteins embedded in the lipid bilayer, the matrix protein (M) lining the inside of the viral envelope, and the helical nucleocapsid (NP), which contains the RNA and polymerase and translation proteins (L,P). From ref. 12.

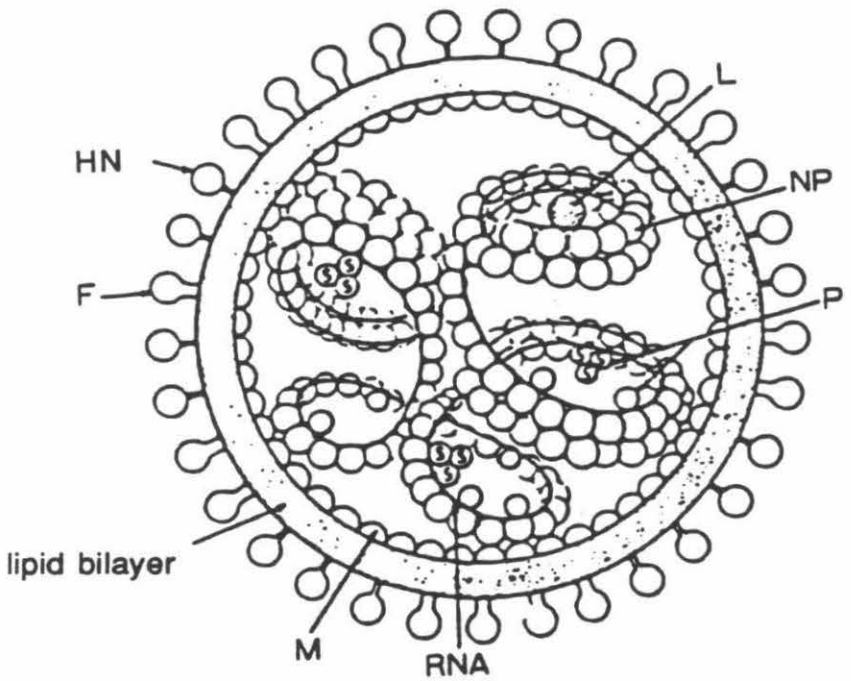

HN
F
lipid bilayer
M
RNA
L
NP
P

**Figure 2**

Pathways of entry of enveloped viruses into cells. Paramyxoviruses, such as Sendai virus enter by fusion with the cell's plasma membrane (pathway A) after binding to cellular receptors. Many other viruses, including influenza virus, are endocytosed (pathway B), and enter the cell by fusion from within the endosome, as the pH triggers protein conformational changes and activates fusion proteins (C).

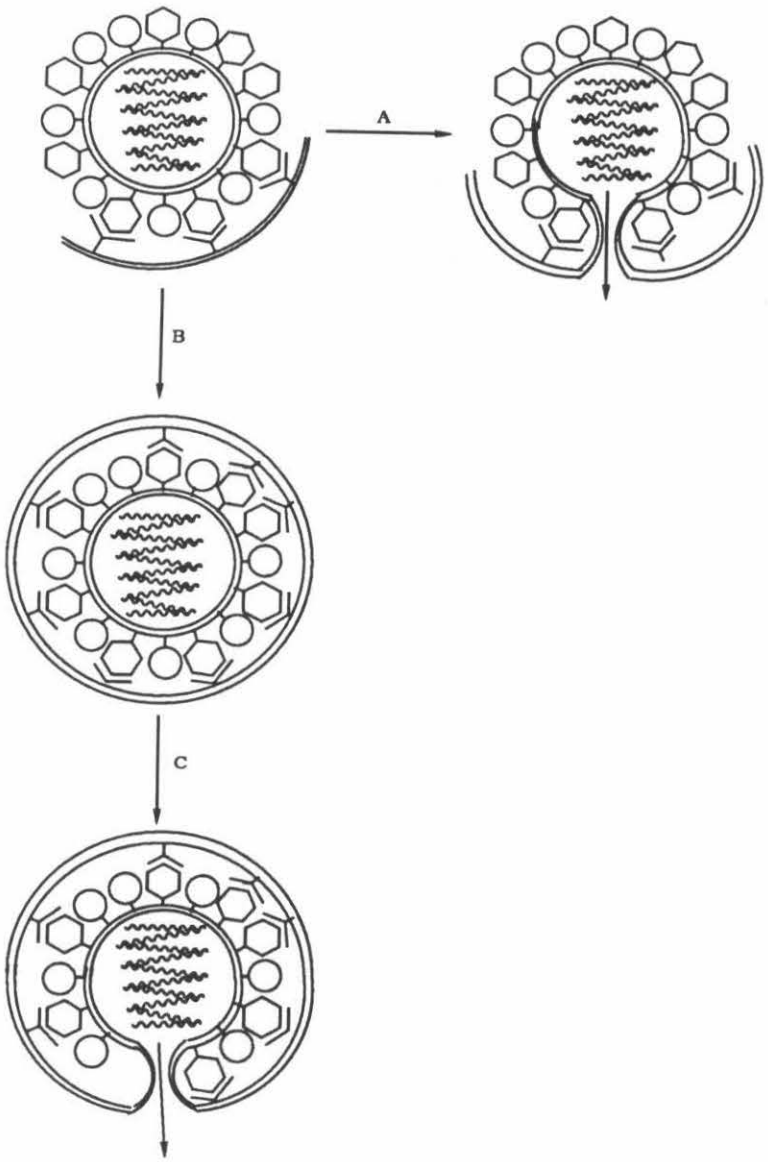

A
B
C

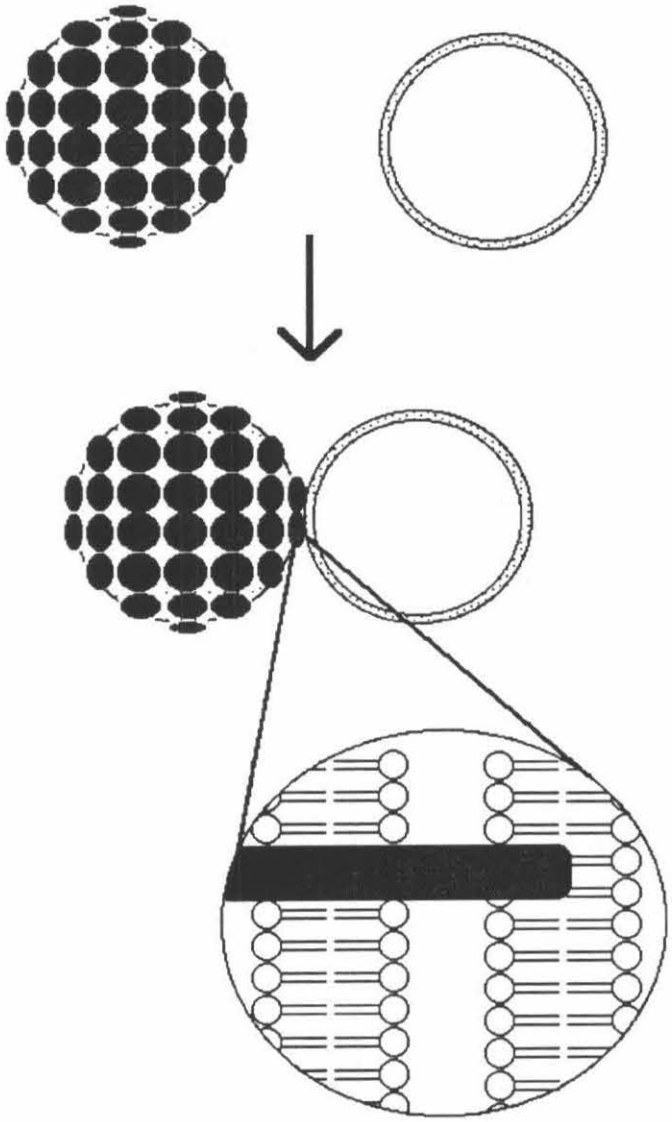
**Table 1**

Prevalence of hydrophobic sequences in the primary structure of viral fusion proteins. Many segments occur at the protein N-termini, but some (Togaviridae and Rhabdoviridae) are internal segments. From refs. 19,20.

| | |
|---|---|
| **PARAMYXOVIRIDAE** | |
| Paramyxovirus F1 | |
| Sendai | FFGAVIGTIALGVATSAQITAGIALAEAR |
| SV5 | FAGVVIGLAALGVATAAQVT |
| Newcastle Disease | FIGAIIGGVALGVATAAQIT |
| Mumps | FAGIAIGIAALGVATAAQVT |
| Pneumovirus F1 | |
| RS | FLGFLLGVGSAIASGVAVSK |
| **ORTHOMYXOVIRIDAE** | |
| Influenza virus HA2 | |
| A/PR/8/34 | GLFGAIAGFIEGGWTGMIDGWYGYH |
| A/Japan/305/57 | GLFGAIAGFIEGGWQGMVDGWYGYH |
| A/Aichi/2/68 | GLFGAIAGFIENGWEGMIDGWYGFR |
| A/FPV/Rostock/34 | GLFGAIAGFIENGWEGLVDGWYGFR |
| B/Lee/40 | GFFGAIAGFLEGGWEGMIAGWHGTY |
| Influenza C virus | |
| C/Cal/18 | IFGIDDLIIGLLFVAIVETGIGGYLLGSR |
| **TOGAVIRIDAE** | |
| Alphavirus E1 | |
| Semliki Forest 79-110 | KVYTGVYPFMWGGAYCFCDSENTQLSEAYVDR |
| Sindbis 79-110 | KVFGGVYPFMWGGAQCFCDSENSQMSEAYVEL |
| **RHABDOVIRIDAE** | |
| Vesicular stomatitis virus | |
| VSV Ind 100-132 | KQGTWLNPGFPPQSCGYATVTDAEAVIVQVTPH |
| VSV NJ 100-132 | KDGVSFNPGFPPQSCGYGTVTDAEAHIVTVTPH |
| VSV Ind 174-200 | KGLCDSNLISMDITFFSEDGELSSLGK |
| VSV NJ 174-200 | ESVCSQLFTLVGGIFFSDSEEITSMGL |
| **RETROVIRIDAE** | |
| Type B oncovirus | |
| MMTV gp36 | FVAAIILGISALIAIITSFAVATTALVK |

**Figure 3**

Diagram showing hypothetical insertion of a viral fusion peptide (darkened) into the hydrophobic core of a target membrane, initiating fusion.



capable of inducing membrane fusion contain similar hydrophobic segments, and interact hydrophobically with membranes (21-25).

From the standpoint of drug delivery, both targeting to appropriate cells and efficient delivery of liposomal contents across the plasma membrane are important. It has been demonstrated that attachment to a cell surface does not in itself insure efficient delivery of a liposome's contents into a cell (26). Thus, incorporation of a fusogenic molecule into the liposomal membrane would greatly aid the ability to deliver liposomal contents into cells.

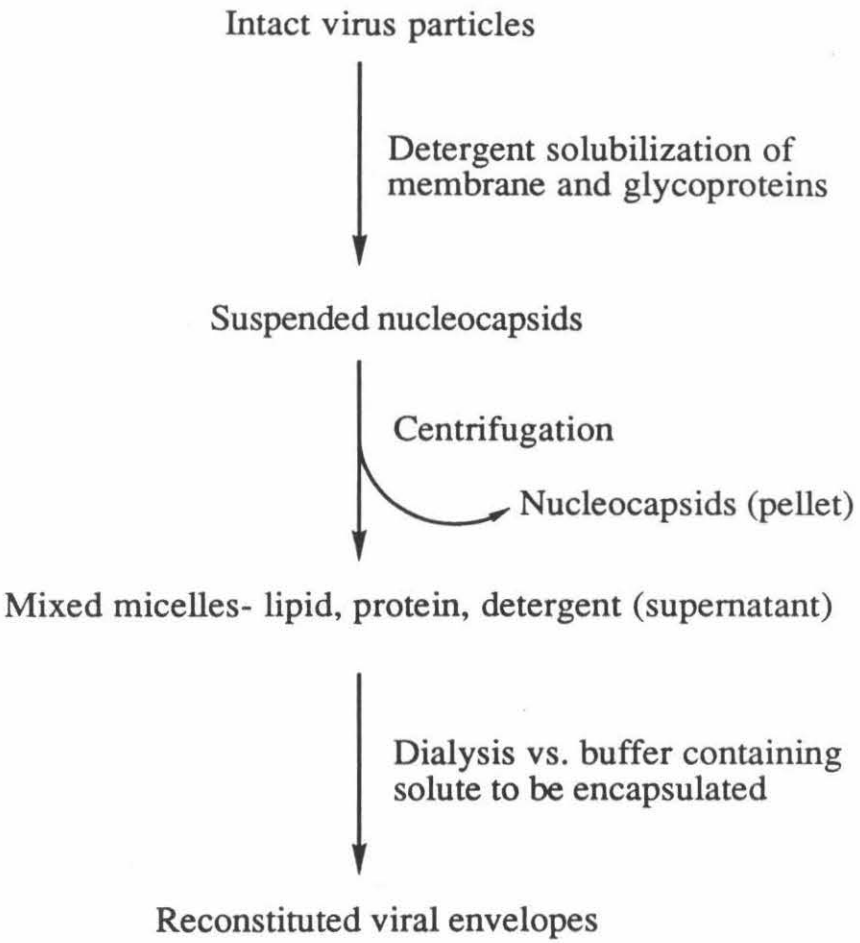
Some early attempts to make liposomes containing viral binding and fusion proteins by detergent removal dialysis have resulted in successful formation of reconstituted viral membranes (Figure 4) of Sendai and other viruses (27,28). Frequently, however, nonfunctional particles are formed, and the selection of the proper reconstitution conditions are quite unclear. Applications of this type are limited by the lack of mechanistic understanding of the structure and function of the viral proteins. The studies in this thesis are aimed at increasing this understanding and, hopefully as a result, to further attempts at efficient drug delivery.

## VIRUS-MIMETIC DRUG DELIVERY

Targeted fusogenic liposomal membranes are functionally analogous to viral membranes. One way to make the liposomes is to reconstitute viral targeting and fusion proteins (cf. Figure 4 and above). Solutes can be encapsulated by inclusion in the dialysis buffer. Reconstituted Sendai virus envelopes (RSVE) entrapping DNA or RNA by this method are capable of fusing with and transfecting cells (28-30). Hydrophobic molecules, such as integral membrane proteins, can also be reconstituted into the viral envelope by inclusion in the dialysate. If antibodies or ligands for cellular receptors are used, the targeting property of the viral envelope can be altered (28,31-

**Figure 4**

Method of viral envelope reconstitution. Viral membranes are detergent solubilized, and nucleocapsids are removed by centrifugation. Dialysis removal of detergent yields reformed viral envelopes.


Intact virus particles
Detergent solubilization of membrane and glycoproteins
Suspended nucleocapsids
Centrifugation
Nucleocapsids (pellet)
Mixed micelles- lipid, protein, detergent (supernatant)
Dialysis vs. buffer containing solute to be encapsulated
Reconstituted viral envelopes

33). This makes it possible to infect a cell with a virus not normally capable of infecting that particular cell type. Functional membrane proteins can be inserted into cell plasma membranes by reconstitution of the proteins into RSVE followed by RSVE-cell fusion (34). Theoretically, any membrane protein receptor can be incorporated into cell membranes. It is therefore conceivable to use vectors such as bacteriophages, which lack receptors on eukaryotic cells but package DNA very efficiently, as DNA delivery vehicles.

Bacteriophage λ (Figure 5) is commonly used as a cloning vector in molecular biology and biochemistry (35). The bacteriophage packages double-stranded DNA very efficiently, normally containing a 48.5 kbp genome, and the packaging function can be reconstituted *in vitro* (37,38), with a much higher efficiency than that attainable by detergent dialysis encapsulation of large DNA molecules in liposomes. Receptor binding and DNA injection by the phage into liposomes bearing reconstituted receptors (LamB) is also very specific (39; see Section II of this thesis). By implanting reconstituted bacteriophage receptors into cell membranes via RSVE-mediated fusion, the cells should be rendered susceptible to transfection by the bacteriophage (Figure 6).

The studies described in this thesis are basic studies preliminary to these applications. The mechanistic information obtained should be of benefit toward achieving these goals. Basic understanding of the viral infection process may also help in developing new strategies to combat viral disease.

**Figure 5**

Diagram of bacteriophage λ structure. The head (50-60 nm diameter) contains a double-stranded genome of 48.5 kbp. Unlike an enveloped virus, the phage is surrounded entirely by a proteinaceous exterior. From ref. 36.

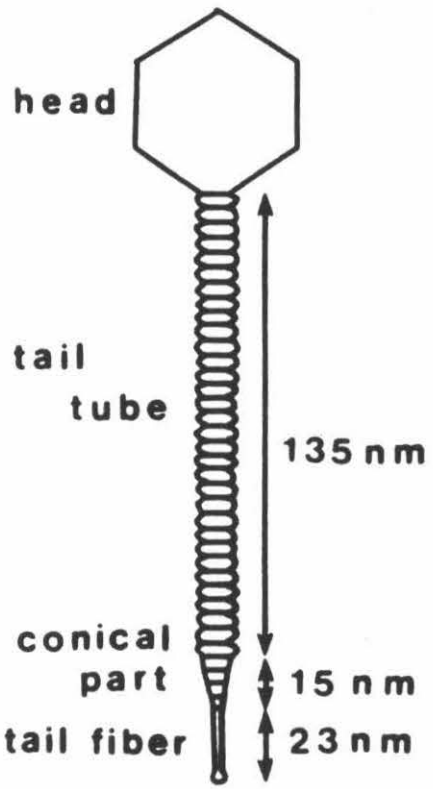
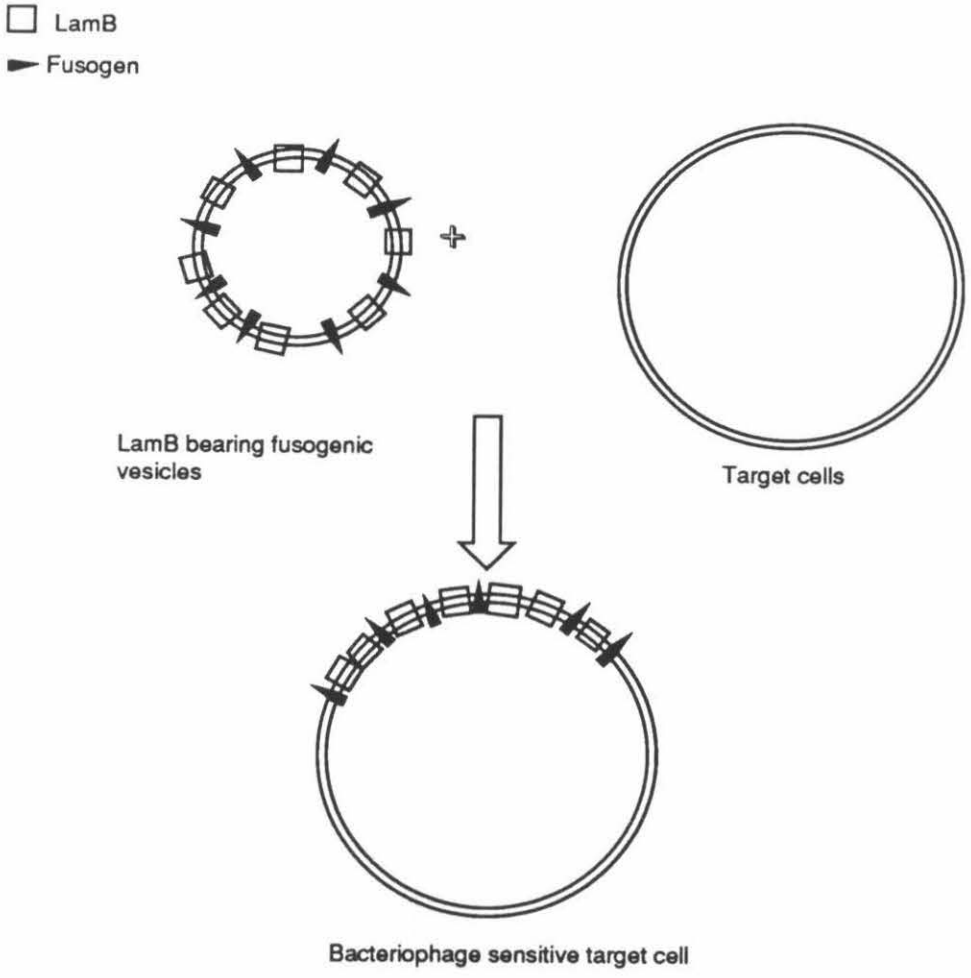

head
tail
tube
135 nm
conical
part
15 nm
tail fiber
23 nm

**Figure 6**

Strategy for gene delivery by bacteriophage λ to mammalian cells. Bacteriophage receptors (LamB) are reconstituted into fusogenic viral envelopes, and implanted into cell membranes by fusion of the reconstituted viral envelopes with the cells. The cells should then be susceptible to bacteriophage attachment and DNA injection.


LamB
Fusogen
LamB bearing fusogenic vesicles
Target cells
Bacteriophage sensitive target cell

## THESIS OVERVIEW

With the aforementioned goals and perspective in mind, studies involving mechanistic aspects of both Sendai virus membrane fusion and bacteriophage λ DNA injection were undertaken.

*Part I* of the thesis describes studies aimed at elucidating the nature of the interactions between the Sendai virus fusion (F) protein and the lipid bilayer of a cell which the virus would infect.

In particular, it was hypothesized that hydrophobic interactions of the N-terminus of the $F_1$ subunit with the target membrane trigger the fusion reaction (13,14). This hypothesis was based on conservation of hydrophobic amino acids in viral fusion proteins (Table I).

In order to probe these hydrophobic interactions in a way that would allow mechanistic conclusions to be drawn about the role of hydrophobic effects, experiments were designed using hydrophobic photoaffinity labels to provide greater insight into the role of hydrophobic interactions as a function of the time course of the reaction.

*Chapter 1* (this chapter) briefly describes the viral systems under study and the motivation and approach for the work presented.

*Chapter 2* describes the uses of hydrophobic photoaffinity labeling, and presents relevant background material. The experimental approach of using a photoaffinity probe solubilized in target membranes is described, including the rationale for choice of the probe. Some practical considerations in the experimental design are also presented. The key distinction which allows much greater mechanistic analysis in these experiments than was possible in earlier hydrophobic photoaffinity labeling, the use of time-resolved hydrophobic photoaffinity labeling, is described. This distinction gives these experiments a fundamentally different approach from previous uses of the

technique and provides a much clearer view of the role of hydrophobic interactions in membrane fusion.

*Chapter 3* presents experiments demonstrating the use of the hydrophobic photoaffinity labeling technique in a model system. Sendai virus fusion with liposomes was examined in order to determine whether hydrophobic photoaffinity labeling was capable of detecting hydrophobic interactions during the early stages of virus-liposome fusion. The use of relatively simple model membranes also allowed examination of the effect of target membrane composition and physical parameters such as temperature and pH on the occurrence of the hydrophobic interactions. Comparison of the photoaffinity labeling data with the independently monitored kinetics of the fusion reaction allowed mechanistic conclusions to be made about the role of these interactions in fusion.

*Chapter 4* describes fully the time-resolved hydrophobic photoaffinity labeling of the Sendai virus glycoproteins during fusion with liposomes. Hydrophobic interactions were investigated as a function of temperature, pH, and target membrane packing. In addition to examining F protein interactions during fusion at neutral pH, the interactions of the viral binding protein, the hemagglutinin/neuraminidase (HN), were examined during fusion at pH 5.0. Hydrophobic interactions of HN were also investigated during the viral binding event. With particular relevance to future drug delivery applications, hydrophobic interactions during fusion of reconstituted Sendai virus envelopes (RSVE) with liposomes were also examined.

*Chapter 5* presents experiments investigating hydrophobic interactions during Sendai virus fusion with erythrocyte membranes. The hydrophobic photoaffinity labeling approach was established in the liposomal system already, and it was then appropriate to test the hypothesis that hydrophobic interactions also are responsible for triggering fusion of two complex biological membranes. Based on the results obtained

in these studies, models of the fusion event and the roles of hydrophobic interactions are discussed, and are compared with existing models.

*Chapter 6* presents additional discussion of the significance of hydrophobic interactions in membrane fusion, comparing data obtained during liposome fusion, virus-liposome fusion, and virus-erythrocyte membrane fusion. Analysis of the fusion reaction using a mass action kinetic model is also discussed. A brief section reviews techniques used for measurements of viral fusion. Some of the hydrophobic photoaffinity labeling data from the virus-liposome fusion experiments is also discussed in the context of membrane packing and hydration of the membrane surface.

*Part II* of the thesis presents experiments aimed at further understanding the DNA injection process of bacteriophages, which could also be important for gene delivery applications.

In order to directly measure the kinetics of DNA injection, a fluorescence spectroscopic assay was developed. The direct observation of the injection process, rather than measurement by indirect methods such as plaque inhibition assays, allowed resolution of the kinetics which showed a much faster process than previously reported.

*Chapter 7* is a brief introduction describing some aspects of the model phage used in these studies, bacteriophage λ. A brief description of its cellular receptor is also given. The motivation for the study and experimental approach are described, and a brief discussion comparing models of bacteriophage DNA packaging and injection is given based on the study in this part of the thesis. The indirect method for kinetic measurements used in earlier studies (plaque inhibition assay) is also described.

*Chapter 8* presents the experiments conducted to measure the kinetics of DNA injection by bacteriophage λ, using fluorescence spectroscopy. The DNA binding dye, ethidium bromide, was entrapped in liposomes containing reconstituted receptors. The

enhanced fluorescence resulting from ethidium binding to injected DNA was monitored. Direct observation of injection shows that injection kinetics are much faster than that reported in earlier indirect studies. Implications for existing DNA injection models are also presented.

*Chapter 9* gives a summary of the thesis, briefly describing the conclusions of the experiments presented.

The *Appendix* gives a further discussion of the role of hydrophobic interactions in membrane fusion, and describes the effects of dehydration of the membrane surface on membrane fusion. This discussion is appropriate in the context of our current hypothesis, that the hydrophobic penetration of the fusion protein may serve to bring apposed bilayers together and overcome the short-range repulsive hydration forces by dehydrating the membrane surface.

## REFERENCES

1. Gabizon, A. (1989) in *Drug Carrier Systems* (Roerdink, F.H.D. and Kroon, A.M., Eds.), Wiley: N.Y., 185-211.

2. Fidler, I.J. (1989) in *Drug Carrier Systems* (Roerdink, F.H.D. and Kroon, A.M., Eds.), Wiley: N.Y., 213-247.

3. Juliano, R.L. (1989) in *Drug Carrier Systems* (Roerdink, F.H.D. and Kroon, A.M., Eds.), Wiley: N.Y., 249-279.

4. Gregoriadis, G. (1985) *Trends Biotech.* **3**, 235-241.

5. Lopez-Berestein, G. (1989) in *Liposomes in the Therapy of Infectious Diseases and Cancer* (Lopez-Berestein, G. and Fidler, I.J., Eds.), Liss: N.Y., 317-327.

6. Szoka, F. and Papahadjopoulos, D. (1980) *Ann. Rev. Biophys. Bioeng.* **9**, 467-508.

7. Lichtenberg, D. and Barenholz, Y. (1988) *Meth. Biochem. Anal.* **33**, 337-462.

8. Baldeschwieler, J.D. (1985) *Macromolecules as Drugs and as Carriers for Biologically Active Materials* (Tirrell, D.A., Donaruma, L.G. and Turek, A.B., Eds.), N.Y. Acad. Sci.: N.Y., 349-367.

9. Connor, J., Sullivan, S. and Huang, L. (1985) *Pharmac. Ther.* **28**, 341-365.

10. Matthay, K.K., Heath, T.D., Badger, C.C., Bernstein, I.D. and Papahadjopoulos, D. (1986) *Cancer Res.* **46**, 4904-4910.

11. Murphy, F.A. (1985) in *Virology* (Fields, B.N., Ed.), Raven Press: N.Y., 7-25.

12. Asano, A. (1983) *Saibo Kogaku* **2**, 852-859.

13. Choppin, P.W. and Scheid, A. (1980) *Rev. Infect. Dis.* **2**, 40-61.

14. White, J., Kielian, M. and Helenius, A. (1983) *Q. Rev. Biophys.* **16**, 151-195.

15. Homma, M. and Ohuchi, M. (1973) *J. Virol.* **12**, 1457-1465.

16. Scheid, A. and Choppin, P.W. (1974) *Virology* **57**, 475-490.

17. Hsu, M.-C., Scheid, A. and Choppin, P.W. (1981) *J. Biol. Chem.* **256**, 3557-3563.

18. Asano, A. and Asano, K. (1984) *Tumor Res.* **19**, 1-20.

19. Ohnishi, S.-I. (1988) *Curr. Topics Membr. Transp.* **32**, 257-296.

20. Server, A.C., Smith, J.A., Waxham, M.N., Wolinsky, J.S. and Goodman, H.M. (1985) *Virology* **144**, 373-383.

21. Fox, R.O. and Richards, F.M. (1982) *Nature* **300**, 325-330.

22. Terwilliger, T.C., Weissman, L. and Eisenberg, D. (1982) *Biophys. J.* **37**, 353-361.

23. Bernheimer, A.W. and Ruby, B. (1986) *Biochim. Biophys. Acta* **864**, 123-141.

24. Kim, J. and Kim, K. (1986) *Biochemistry* **25**, 7867-7874.

25. Maezawa, S., Yoshimura, T., Hong, K., Düzgünes, N. and Papahadjopoulos, D. (1989) *Biochemistry* **28**, 1422-1428.

26. Weinstein, J.N., Blumenthal, R., Sharrow, S.O. and Henkart, P.A. (1978) *Biochim. Biophys. Acta* **509**, 272-288.

27. Harmsen, M.C., Wilschut, J., Scherphof, G., Hulstaert, C. and Hoekstra, D. (1985) *Eur. J. Biochem.* **149**, 591-599.

28. Loyter, A. and Volsky, D.J. (1982) in *Membrane Reconstitution* (Poste, G. and Nicolson, G.L., Eds.), Elsevier: Amsterdam, 215-266.

29. Volsky, D.J., Gross, T., Sinangil, F., Kuszynski, C., Bartzatt, R., Dambaugh, T. and Kieff, E. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5926-5930.

30. Arad, G., Hershkovitz, M., Panet, A. and Loyter, A. (1986) *Biochim. Biophys. Acta* **859**, 88-94.

31. Gitman, A.G. and Loyter, A. (1984) *J. Biol. Chem.* **259**, 9813-9820.

32. Gitman, A.G., Graessmann, A. and Loyter, A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7309-7313.

33. Shapiro, I.M., Klein, G. and Volsky, D.J. (1981) *Biochim. Biophys. Acta* **676**, 19-24.

34. Volsky, D.J., Cabantchik, Z.I., Beigel, M. and Loyter, A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5440-5444.

35. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 17-49.

36. Katsura, I. (1983) in *Lambda II* (Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A., Eds.), Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 331-346.

37. Hohn, B., Wurtz, M., Klein, B., Lustig, A. and Hohn, T. (1974) *J. Supramol. Struct.* **2**, 302-317.

38. Enquist, L. and Sternberg, N. (1979) *Meth. Enzymol.* **68**, 281-298.

39. Roessner, C.A., Struck, D.K. and Ihler, G.M. (1983) *J. Biol. Chem.***258**, 643-648.

# Chapter 2
# Background and Design of Hydrophobic Photoaffinity Labeling Experiments

## DEFINITION

Photoaffinity labeling is the covalent labeling of a molecule in a defined receptor site by a probe localized to the receptor and activated by light (1). Photolysis of the probe generates a highly reactive species capable of forming covalent bonds with moieties in the receptor site.

The use of a photoaffinity label allows a high degree of control of the labeling reaction, by generating the reactive species at defined times during the course of a reaction, and under specific conditions such as pH and temperature. This approach is useful for many applications, including analysis of receptor sites and identification of ligands (2), and exposure of proteins to hydrophobic environments such as membranes (3,4).

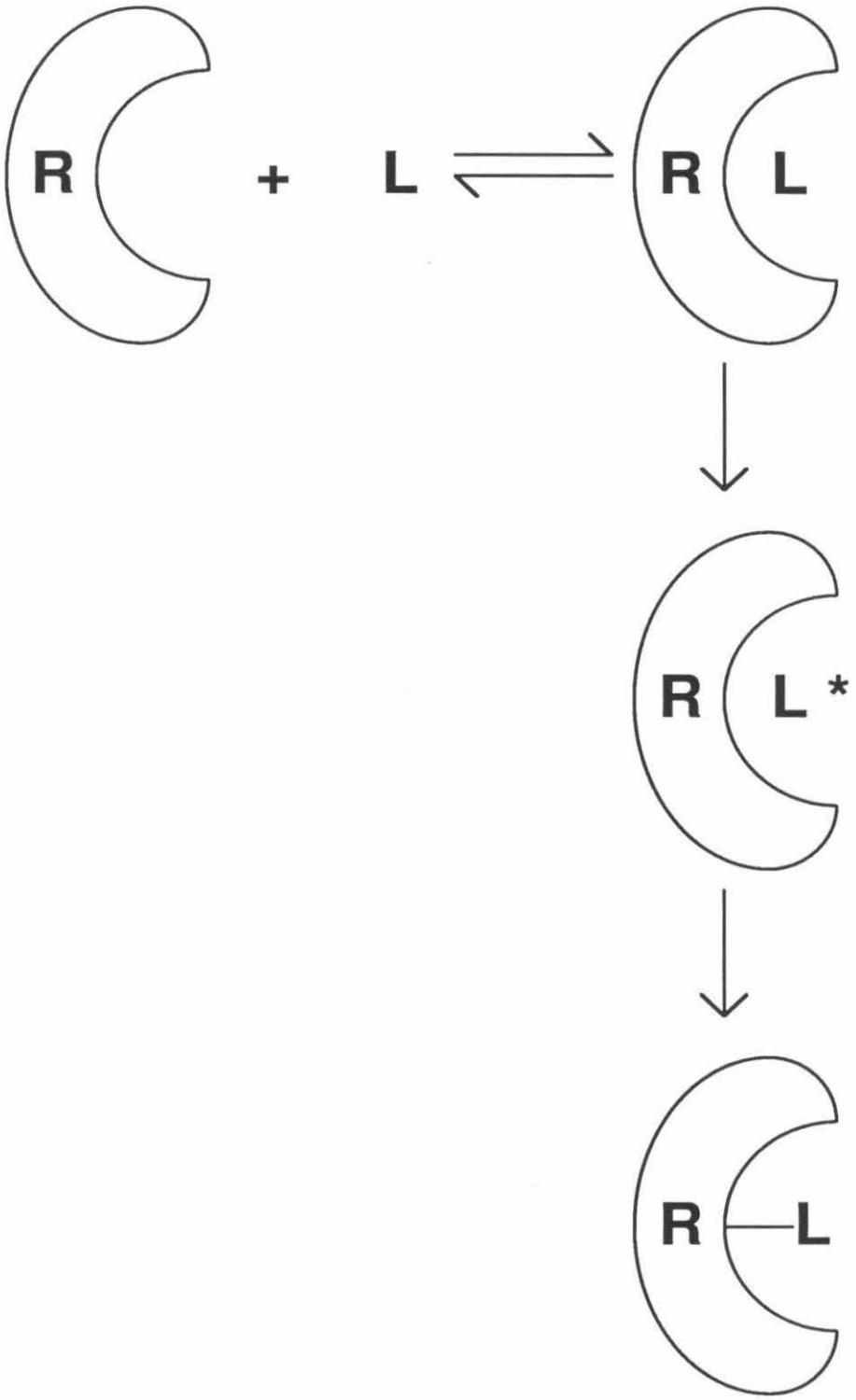
## PRACTICAL ASPECTS OF PHOTOAFFINITY LABELING

### A. Types of experiments

One of the common goals of photoaffinity labeling in biological systems is to identify intermolecular and ligand-receptor interactions. The approach is shown schematically in Figure 1. One of the components bears a photoreactive group. After allowing binding of both components, unbound material can be removed. The reactive group is then photolyzed, forming a highly reactive intermediate capable of making a covalent bond with the target molecule. The label usually also bears a spectroscopic or radioactive tag, for subsequent analysis of the label's location within the macromolecule. To examine intermolecular interactions of macromolecules, photoaffinity crosslinking reagents are often used. These molecules are composed of a group which is chemically reactive with, e.g., protein functional groups tethered to a photosensitive group for labeling a molecule interacting with the derivatized protein.

**Figure 1**

Scheme of a photoaffinity labeling experiment, using a photoreactive ligand analog to label the receptor. After reversible binding, the photoreactive group is activated, forming a covalent bond to the receptor.


R
+
L
R L
R L*
R L

The tether often contains a cleavage site for subsequent analysis of the crosslinked molecules.

In many instances the interaction of interest is localized, and can be described as a ligand-receptor interaction. In order to identify a receptor site of a known ligand, photoreactive ligand analogs are frequently employed ( cf. Figure 1). Conversely, ligands can be identified using photoreactive groups which are incorporated into the receptor site, using a derivatized group in the receptor site, e.g., a derivatized amino acid (5,6).

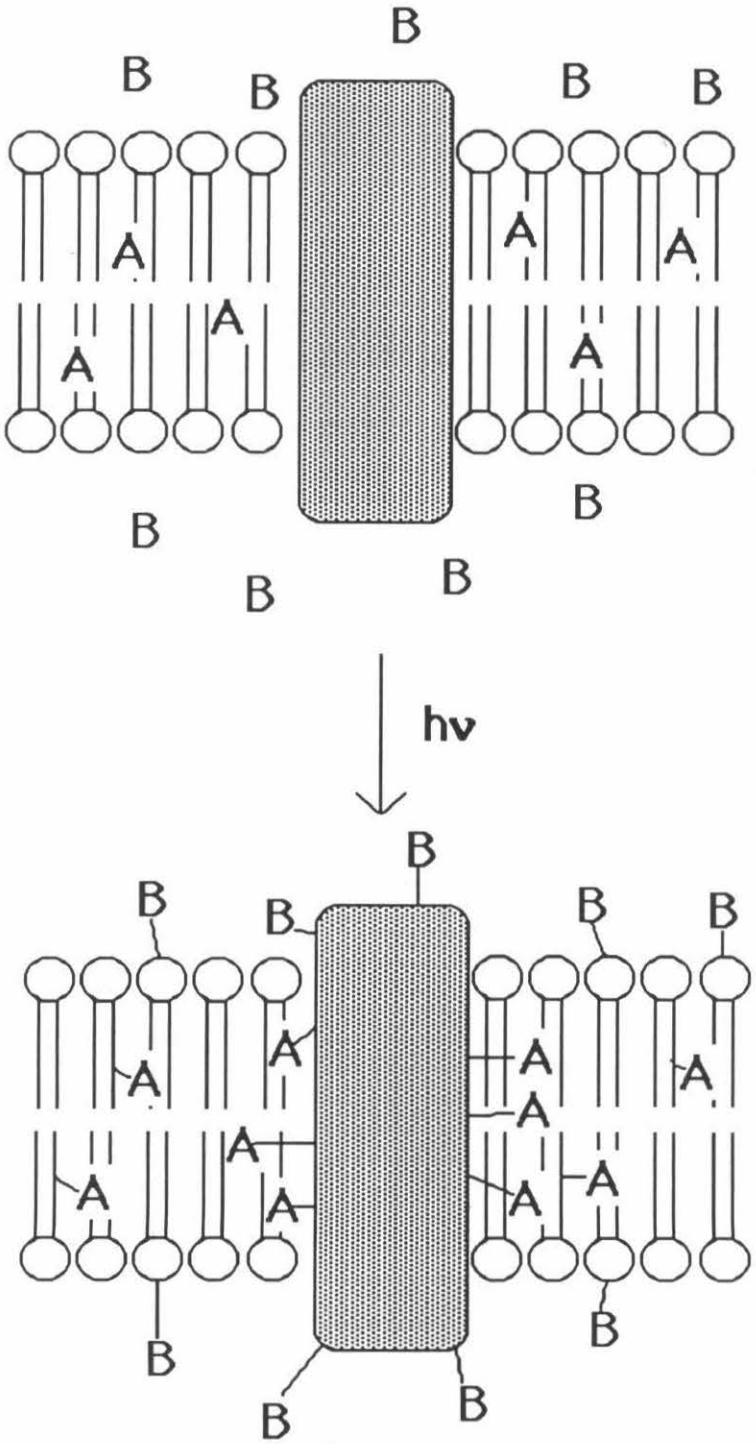
Both the transmembrane and aqueous segments of integral membrane proteins have been localized using photoaffinity labels which are soluble in the lipid or aqueous phase, respectively (3,4). Combined with spectroscopic data, photoaffinity labeling data are one of the major types of evidence for understanding protein membrane topology. The experimental scheme is shown in Figure 2. These probes are chosen for their high partition coefficients in either the lipid or aqueous phase, and for the high reactivity and low selectivity of their photoreactive groups (2).

### B. Reactive intermediates

Nitrenes and carbenes are short-lived reactive species which can be generated photochemically and satisfy the criteria above. Most applications of photoaffinity labeling have employed nitrene generating molecules, largely due to their ease of synthesis and handling (2). Aryl azides have been the probes of choice in these studies. Upon photolysis, the azide generates a nitrene, with loss of $N_2$. The nitrene's reactivity depends on the presence of reactive functional groups in its environment, its electronic state (singlet vs. triplet), and its tendency to undergo intramolecular rearrangement (2,7).

**Figure 2**

Use of photoaffinity labeling to determine transmembrane protein organization. Lipid-soluble (A) and water-soluble probes (B) can be used to covalently label protein domains which are embedded in the membrane and exposed at the membrane surface, respectively.


B
B
B
B
B
B
A
A
A
A
A
B
B
B
B
hν
B
B
B
B
B
A
A
A
A
A
A
A
A
B
B
B
B
B

Carbenes have properties which make them more desirable intermediates for photoaffinity labeling than nitrenes. Shorter lifetimes, higher reactivity and lower selectivity, coupled with good chemical and thermal stability, have led to the increasing use of carbene generating probes in recent studies (3,4,5,7). Many carbenes in use have lifetimes in the picosecond to nanosecond range, in contrast to the millisecond lifetimes which can be observed for aryl nitrenes (8). Short lifetimes are helpful in analyzing events on the molecular timescale.

In the study of interactions at the molecular level, high reactivity and low specificity are helpful in localizing parts of a molecule in receptor sites or phases containing the reactive intermediate. Amino acid specific probes are also useful for analysis of membrane topology, however. While some differences in the selectivities of carbenes have been reported (9,10), they are generally far more reactive and less selective than their nitrene counterparts (3,4,7,8,10).

Many carbene generators, including 3-trifluoromethyl-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID; Figure 3), generate singlet carbenes upon photolysis at or near room temperature in the absence of triplet sensitizers (10,11). For the present studies, whose goals include the labeling of protein functional groups in preference to an excess of phospholipid acyl chains (see following section), singlet carbene formation is an important advantage. The "zwitterion-like" singlet carbene kinetically prefers addition to multiple bonds and insertion into heteroatomic single bonds over formal triplet "radical-like" insertion into C-H bonds (10,11; see Figure 4). This preference in reactivity enables the labeling of minor membrane components.

Finally, the intramolecular rearrangement of the active species to form intermediates capable of side reactions competes with the desired labeling process. Many azides (used to generate nitrenes) rearrange to form imines, or cyclize and lead to ring expansion or other reactions (2,7). Diazirines, used as carbene generators, can

**Figure 3**

Structure of 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID), a hydrophobic photoaffinity label.

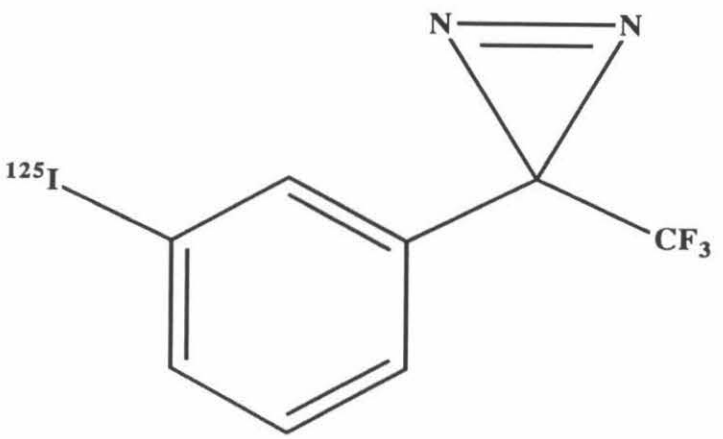
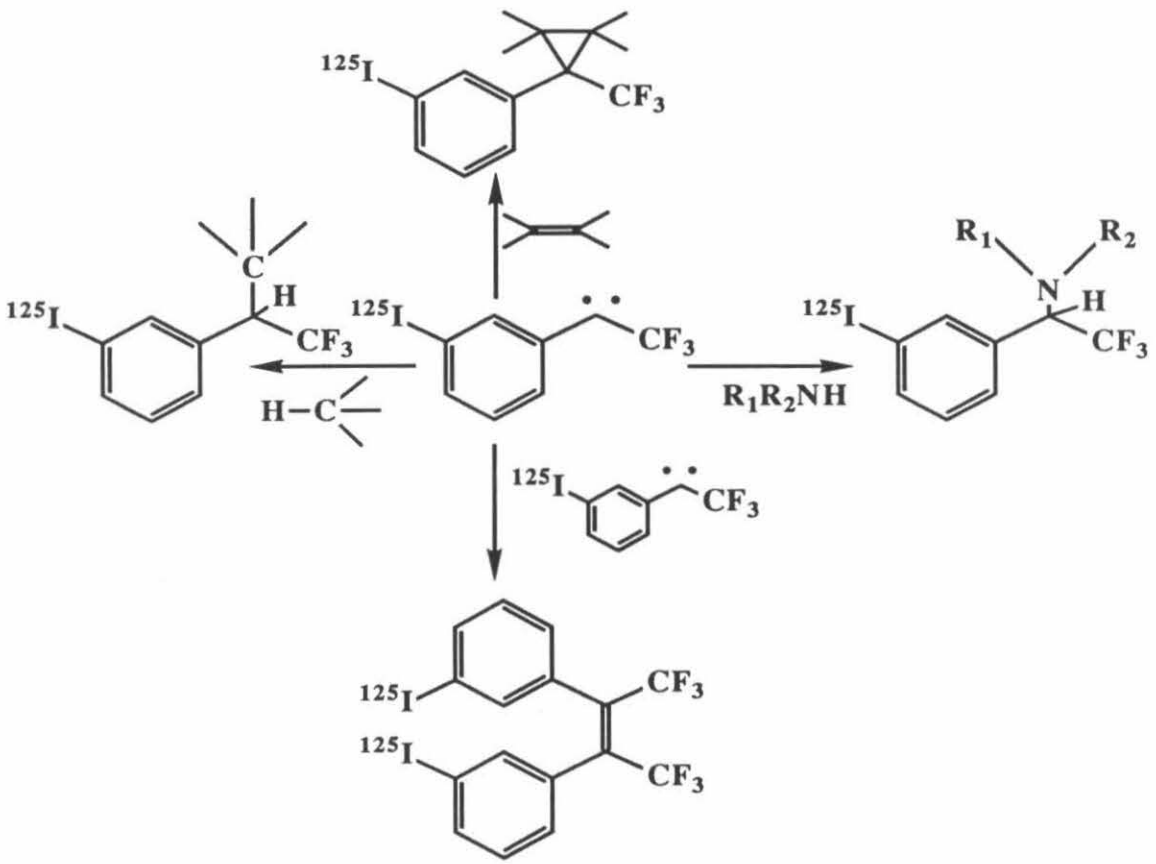

N
N
$^{125}$I
CF$_3$

**Figure 4**

Reactive pathways available to the carbene after TID photolysis. Near room temperature, the singlet carbene kinetically favors addition to multiple bonds (top) and insertion into heteroatomic single bonds (right) over formal triplet insertion into C-H bonds (left). An additional possible side reaction is probe dimerization (bottom).


125I
CF3
C
H
125I
CF3
125I
CF3
H–C
R1R2NH
R1
R2
N
H
125I
CF3
125I
CF3
125I
CF3

rearrange to form diazo compounds (Figure 5). This increases the time for carbene generation, but the reactivity is not altered, since upon photolysis the diazo group also generates the carbene (2,3,7). The presence of the 3-trifluoromethyl group in TID helps to minimize rearrangement and makes the rearranged compound less reactive (2,7,12). The aforementioned considerations make carbenes more favorable probes for the types of studies described in this thesis.

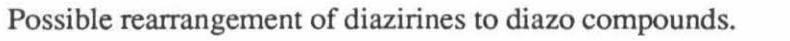
### C. Choice of probe for hydrophobic photoaffinity labeling

The criteria in the preceding description of diazirines and carbenes as photoaffinity probes led to the choice of TID (Figure 3) as the probe of choice. As a trifluoromethyldiazirine, TID is less likely to undergo intramolecular rearrangement than many other diazirines (7,8). In addition, the rearranged molecule is unreactive and does not substantially compete with the carbene reaction.

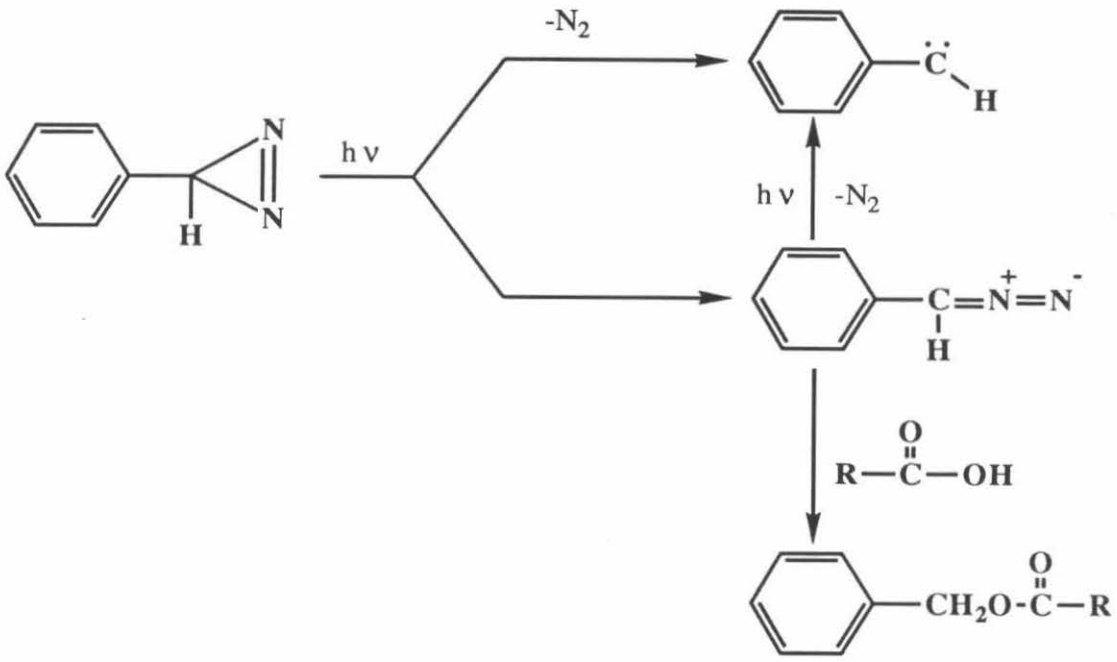
The TID is very chemically and thermally stable, and is commercially available. In addition, the fact that the carbene could be generated by photolysis at 353 nm meant that photolysis would not degrade biological molecules which absorb more strongly in the short wavelength ultraviolet region.

Labeling of molecules present in the hydrophobic bilayer core requires a probe with a high partition coefficient. This requirement was satisfied for TID, with very high partition coefficients in both natural and artificial membranes (12,15). Earlier studies showed that addition of aqueous scavengers to membranes containing TID did not affect the labeling of transmembrane segments of integral membrane proteins, and gave transmembrane information consistent with that obtained by other techniques (12-16).

**Figure 5**



Possible rearrangement of diazirines to diazo compounds.


-N2
hν
hν
-N2
R—C—OH
CH2O-C—R

## D. Experimental design for hydrophobic photoaffinity labeling

### i. Classical design: hydrophobic photoaffinity labeling at equilibrium

The dominant use of hydrophobic photoaffinity labeling has been the identification of transmembrane segments of integral membrane proteins and membrane penetrating segments of amphipathic peptides (3,4,7,12-17). It is important in these studies that both the photoaffinity label and the protein components be equilibrated in the membrane system prior to photolysis. Consequently, the experimental design reflects this need. In general, a lipid-soluble photoaffinity label (such as TID) is added to a membrane preparation and is allowed to equilibrate in the dark. Label present in the aqueous phase is removed prior to photolysis. This type of experiment has also proven useful in identifying the transmembrane organization of viral glycoproteins (18,19).

In some instances, this experimental approach has been applied to studies of the interaction of fusogenic proteins with "target" membranes. The hydrophobic segment of a viral fusion protein may interact with the bilayer core of a target membrane, initiating membrane fusion. If this is the case, the protein should be covalently labeled by a photoaffinity label present within the target membrane. Such experiments have suggested that the fusion of Sendai virus (20) and influenza virus (21-23) initiate fusion in this manner.

There is an important limitation on the interpretation of these experiments, however. According to the hypothesis of (viral) protein induced membrane fusion (see first section of this chapter), the hydrophobic protein-target membrane interaction occurs at the *beginning* of the fusion reaction. The experiments mentioned above (20-23) were carried out under conditions of equilibrium, i.e., *after* fusion proceeded for a

lengthy period of time, ranging from minutes to hours (Figure 6). By this time, most of the membrane components should have reorganized within the newly formed fusion product. This implies that these experiments identify protein-membrane interactions *after* fusion, and do *not* reflect the type of interactions which are involved in initiating membrane fusion.

In order to "catch" these early interactions leading to membrane fusion, it is necessary to carry out photolysis *before* the system reaches equilibrium. This experimental scheme is described in the following section.

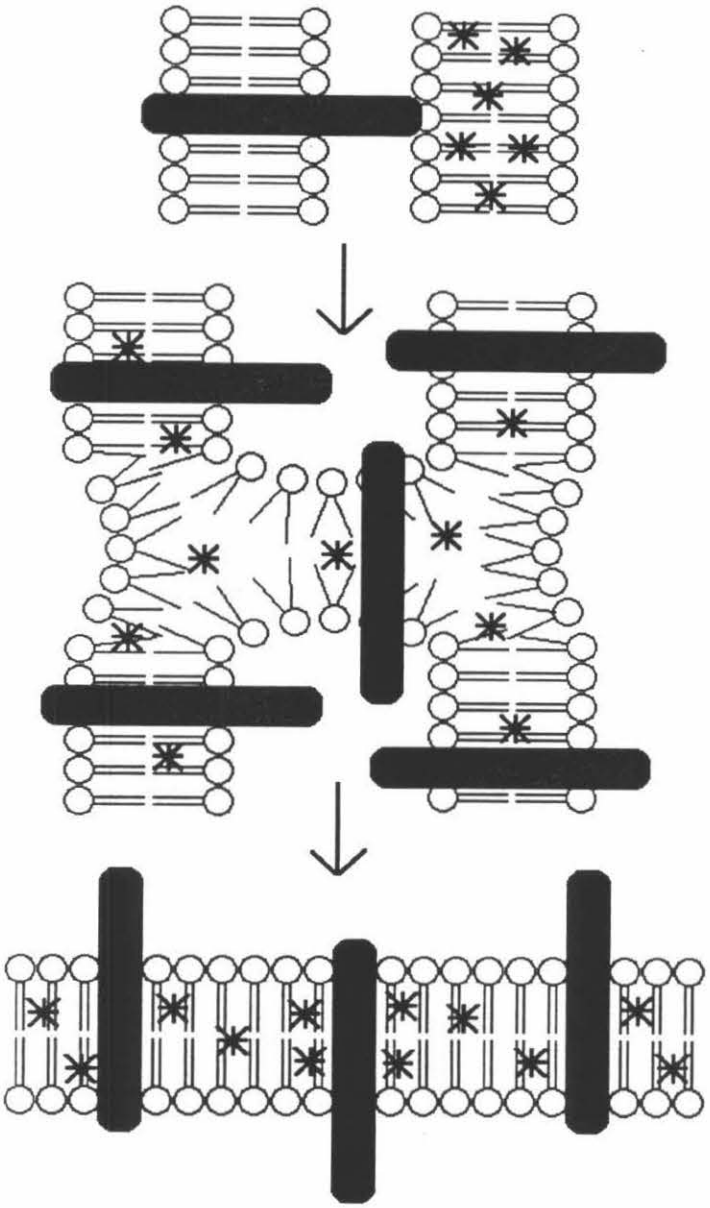
### ii. Design of time-resolved hydrophobic photoaffinity labeling experiments

Due to the inability of conventional hydrophobic photoaffinity labeling at equilibrium to resolve the protein-lipid interactions responsible for initiating membrane fusion (see preceding section), it was necessary to conduct the experiments in a time-dependent manner. Varying the reaction times prior to photolysis yields a series of "snapshots" of the hydrophobic protein-target membrane interactions taking place throughout the course of the fusion reaction (Figure 6). Briefly, virus is added to target membranes containing TID and allowed to react for various periods of time in the dark. After the appropriate reaction time, the sample is photolyzed, and the reaction is stopped. Labeling after short reaction times shows qualitative differences in the protein-lipid interactions occurring during initial and later stages in fusion. Subsequently, viral proteins are separated and analyzed for label incorporation. Details of the procedure are given in the following chapters.

Using this approach, it is possible to correlate hydrophobic protein-target membrane interactions with each step in the fusion process. This type of analysis is

**Figure 6**

Design of a conventional hydrophobic photoaffinity labeling experiment. Membranes with and without the label (*) are mixed and allowed to equilibrate. After equilibration, the sample is photolyzed. If the process under study is membrane fusion, initiating events will be completed before photolysis, and membrane components such as proteins (darkened) and lipids will have already reorganized.



necessary to a better understanding of the mechanism and physical parameters governing membrane fusion.

## REFERENCES


1. Singh, A., Thornton, E.R. and Westheimer, F.H. (1962) *J. Biol. Chem.* **237**, PC3006-PC3008.

2. Bayley, H. and Staros, J.V. (1984) in *Azides and Nitrenes: Reactivity and Utility* (Scriven, E.F.V., Ed.), Academic Press: Orlando, 433-490.

3. Richards, F.M. and Brunner, J. (1979) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Academy of Science: N.Y., 145-164.

4. Sigrist, H. and Zahler, P. (1985) in *The Enzymes of Biological Membranes* **10** (Martonosi, A.N., Ed.), Plenum: N.Y., 333-370.

5. Baldini, G., Martoglio, B., Schachenmann, A., Zugliani, C. and Brunner, J. (1988) *Biochemistry* **27**, 7951-7959.

6. Pollack, S.J., Nakayama, G.R. and Schultz, P.G. (1988) *Science* **242**, 1038-1040.

7. Bisson, R. and Montecucco, C. (1985) in *Progress in Protein-Lipid Interactions* **1**, 259-287.

8. Brunner, J., Senn, H. and Richards, F.M. (1980) *J. Biol. Chem.* **255**, 3313-3318.

9. Moss, R.A. (1980) *Acc. Chem. Res.* **13**, 58-64.

10. Lowry, T.H. and Richardson, K.S. (1987) *Mechanism and Theory in Organic Chemistry*, 3rd ed., Harper & Row: N.Y., 552-562.

11. Turro, N.J. (1978) *Modern Molecular Photochemistry*, Benjamin/Cummings: Menlo Park, CA, 551-557.

12. Brunner, J. and Semenza, G. (1981) *Biochemistry* **20**, 7174-7182.

13. Spiess, M., Brunner, J. and Semenza, G. (1982) *J. Biol. Chem.* **257**, 2370-2377.

14. Hoppe, J., Brunner, J. and Jørgensen, B.B. (1984) *Biochemistry* **23**, 5610-5616.

15. Brunner, J., Franzusoff, A.J., Lüscher, B., Zugliani, C. and Semenza, G. (1985) *Biochemistry* **24**, 5422-5430.

16. Meister, H., Bachofen, R., Semenza, G. and Brunner, J. (1985) *J. Biol. Chem.* **260**, 16326-16331.

17. Boggs, J.M., Rangaraj, G. and Koshy, K.M. (1988) *Biochim. Biophys. Acta* **937**, 1-9.

18. Moscufo, N., Gallina, A., Schiavo, G., Montecucco, C. and Tomasi, M. (1987) *J. Biol. Chem.* **262**, 11490-11496.

19. Zakowski, J.J. and Wagner, R.R. (1980) *J. Virol.* **36**, 93-102.

20. Asano, A. and Asano, K. (1984) *Tumor Res.* **19**, 1-20.

21. Boulay, F., Doms, R.W. and Helenius, A. (1987) in *Positive Strand RNA Viruses* (Brinton, M.A. and Rueckert, R.R., Eds.), Liss: N.Y., 103-112.

22. Harter, C., Bächi, T., Semenza, G. and Brunner, J. (1988) *Biochemistry* **27**, 1856-1864.

23. Harter, C., James, P., Bächi, T., Semenza, G. and Brunner, J. (1989) *J. Biol. Chem.* **264**, 6459-6464.

# Chapter 3
# Membrane Penetration of Sendai Virus Glycoproteins During the Early Stages of Fusion with Liposomes as Determined by Hydrophobic Photoaffinity Labeling

The text of this chapter was co-authored with Dick Hoekstra and has been published in the *Proceedings of the National Academy of Sciences of the United States of America*, Volume 85, pages 7433-7437, October 1988.

# Membrane Penetration of Sendai Virus Glycoproteins During the Early Stages of Fusion with Liposomes as Determined by Hydrophobic Photoaffinity Labeling (membrane fusion/ virus infection)

Steven L. Novick* and Dick Hoekstra†‡

*Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125; and †Laboratory of Physiological Chemistry, State University of Groningen, Bloemsingel 10, 9712KZ Groningen, The Netherlands

*Communicated by John D. Baldeschwieler, May 19, 1988*

Abbreviations: HN, hemagglutinin/neuraminidase; F protein, fusion protein; TID, 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine.

‡To whom reprint requests should be addressed.

## ABSTRACT

The hydrophobic photoaffinity label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine was used to label Sendai virus proteins during fusion with cardiolipin and phosphatidylserine liposomes. Preferential labeling of the viral fusion protein during the initial stages of fusion demonstrated that this protein interacts with the hydrophobic core of the target membrane as an initiating event of virus-liposome fusion. Labeling showed time, temperature, and pH dependence consistent with earlier fluorescent measurements of fusion kinetics. The present method provides conclusive evidence supporting the hypothesis that hydrophobic interaction of the fusion protein with the target bilayer is an initial event in the fusion mechanism of viral membranes.

## INTRODUCTION

The infectious entry of enveloped viruses is accomplished by a mechanism involving membrane fusion (1-3). Sendai virus, a paramyxovirus, enters host cells by fusion of the viral envelope with the cell's plasma membrane, mediated by the two Sendai envelope glycoproteins (1,3). The hemagglutinin/neuraminidase (HN) mediates viral attachment to sialic acid-containing cell surface receptors, while the fusion (F) protein, which consists of two disulfide-linked subunits, $F_1$ and $F_2$ (4), triggers the actual fusion reaction. It has been proposed that fusion is initiated as a result of the insertion of the hydrophobic $F_1$ $NH_2$ terminus, consisting of about 20 amino acids, into the target membrane (1,3,5,6).

Hydrophobic protein-lipid interactions (7-10) and some proteins that cause membrane fusion (11,12) have been investigated by using photoaffinity labels. Such studies typically involve labeling of both protein and lipid after an incubation period, allowing identification of the transmembrane segments of proteins or a distinction between subunits potentially interacting with membranes. Protein-induced fusion involves an initial local interaction between fusogen and apposed membranes, rapidly followed by randomization of membrane components in the lateral plane of the newly formed (i.e., fused) membrane. By focusing on the very early events at the onset of fusion, i.e., those prior to membrane randomization, the proteins penetrating the target membrane as fusion initiators can be selectively labeled. In the case of Sendai virus fusion, such an experiment would allow analysis of the hypothesis that fusion is initiated by insertion of the hydrophobic $F_1$ $NH_2$ terminus into the target membrane. In order to examine exclusively these initial interactions, photolabeling must be done for limited periods of time, i.e., while fusion is in progress, before the proteins have reoriented in the fused membrane. Obviously, this requires a detailed knowledge of the kinetics of the fusion reaction.

Although circumstantial evidence has been collected (1,3,5,13,14), no chemical evidence of the penetration of viral glycoproteins into target membranes during the initial moments of fusion has been reported thus far. The present study shows preferential labeling of the Sendai virus F protein at the initiation of the fusion reaction with negatively charged liposomes containing 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID). Although the fusion between a virus and a liposome may not resemble in every respect the fusion that occurs under biological conditions (14), it is our contention that this approach provides a unique opportunity to identify the fusion-initiating proteins and permits greater insight into the mechanisms of viral entry and membrane fusion.

## METHODS

**Virus.** Sendai virus (Z strain) was grown for 72 h in the allantoic compartment of ten-day-old fertilized chicken eggs. The virus was purified by differential ultracentrifugation and stored in 150 mM NaCl/5 mM Hepes, pH 7.4, at -70°C (15). Viral protein concentration was determined by the Peterson modification of the Lowry method (16).

**Liposomes.** Large unilamellar vesicles (sized through 0.1-μm polycarbonate membranes) were prepared from bovine heart cardiolipin or bovine brain phosphatidylserine (Avanti Polar Lipids) by the reverse-phase evaporation method (14). Lipid phosphorus concentration was determined by the modification of the Bartlett assay described by Böttcher et al. (17).

**Fusion and Photolabeling.** A 1-mCi/ml solution of TID (Amersham, 10 Ci/mmol, 90% radiochemical purity, 1 Ci = 37 GBq) in 10% ethanol (10 μl) was added to 200 nmol of liposomes of appropriate composition in 940 μl of buffer. The mixture was mixed on a Vortex and incubated for 1 h on ice in the dark. Additional details are

provided in *Results*. Sendai virus (80 μg) was added at the specified temperature and the mixture (final volume, 1 ml) was stirred continuously. The sample was irradiated for 30 sec by an Osram HBO 100W/2 super-pressure mercury lamp at 10 cm, with a Schott Glass Technology WG-360 high-pass cutoff filter (9). The reaction was stopped by immersing the sample in ice in the dark. The kinetics of fusion between Sendai virus and liposomes under the described conditions were monitored continuously by the $R_{18}$ (octadecylrhodamine B chloride) fusion assay, as described elsewhere (18).

**Analysis of Labeled Viral Proteins.** Proteins were precipitated by addition of 75 μl of cold 72% (wt/vol) trichloroacetic acid, resuspended in reducing electrophoresis sample buffer (5% sodium dodecyl sulfate/5% 2-mercaptoethanol/8 M urea/62.5 mM Tris•HCl, pH 6.8/0.01% bromophenol blue), and denatured and separated by PAGE (19). Protein bands were stained with 0.2% Coomassie brilliant blue R-250. Dried gels were autoradiographed at -70°C with Kodak XAR-5 film and a calcium tungstate intensifying screen. Scintillation counting was conducted after solubilizing 2- to 4-mm gel slices in 0.5 ml of Lumasolve (Lumac, Landgraaf, The Netherlands) and adding 10 ml of Hydrocount scintillation fluid (Baker) per sample.

## RESULTS

**TID Incorporation into Liposomes.** TID incorporation, assayed by liquid scintillation counting, was 80 ± 2% of added activity in all cases (data not shown) with the exception of addition to phosphatidylserine vesicles at pH 7.4. In that case only 59 ± 2% of added activity was incorporated, perhaps because the smaller inter-headgroup distances in phosphatidylserine compared to cardiolipin (20) hamper the ability of the probe to penetrate and insert into the hydrophobic core of the lipid bilayer (see ref. 14). In the course of these experiments, we noted that unbound probe

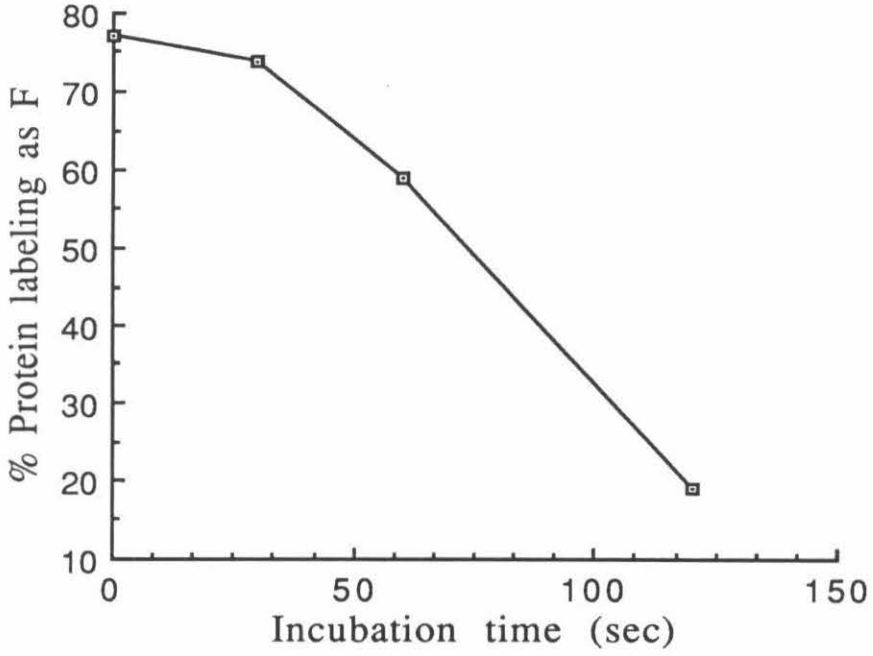
sticks very efficiently to the plastic Eppendorf tubes used, so that it was not necessary to routinely chromatograph each sample prior to use. However, the sticking of probe to the tubes may have contributed to the 20% loss in the other cases.

**Preferential Association of F Protein with Cardiolipin Vesicles.** Sendai virus fuses readily with negatively charged cardiolipin or phosphatidylserine vesicles (14). At neutral pH the fusion event is dependent on the F protein, as trypsinization of the virus, which removes specifically the F protein, inhibits fusion by about 80% (14). The hydrophobic interaction of the F protein with cardiolipin vesicles during early stages of fusion is preferential (Fig. 1). Nearly 80% of all labeling immediately after addition of virus is of the F protein. As the reaction continues, labeling of other proteins increases, with a concomitant decrease in F labeling, presumably due to later interactions of these proteins with the target membrane during membrane mixing and protein reorientation. Hence, the transmembrane parts of both the F and HN peptide chains will also become labeled. Typically, a protein labeling efficiency of 0.1-0.3% was obtained. Although the 30-sec photolysis was the minimum period necessary for sufficient labeling with the light source used, shorter photolysis periods can be used if the photon flux is increased (data not shown). Approximately 80% of the TID is photolyzed during the 30-sec period (9). The presence of larger amounts of TID in the membrane did not significantly increase the amount of labeling, indicating that the amount of probe is not limiting (ref. 9 and data not shown).

Carbenes formed by photolysis of diazirines are more reactive and less selective than nitrenes (8,21,22), but it appeared that protein was preferentially labeled over lipid. This gave the appearance of a "competition" among proteins for label, such that if fusion and F labeling were impeded, labeling of other viral proteins increased without any change in the magnitude of lipid labeling. This is explained by the preferential

## Figure 1

Preferential association of F protein with cardiolipin vesicles during initial membrane interactions. Sendai virus (80 μg of viral protein) was added to 200 nmol of cardiolipin vesicles, containing TID, in a total volume of 1 ml. The mixture was incubated at 37°C in 150 mM NaCl/5 mM Hepes, pH 7.4, with a magnetic stirrer. After various incubation times, the samples were photolyzed for 30 sec. Protein label incorporation was determined by liquid scintillation counting of 2-mm gel slices after SDS-PAGE. The labeling of F protein, as a percentage of total protein labeled, was calculated and plotted as a function of the incubation time before photolabeling.


80
70
60
50
40
30
20
10
% Protein labeling as F
0
50
100
150
Incubation time (sec)

addition of singlet carbenes formed upon diazirine photolysis to the commonly occurring double bonds and heteroatomic single bonds of the proteins, rather than insertion into the C-H bonds in the lipid core (21,22).

**F Protein Labeling During Fusion Between Sendai Virus and Cardiolipin Vesicles.** In addition to the time-dependent preference in F labeling relative to labeling of the other proteins, the incorporation of label into the F protein showed a time dependence with striking similarity to the kinetics of virus-cardiolipin vesicle fusion (14). When fusion was allowed to proceed at 37°C before photolabeling, the percentage of radioactivity in F decreased sharply (Fig. 2). This corresponds to a hydrophobic interaction of the F protein with the target membrane as an initiating event in protein-mediated membrane fusion, followed by randomization of viral and target membranes, causing the labeling of viral proteins other than the F protein.

**$F_1$ vs. $F_2$ Labeling.** The $F_1$ subunit's hydrophobic $NH_2$ terminus has been proposed to be the fusion-initiating peptide (5), whereas the $F_2$ subunit, located outside the viral membrane (23), does not participate in fusion. To ascertain the exclusive involvement of $F_1$ in viral fusion, TID labeling was conducted as described earlier. Proteins were separated by gel electrophoresis and analyzed for label incorporation. Label was concentrated in the $F_1$ subunit, with little or no detectable label showing up with the $F_2$ subunit (Fig. 3).

**Effect of Temperature.** Sendai virus fusion with both biological and artificial membranes is temperature-dependent (14,24). Similarly, F protein labeling showed a strong temperature dependence (Fig. 4), consistent with earlier reports that fusion at neutral pH is related to a temperature-dependent increase in rotational mobility of the F protein (24,25). The temperature dependence of F labeling corresponds closely to the initial rates of fusion (14). An interesting and perhaps significant observation is that under conditions in which fusion does not occur (2°C), substantial

**Figure 2**

Hydrophobic interaction of F protein with cardiolipin vesicles as a function of time. TID labeling was conducted during fusion of Sendai virus (80 μg of protein) and 200 nmol of cardiolipin vesicles at 37°C. Labeling of F protein as a percentage of total sample activity (open squares) was calculated and plotted as a function of time before photolabeling. Data are compared with the kinetics of fusion (% fluorescence) of Sendai virus and cardiolipin vesicles (solid circles) as determined by the $R_{18}$ lipid-mixing assay for fusion. The kinetics measurements were carried out in a parallel experiment under otherwise identical conditions as the photolabeling experiments.

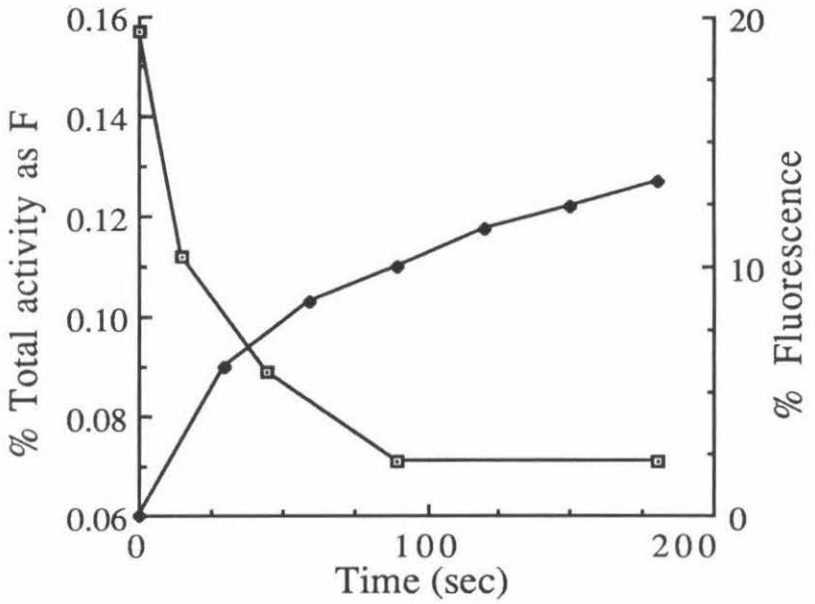

0.16
0.14
0.12
0.10
0.08
0.06
% Total activity as F
20
10
0
% Fluorescence
0
100
200
Time (sec)

**Figure 3**

$F_1$ vs. $F_2$ labeling. Sendai virus and cardiolipin vesicles were mixed under conditions as described in the legend to Figure 2. Photolysis was conducted as described in METHODS. Proteins were separated by PAGE and label incorporation was determined by $\gamma$ counting of gel slices.

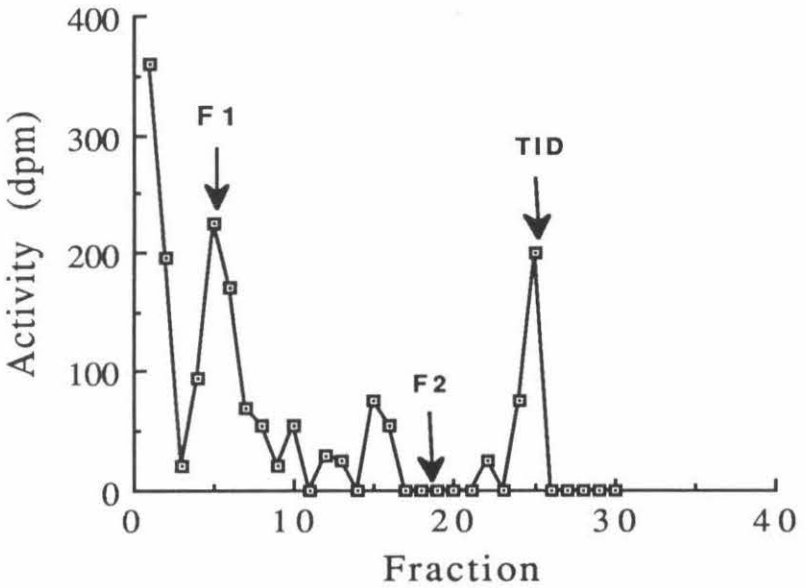
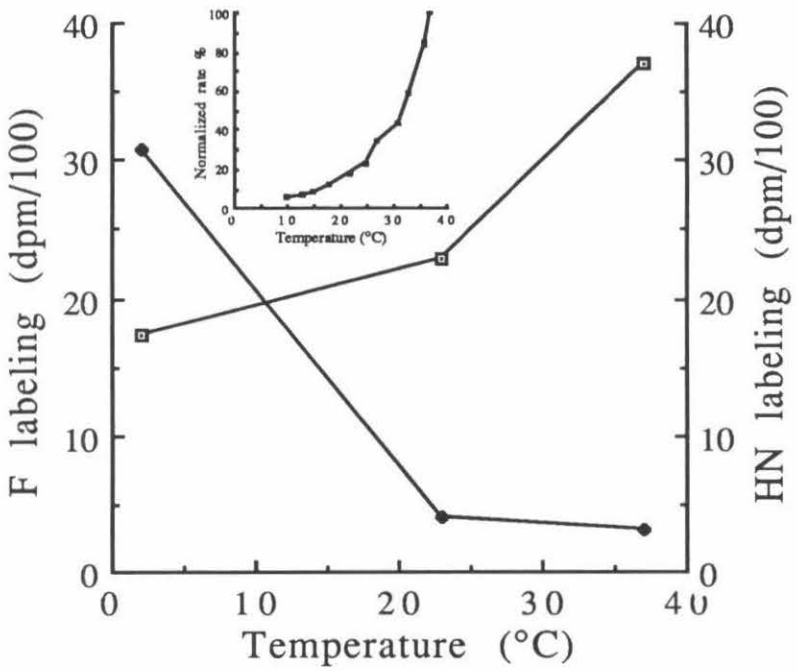

400
300
200
100
0
Activity (dpm)
F 1
F 2
TID
0
10
20
30
40
Fraction

**Figure 4**

Temperature dependence of F and HN labeling and initial rates of Sendai virus-cardiolipin vesicle fusion. TID labeling during fusion of Sendai virus (80 μg of protein) and 200 nmol of cardiolipin vesicles was conducted as described for Figure 2. Labeling of F (open squares) and HN (solid circles) were determined and plotted as a function of temperature. The temperature dependence of the initial rates of fusion is also shown (*Inset*). Initial fusion rates at various temperatures [$V_i(t)$] are normalized to the corresponding initial rate at 37°C [$V_i$(37°C)].


F labeling (dpm/100)
HN labeling (dpm/100)
Temperature (°C)
Normalized rate %
Temperature (°C)

labeling of the viral binding protein, HN, was observed (Fig. 4). This suggests that in addition to electrostatic interactions, hydrophobic interaction between HN and the target membrane may be important in viral attachment. HN labeling decreased with increasing temperature, as F protein-liposome hydrophobic interactions increased. The increase in F labeling and the concomitant increase in the initial fusion rate with temperature (Fig. 4 *Inset*) further support the view that hydrophobic interaction of F with the target membrane represents the ultimate trigger of viral fusion activity.

At present, we assume that after the initial penetration of the hydrophobic $F_1$ $NH_2$ terminus, subsequent randomization of viral and TID-labeled liposomal membranes during fusion causes redistribution of the label. This also causes TID to hydrophobically interact with viral proteins other than the membrane glycoproteins. These other proteins are not believed to play critical roles in membrane fusion, but chemical evidence has been found for close interaction of nucleocapsid and matrix proteins with the viral membrane glycoproteins (26).

**Effect of Lipid Composition of Target Vesicles and pH.** Labeling of F protein during fusion of Sendai virus with cardiolipin vesicles was consistently higher than that observed during fusion with phosphatidylserine vesicles (data not shown). This observation is entirely consistent with the higher kinetics and extent of virus fusion with cardiolipin vesicles (14). Labeling of both F and HN showed similar temperature dependence for both vesicle types, with F labeling increasing and HN labeling decreasing with increasing temperature, corresponding to the amount of fusion occurring (Fig. 4). The F/HN labeling ratios give some indication, then, of the type of interaction occurring, in terms of fusion vs. attachment. With both cardiolipin and phosphatidylserine vesicles, F/HN labeling ratios were similar, indicating the same relative amounts of hydrophobic interaction of F and HN in the early stages of fusion.

This is also suggestive of a common fusion mechanism, in spite of differences in the kinetics and extent of fusion (14).

In addition, F and HN labeling during fusion of Sendai virus with both vesicle types under various conditions of pH showed similar labeling ratios, further supporting a common fusion mechanism. The ratio of F/HN labeling at pH 7.4 was much higher than that seen at pH 5.0 for either vesicle type, in agreement with the earlier finding that fusion at low pH is mediated to a large extent by HN (14).

## DISCUSSION

The experiments in the present study demonstrate the use of a hydrophobic photoaffinity probe for covalent labeling of viral proteins that interact with the hydrophobic core of target membranes at the onset of membrane fusion. By limiting the time of irradiation and by commencing photolysis simultaneously with virus addition to liposomes, labeling during initial interactions can be isolated from that which might occur during subsequent events. The results provide strong, direct support for the hypothesis that the Sendai virus $F_1$ peptide mediates fusion at neutral pH by hydrophobic penetration into the target membrane (5,6).

Although this method is clearly useful as a probe of the fusion mechanism, practical considerations dictate that the results obtained from this type of experiment be treated carefully. Hydrophobic probes such as TID partition with great preference into the inner core of membranes (9) but may diffuse out and bind to hydrophobic domains of proteins in the aqueous phase (23). This limitation imposes restrictions on the interpretation of absolute amounts of label incorporated, but relative labeling patterns of different proteins under a given set of conditions yield a profile of protein-lipid interactions consistent with existing models. To eliminate the possibility of viral protein labeling due to diffusion of probe outside the bilayer, an experiment in the

presence of reducing agents such as glutathione and dithiothreitol would have been desirable (27). These agents inhibit labeling of the proteins by probe diffusion through the aqueous phase. Unfortunately, at the concentrations required, both compounds immediately inhibit the fusion activity of the virus (15). As a control, we therefore examined the extent of labeling of Sendai virus proteins upon incubation of the virus with "free" TID. The results showed a labeling pattern entirely different from that seen when the virus had interacted with membrane-inserted TID [in the former case, 28% of the label was associated with F, 14% with HN, and 57% (± 2%) with other viral proteins, independent of conditions]. Hence, in conjunction with the results in Fig. 1, showing an almost exclusive labeling of F under appropriate conditions, and those in Fig. 4, demonstrating a remarkably distinct labeling pattern as a function of temperature, we exclude the possibility that (at least during the early interactions), a significant contribution of the labeling occurred as a result of processes other than the penetration of viral proteins into the target membrane.

The present approach cannot provide insight into the depth of protein penetration into the target membrane (23). Such information could be obtained by using photoaffinity probes that are lipid-bound.

The strong preference of F labeling at early times during fusion (Fig. 1) provides conclusive evidence that the hydrophobic interaction between the F protein and the target membrane occurs prior to other interactions during fusion at neutral pH with cardiolipin vesicles and that this interaction constitutes the initiating event in fusion. This result thus provides direct chemical evidence supporting the hypothesis that viral fusion is initiated by hydrophobic interaction with the target membrane (5). The time dependence of F labeling during Sendai virus-cardiolipin vesicle fusion at neutral pH closely parallels the kinetics of fusion (Fig. 2 and ref. 14). This result lends further support for the occurrence of a hydrophobic penetration of F protein into the

target membrane as a key event in the triggering of viral fusion activity. Although the exact location of the probe in the F protein remains to be identified, the result that TID labels the $F_1$ polypeptide specifically (Fig. 3) is highly suggestive for labeling of the hydrophobic $NH_2$ terminus. Such hydrophobic sequences have been found in a variety of virus families, leading to the proposal that penetration of these peptides into the target membrane may represent the universal trigger of viral fusion (3,11). In fact, penetration of hydrophobic peptides into membranes at neutral or low pH may be a common theme in protein-induced fusion.

The temperature dependence of F labeling (Fig. 4) is also in agreement with requirements for increased protein rotational mobility during fusion at neutral pH (24,25) and mirrors the temperature-dependent initial rates of fusion (ref. 14 and Fig. 4 *Inset*). The strong temperature dependence of HN labeling at neutral pH (Fig. 4) indicates that hydrophobic interactions, in addition to electrostatic interactions, may play a role in viral attachment by HN. We cannot at this point rigorously exclude the possibility that HN labeling at low temperature might be due to the transfer of probe to bound virus. However, the lack of HN labeling at elevated temperatures makes this seem an unlikely possibility.

The extrapolation of mechanistic studies of fusion with liposomes to biological membranes must be done cautiously, since evidence suggesting that liposomal membranes may not be suitable models for physiological membrane fusion has been obtained (14). Liposomal models are useful, however, for creating well-defined membranes in which particular structural elements may be isolated for study. With both cardiolipin and phosphatidylserine vesicles it has been demonstrated that at neutral pH, fusion of Sendai virus with such vesicles is largely dependent on F protein. The almost exclusive labeling of F during initial fusion events with these vesicles is consistent with this notion.

Labeling during fusion of virus with cardiolipin vesicles was higher than that seen during fusion with phosphatidylserine vesicles, consistent with the higher extent and faster kinetics of fusion observed for cardiolipin (14). The very similar F/HN labeling ratios observed under various conditions of temperature and pH suggest a common fusion mechanism for both vesicle types. Furthermore, the higher HN labeling relative to F observed at low pH supports the hypothesis that HN mediates fusion at pH 5.0 by a low-pH-induced conformational change allowing hydrophobic interaction with the target membrane (14,28), and is consistent with the model for fusion mediated by water-soluble proteins at low pH (12,29,30).

These studies provide a means of obtaining direct chemical evidence leading to a structural and mechanistic understanding of the protein-lipid interactions that lead to membrane fusion. In addition to studies of fusion initiated by other viral and cellular proteins, continuing studies are underway to identify the fragments of fusion-initiating proteins which are labeled. Having set up the principle of the approach in a semi-artificial system, the following challenge is to evaluate this approach in a pure biological system. It is our contention that such studies will help in gaining an understanding of the molecular mechanism of viral fusion activity, as well as of peptide-lipid interactions in other biological systems that initiate fusion.

## ACKNOWLEDGMENT

We wish to thank Karin Klappe for expert technical assistance and to acknowledge the helpful suggestions of Dr. Peter Zahler (Laboratory of Biochemistry, University of Bern, Bern, Switzerland) during the early stages of this work and Drs. John D. Baldeschwieler and Robert A. Copeland for critical readings of the manuscript. This work was supported by U.S. Army Research Office Grant DAAG29-83-K-0128, National Institutes of Health Biomedical Research Support Grant RR07003

administered by the Division of Research Resources, and grants from Monsanto and Amersham. S.L.N. is the recipient of a National Science Foundation Predoctoral Research Fellowship.

## REFERENCES

1. Choppin, P.W. and Scheid, A. (1980) *Rev. Infect. Dis.* **2**, 40-61.

2. Loyter, A. and Volsky, D.J. (1982) *Cell Surf. Rev.* **8**, 215-266.

3. White, J., Kielian, M. and Helenius, A. (1983) *Q. Rev. Biophys.* **16**, 151-195.

4. Scheid, A. and Choppin, P.W. (1977) *Virology* **80**, 54-66.

5. Hsu, M.-C., Scheid, A. and Choppin, P.W. (1981) *J. Biol. Chem.* **256**, 3557-3563.

6. Asano, A. and Asano, K. (1984) *Tumor Res.* **19**, 1-20.

7. Brunner, J. (1981) *Trends Biochem. Sci.* **6**, 44-46.

8. Sigrist, H. and Zahler, P. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A.N., Ed.), Vol. 10, Plenum: N.Y., 333-370.

9. Brunner, J. and Semenza, G. (1982) *Biochemistry* **20**, 7174-7182.

10. Lecompte, M.-F., Krishnaswamy, S., Mann, K.G., Nesheim, M.E. and Gitler, C. (1987) *J. Biol. Chem.* **262**, 1935-1937.

11. Boulay, F., Doms, R.W. and Helenius, A. (1986) in *Positive Strand RNA Viruses* (Brinton, M.A. and Rueckert, R.R., Eds.), Liss: N.Y., 103-112.

12. Kim, J. and Kim, H. (1986) *Biochemistry* **25**, 7867-7874.

13. Eidelman, O., Schlegel, R., Tralka, T.S. and Blumenthal, R. (1984) *J. Biol. Chem.* **259**, 4622-4628.

14. Klappe, K., Wilschut, J., Nir, S. and Hoekstra, D. (1986) *Biochemistry* **25**, 8252-8260.

15. Hoekstra, D. and Klappe, K. (1986) *J. Virol.* **58**, 87-95.

16. Peterson, G.L. (1977) *Anal. Biochem.* **83**, 346-356.

17. Böttcher, C.J.F., Van Gent, C.M. and Fries, C. (1961) *Anal. Chim. Acta* **24**, 203-204.

18. Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) *Biochemistry* **23**, 5675-5681.

19. Laemmli, U.K. (1970) *Nature (London)* **227**, 680-685.

20. Boggs, J.M., Stollery, J.G. and Moscarello, M.A. (1980) *Biochemistry* **19**, 1226-1234.

21. Richards, F.M. and Brunner, J. (1980) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 144-164.

22. Turro, N.J. (1978) *Modern Molecular Photochemistry*, Benjamin/Cummings: Menlo Park, CA, 551-557.

23. Moscufo, N., Gallina, A., Schiavo, G., Montecucco, C. and Tomasi, M. (1987) *J. Biol. Chem.* **262**, 11490-11496.

24. Hoekstra, D., Klappe, K., De Boer, T. and Wilschut, J. (1985) *Biochemistry* **24**, 4739-4745.

25. Lee, P.M., Cherry, R.J. and Bächi, T. (1983) *Virology* **128**, 65-76.

26. Markwell, M.A.K. and Fox, C.F. (1980) *J. Virol.* **33**, 152-166.

27. Schroit, A.J., Madsen, J. and Ruoho, A. (1987) *Biochemistry* **26**, 1812-1819.

28. Doms, R.W., Helenius, A. and White, J. (1985) *J. Biol. Chem.* **260**, 2973-2981.

29. Arvinte, Hildenbrand, K., Wahl, P. and Nicolau, C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 962-966.

30. Hong, K., Yoshimura, T. and Papahadjopoulos, D. (1985) *FEBS Lett.* **191**, 17-23.

# Chapter 4
# Initiation of Fusion of Sendai Virus and Reconstituted Sendai Virus Envelopes with Liposomes by Viral Glycoproteins Determined by Time-Resolved Hydrophobic Photoaffinity Labeling

The text of this chapter was co-authored with John D. Baldeschwieler and Dick Hoekstra and is being submitted for publication.

# INITIATION OF FUSION OF SENDAI VIRUS AND RECONSTITUTED SENDAI VIRUS ENVELOPES WITH LIPOSOMES BY VIRAL GLYCOPROTEINS DETERMINED BY TIME-RESOLVED HYDROPHOBIC PHOTOAFFINITY LABELING†

Steven L. Novick‡,§, John D. Baldeschwieler‡, and Dick Hoekstra*,//,
Division of Chemistry and Chemical Engineering, Mail Code 127-72, California Institute of Technology, Pasadena, CA, 91125, and Laboratory of Physiological Chemistry, State University of Groningen, Bloemsingel 10, 9712KZ Groningen, The Netherlands

†This investigation was supported by ARO grant DAAG-29-83-K-0128, NIH Grants RR07003 and AI 25534, and grants from Monsanto and Amersham.

‡California Institute of Technology

//State University of Groningen.

§Recipient of an NSF Predoctoral Research Fellowship

Running title: Initiation of Viral Membrane Fusion

## ABSTRACT

Hydrophobic interactions between the glycoproteins of Sendai virus and target membranes during fusion are presented. Penetration of the proteins into liposomal membranes composed of cardiolipin (CL) and phosphatidylserine (PS) was monitored by covalent labeling with the photoreactive hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID). Hydrophobic interactions were monitored as a function of time, temperature, pH, and liposome composition. The viral fusion (F) protein was preferentially labeled at early time points during virus-liposome fusion at neutral pH, and this labeling closely parallels the kinetics of fusion as monitored independently with the octadecyl Rhodamine B ($R_{18}$) assay based on the relief of fluorescence self-quenching. This result implies that the F protein interacts with the target bilayer as an initial event in fusion, preceding interactions of the other viral proteins. The preference of F labeling was higher at 37°C than at 2°C, implying a requirement for conformational mobility of the protein for fusion activity. During fusion at pH 7.4, the $F_1$ subunit is almost exclusively labeled in the absence of $F_2$ labeling, implying that most likely the hydrophobic $F_1$ N-terminus is the actual fusogenic peptide. At pH 5.0, the hemagglutinin/neuraminidase (HN) was heavily labeled, reflecting the role of HN in fusion with artificial membranes, which is presumably due to exposure of hydrophobic residues by a low pH induced conformational change. At neutral pH, the HN protein is also heavily labeled in the absence of fusion at 2°C, suggesting that besides electrostatic interactions, viral attachment may involve hydrophobic interactions as well. Labeling during virus fusion with CL vesicles was higher than that seen during fusion with PS vesicles, consistent with the notion that membrane packing regulates peptide insertion into the lipid bilayer. Similar labeling patterns were observed during fusion of reconstituted Sendai virus envelopes (RSVE) with liposomes, indicating that the reconstituted

glycoproteins retain their capacity to mediate fusion with liposomes via hydrophobic interactions. By using a high photon flux for photolysis, it was revealed that the hydrophobic interaction of F with the target membranes actually precedes membrane fusion, which strongly supports its role as the actual trigger of viral fusion.

## INTRODUCTION

Enveloped viruses enter cells targeted for infection by fusion with either the cell's plasma membrane or with the endosomal membrane (1-3). In the case of Sendai virus, a paramyxovirus, this is accomplished by fusion of the viral envelope with the plasma membrane, at neutral pH. The overall process is mediated by two viral membrane glycoproteins, the hemagglutinin/neuraminidase (HN) and the fusion protein (F) (1,3,4). Virus attachment to cellular sialic acid containing receptors is HN dependent, and the F protein is responsible for the fusion event itself. The inactive F protein precursor, $F_0$, is cleaved post-translationally by a host cell enzyme to yield two disulfide-linked subunits, $F_1$ and $F_2$ (5). As a result, the $F_1$ hydrophobic N-terminus is unmasked. This hydrophobic peptide, about twenty amino acids in length, is believed to trigger the fusion reaction by hydrophobic interaction with the target membrane (1,3,6).

Hydrophobic photoaffinity labeling has proved a valuable method in identifying the membrane-spanning regions of proteins (7,8). Labeling is usually conducted with hydrophobic lipid soluble probes (9-11) or probes covalently linked to the acyl chains of phospholipids (12-17). As a further step in identifying the molecules involved in membrane fusion, some investigators have used hydrophobic photolabeling to covalently label potentially fusogenic proteins which are believed to interact with the hydrophobic core of membranes (15,16,18-20).

In order to elucidate protein-lipid interactions leading to fusion in a mechanistic manner, we have carried out time-dependent photolabeling of Sendai virus proteins during fusion with liposomes. The approach involves labeling proteins interacting hydrophobically with the target membrane containing the photoreactive probe during initial events in the fusion process. By labeling only during early interactions in fusion, subsequent protein-lipid interactions taking place during reorganization of the newly fused membrane are excluded.

We report the preferential time-dependent labeling of the F protein during initial stages of fusion, thus providing evidence that directly supports the hypothesis that the F protein triggers membrane fusion by hydrophobic interaction with the target membrane. We also show that the hydrophobic penetration of the F protein, which we believe initiates fusion, occurs prior to the fusion event. These results are discussed in the context of a mechanistic analysis of membrane fusion. In addition, we show that functional reconstitution of the virus involves retention of the ability of the glycoproteins to undergo hydrophobic interactions with target membranes. A report establishing the method of time-resolved photoaffinity labeling has been published (21).

## METHODS

**Reagents.** The photoaffinity label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID, specific activity 10 Ci/mmol, 95% radiochemical purity) was obtained from Amersham. Bovine heart cardiolipin (CL) and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids, Birmingham, AL. β-D-Octylglucopyranoside was purchased from Calbiochem, and Spectrapor-2 dialysis

tubing was obtained from Spectrum Medical Industries (Los Angeles, CA). Octadecyl Rhodamine B chloride was purchased from Molecular Probes (Eugene, OR).

**Virus.** Sendai virus (Z strain) was grown in the allantoic cavity of ten-day-old embryonated chicken eggs. Virus was harvested 72 h after infection, and purified as described previously (22,23). Afterwards, virus was suspended in 150 mM NaCl/ 5 mM Hepes buffer, pH 7.4, and stored at -70°C. Concentrations of viral proteins were determined by the Peterson modification of the Lowry method (24).

**Liposomes.** Large unilamellar vesicles (LUVs) were prepared by the reverse-phase evaporation method (25). Liposomes were sized to an average diameter of 0.1 μm by extrusion through polycarbonate membranes (Nucleopore, Pleasanton, CA) in a Lipex Biomembranes extruder (Vancouver; 26). Lipid phosphorus concentrations were determined by the Böttcher modification of the Bartlett assay (27).

**Reconstituted Sendai virus envelopes (RSVE).** Sendai virus envelopes were reconstituted by octylglucoside solubilization and dialysis (28). Briefly, virus was washed with NaCl/Hepes buffer (pH 7.4) and centrifuged at 100,000 x *g* for 30 min at 4°C. Viral membranes were solubilized in NaCl/Hepes buffer containing 50 mM octylglucoside by shaking for 1 h at room temperature, giving a turbid suspension of nucleocapsid particles. Insoluble nucleocapsids were pelleted by centrifuging at 100,000 x *g* for 1 h at 4°C. Octylglucoside was removed from the supernatant by dialyzing in a three-step procedure. The sample was dialyzed against two 250 ml volumes of NaCl/Hepes buffer for 1 h at room temperature, followed by dialysis against 500 ml of buffer for 12.5 h. Finally, the solution was dialyzed against a 2.5 l volume of buffer for 18 h at 4°C, with three buffer changes. Protein concentration in the resulting RSVE was determined by the Peterson modification of the Lowry method (24).

**Fusion and photolabeling.** Prior to use, the TID solution supplied by Amersham was diluted to 1 mCi/ml with NaCl/Hepes buffer, pH 7.4, containing 10% ethanol, and was stored at 4°C. Ten ml of this solution were added to 200 nmol LUV composed of CL or PS in 940 μl buffer. The solution was vortexed and incubated for 1 h on ice in the dark. Unincorporated TID was removed from the aqueous phase by adsorption to the plastic Eppendorf tubes used (15,21). TID incorporation was approximately 80% of the added activity for CL vesicles and about 60% in the case of PS vesicles at pH 7.4.

Sendai virus in NaCl/Hepes buffer (1.56 mg protein/ml), preincubated under conditions identical to the liposomes, was added to give a mixture containing 80 μg protein and 200 nmol liposomes in a total volume of 1 ml. The sample was stirred in a cuvette by a magnetic stirrer for varying periods of time, and was then photolyzed by one of two procedures. The sample was irradiated for 30 s by an Osram HBO 100W/2 super-pressure mercury lamp at 10 cm, with a Schott Glass Technology (Duryea, PA) WG-360 high-pass cutoff filter to prevent photoisomerization of the probe (9), as well as absorption of short wavelength light by protein and lipid, which could lead to denaturation.

Alternatively, samples were irradiated for 5 s by an Oriel (Stratford, CT) Model 6140 1000 W xenon arc lamp, with a 10 cm circulating cold water filter and the same high-pass filter, which was placed in a water bath. The samples were thermostatted in a homemade cell, composed of an aluminum plate holder for infrared spectroscopy (Wilks, Foxboro, MA) modified with welded copper tubing for heat exchange, and connected to a thermostatted circulating water bath. The reaction was stopped by immersing the sample in ice, in the dark. In some cases, the reaction was stopped prior to photolysis by two-fold dilution in prechilled 1:1 ethylene glycol/water, and the mixture was caused to undergo a glass transition in a quartz EPR tube (Wilmad, Buena,

NJ) in liquid nitrogen. The sample was then photolyzed at 77 K in a liquid nitrogen dewar with a quartz window.

Fusion kinetics were independently monitored by labeling virus with $R_{18}$, as described elsewhere (29). Fusion was initiated by addition of liposomes to labeled virus in NaCl/Hepes buffer under the appropriate conditions. Measurements were made with an SLM 4800 spectrofluorometer, with excitation and emission wavelengths of 560 and 590 nm, respectively. The sample chamber was thermostatted and equipped with a magnetic stirrer. Fluorescence was calibrated to the background fluorescence of labeled virus as 0%, and 1% Triton X-100 was added to correspond to 100% (infinite dilution of the probe), after correction for sample dilution.

**Analysis of labeled proteins.** Protein was precipitated by addition of 75 µl cold 72% trichloroacetic acid. Samples were vortexed and incubated for 1 h at 0°C, then centrifuged for 15 min at 12,000 x *g* at 4°C. Supernatants were carefully decanted, and precipitates were resuspended in reducing electrophoresis sample buffer (5% sodium dodecyl sulfate/ 5% β-mercaptoethanol/ 8 M urea/ 62.5 mM Tris-HCl, pH 6.8/ 0.01% bromophenol blue), and were then denatured and separated by polyacrylamide gel electrophoresis (PAGE; 30). Proteins were visualized with 0.2% Coomassie Brilliant Blue R-250 in 25% methanol/ 10% acetic acid. Samples were run in duplicate and analyzed for label incorporation by gel drying and autoradiography, or by slicing and counting. Dried gels were autoradiographed at -70°C with Kodak XAR-5 film and a Dupont Cronex intensifying screen. Counting of 2 mm gel slices was conducted with a Beckman Biogamma II gamma counter.

## RESULTS

**Preferential association of F protein with liposomes.** Sendai virus fusion with CL or PS vesicles occurs at neutral pH in an F protein dependent event (29). We have shown previously that the F protein preferentially interacts with the hydrophobic core of target membranes, during a time course consistent with the kinetics of fusion (21). Hydrophobic photolabeling with TID upon 30 s photolysis demonstrated that the hydrophobic interaction occurs at the onset of fusion. The fusion reaction was monitored independently using the octadecyl Rhodamine B chloride ($R_{18}$) assay, based on the relief of fluorescence self-quenching (29). The preliminary results showed that the ratios of F/HN labeling provide a mechanistic profile of hydrophobic interaction of these proteins with target liposomal membranes consistent with the existing hypothesis.

At pH 7.4, labeling of the Sendai virus F protein is preferential at early time points during the course of the fusion reaction (Figure 1). At zero time before photolysis, almost 80% of the total (viral) protein-associated radioactivity comigrates with F upon analysis of total viral proteins by gel electrophoresis (Figure 2). This preferential labeling indicates that hydrophobic interaction of F with the target membrane occurs prior to potential hydrophobic interactions of the other viral proteins with the target membrane, and supports the notion that this event triggers membrane fusion. The labeling drops to half its maximum value in approximately 60 s. Elsewhere, we have shown (29) that Sendai virus fuses avidly with negatively charged vesicles at mild acidic pH, which is in contrast to its assumed physiological behavior. The work revealed that at least part of the fusion reaction at low pH is mediated by HN. Indeed, as shown in Figure 1, at pH 5.0 the F protein labeling is less (about 25%) than that seen at pH 7.4 (initial time point), in spite of the fact that the number of virus

**Figure 1**

Time and pH dependence of F protein labeling during Sendai virus-CL vesicle fusion. Sendai virus (80 μg protein) was added to 200 nmol CL vesicles containing 10 μCi TID, in NaCl/Hepes buffer at 37°C, pH 7.4 (solid circles), or in 140 mM NaCl/10 mM sodium acetate/5 mM Hepes buffer, pH 5.0 (open circles). Fusion was initiated by injection of virus into the stirred cuvette, and was allowed to proceed for various periods of time before photolysis with the Osram HBO 100W/2 lamp. The reaction was stopped by immersing the sample in ice, in the dark. Incorporation of radiolabel in the F protein was assayed by radioactive counting, and was calculated as the percentage of sample activity.

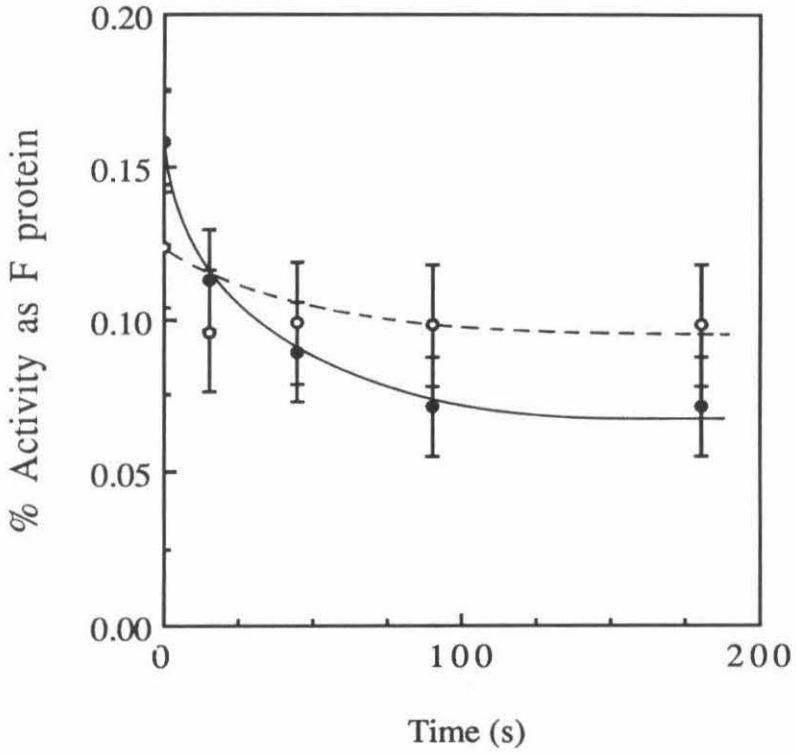
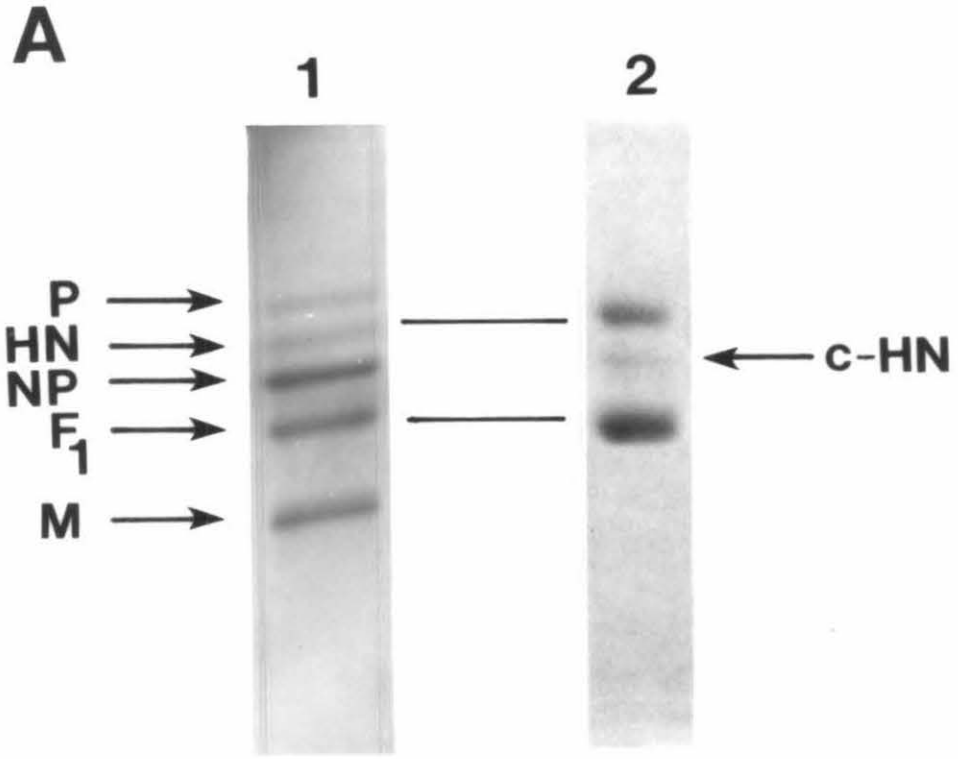
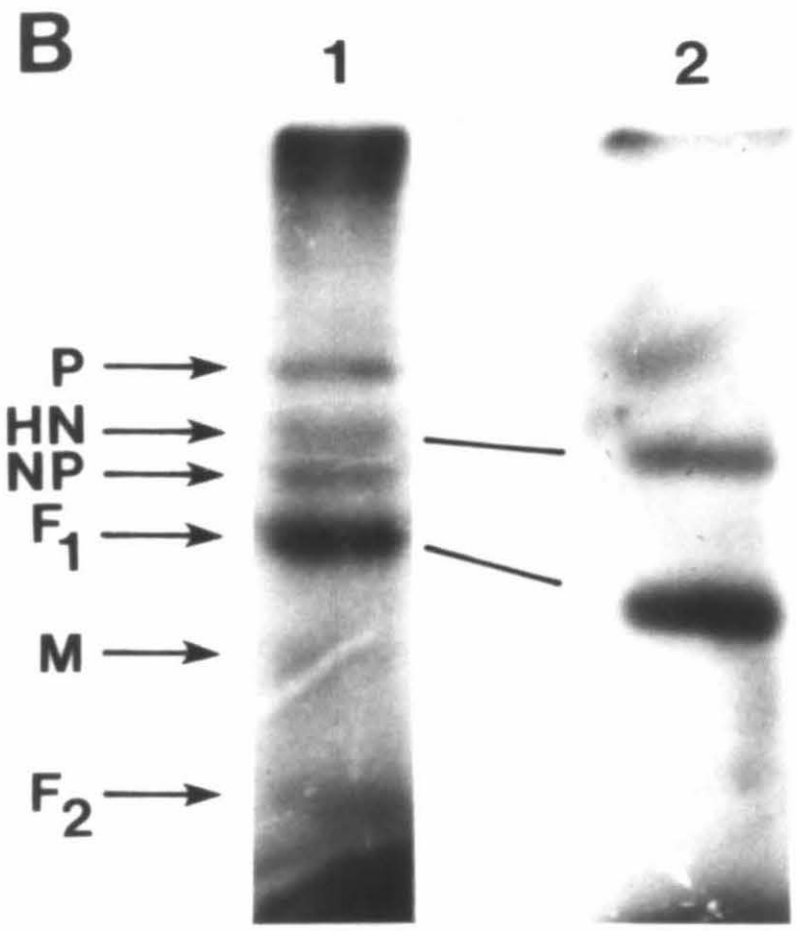

0.20
0.15
0.10
0.05
0.00
% Activity as F protein
0
100
200
Time (s)

**Figure 2**

TID labeling of Sendai virus and RSVE glycoproteins during fusion with liposomes. Following fusion and photolysis (total time interval 30 s at 37°C), viral proteins were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue R-250 or dried and autoradiographed. (*A*) Migration of both intact viral proteins (lane 1) and reconstituted viral glycoproteins (lane 2) stained with Coomassie Brilliant Blue R-250. (*B*) Autoradiogram of labeled proteins after TID labeling during fusion of intact virus (lane 1) and reconstituted viral envelopes (lane 2) with CL vesicles at pH 7.4. In lane 1, faint labeling of the $F_2$ protein can be seen, although this protein was not visible in the Coomassie stained gel. Radioactivity at the top and bottom of the gel corresponds to labeled lipid and free TID, respectively.


A
1
2
P
HN
NP
F1
M
c-HN


B
1
2
P
HN
NP
F1
M
F2

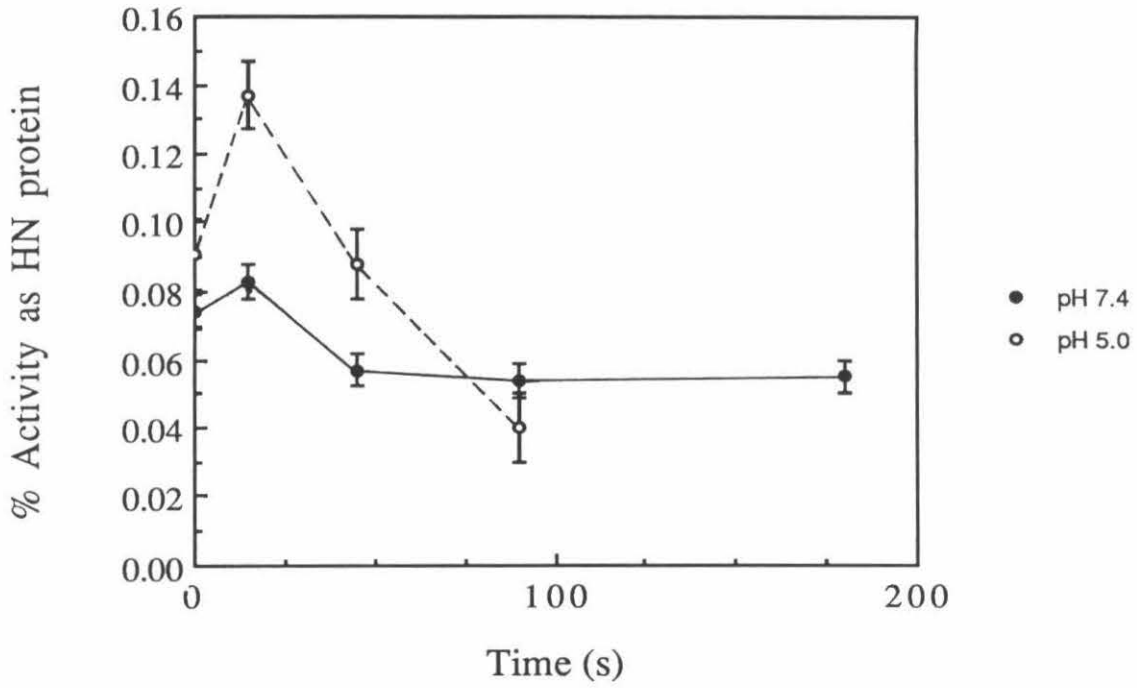
particles fusing at low pH is about 1.5-fold higher. The higher kinetics and extent of fusion observed at low pH may well explain the higher F labeling at longer times at pH 5.0, relative to that at pH 7.4.

The labeling of HN protein during virus-liposome fusion shows a high degree of time-dependence at pH 5.0, but very little time dependence at pH 7.4 (Figure 3). This is also consistent with fusion at low pH mediated by HN. As discussed previously (29), the HN dependent fusion event presumably occurs via a low pH-induced conformational change, exposing hydrophobic segments for interaction with the target membrane, after protonation of acidic amino acids. This mechanism would bear some analogy to that of the influenza virus hemagglutinin (HA; 31). An interesting result can be seen in the rise in HN labeling after 15 s fusion. This increase can be explained by the preferential reaction of the singlet carbene formed upon diazirine photolysis with protein double bonds and heteroatomic single bonds, rather than C-H bond insertion (10,32), and the resultant competition among proteins for photoactivated probe. At the earlier time points, when F labeling is high, less of the activated probe is available for HN labeling. As the membranes are pulled together, the F/HN ratio in contact with the probe-containing membrane decreases, and HN labeling becomes predominant. Even at pH 5.0, the early interaction of a small amount of F with the target membrane dominates the labeling. This suggests that the fusogenic portion of the F peptide is initially in contact with the target membrane to a greater extent than the hydrophobic segments of the HN protein which largely mediate fusion at low pH. This distinction could also be due to differences in the extent of protein penetration which, in turn, would affect the probability of probe-protein interaction.

Under routine labeling conditions (0-37°C), TID photolysis leads to preferential formation of the singlet carbene, but at low temperature (77K) triplet carbenes and nitrenes can be preferentially generated from diazirines and azides (33,34). In

**Figure 3**

Time and pH dependence of HN protein labeling during Sendai virus-CL vesicle fusion. Fusion and photolabeling were conducted as described in the legend to Figure 1. Incorporation of label in HN was measured by counting of radioactivity, and was calculated as the percentage of sample activity.


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
% Activity as HN protein
0
100
200
Time (s)
pH 7.4
pH 5.0

experiments in which we conducted photolysis at 77K, labeling of protein relative to lipid diminished greatly (not shown). This is also consistent with the sequestering of the TID within the target liposomal bilayer, and the lack of diffusion into the aqueous phase. As a further control to predict the labeling of hydrophobic protein pockets due to TID diffusion out of the liposomes which might occur (15), free TID was added to virus particles and photolyzed. Labeling of the viral proteins was altered substantially from that seen during fusion, and was independent of conditions (Table I). This indicates that probe diffusion does not significantly contribute to the labeling observed during fusion. Typical carbene scavenger controls for aqueous probe diffusion using glutathione or dithiothreitol (17) were not possible in this system, as these reagents specifically inactivate the F protein (23). The lack of significant probe diffusion in the present study is also consistent with earlier hydrophobic photolabeling studies using TID (10), and the nitrene precursor [$^{125}$I]-5-iodonaphthalene-1-azide (11). Aqueous probe diffusion must be examined in this type of experiment, since some hydrophobic labels have been shown not to reside entirely within the bilayer core (35).

The preference for protein labeling over lipid labeling allowed us to examine the protein hydrophobic interactions with the target membrane during fusion by measuring the ratio of F to HN labeling. We further examined the fusion reaction in this context.

**Temperature dependence of labeling during Sendai virus-liposome fusion.** The fusion of Sendai virus with both biological membranes and negatively charged liposomes is a temperature dependent process (29,36). Since fusion depends on hydrophobic interactions, we examined these interactions using hydrophobic photolabeling as a function of temperature, pH, and lipid composition of the target membranes.

The F/HN labeling ratios were compared at a temperature at which fusion is at a maximum (37°C) and at a temperature at which fusion is inhibited (2°C). The F/HN

**Table 1**

TID labeling of viral proteins by diffusion through the aqueous phase.

**TID Labeling of Viral Proteins By Diffusion Through the Aqueous Phase**[a]

| Light source | pH | | | | | |
|---|---|---|---|---|---|---|
| | 7.4 | | | 5.0 | | |
| | F | HN | Other | F | HN | Other |
| 100 W | 38 | 11 | 51 | 34 | 10 | 56 |
| 1000 W | 32 | 13 | 54 | 34 | 11 | 55 |

[a] % of protein labeling (± 2%)

labeling ratios observed during virus fusion with both CL and PS vesicles at neutral pH showed a large increase at 37°C, relative to that seen at 2°C (Table II). This is consistent with earlier observations of the temperature dependence of fusion (29), and supports the role of a hydrophobic interaction of the F protein during fusion as the temperature dependent determining event. The F/HN ratios seen at either temperature were similar during virus fusion with both CL and PS vesicles. This suggests a common fusion mechanism with both vesicle types in terms of protein-lipid interactions, in spite of the higher kinetics and extent of fusion with CL vesicles relative to those with PS, as measured by the $R_{18}$ assay for lipid mixing (29). While the temperature dependent labeling trends are consistent within a given set of experiments, the range of F/HN ratios attainable seems to vary with parameters such as virus passage number and length of time between thawing and using frozen virus. The maximum and minimum F/HN ratios we observed at 37°C and 2°C were as high as 12.5 and as low as 0.5, respectively. In spite of these differences, however, the observed labeling within given sets of experiments consistently reflects the model of membrane fusion discussed.

**pH dependence of labeling during Sendai virus-liposome fusion.** Although fusion of Sendai virus at neutral pH is mediated by the F protein, the HN protein largely mediates fusion at pH 5.0. After trypsinization and F protein inactivation, the HN protein sustains the fusion reaction at low pH (29). When TID labeling is conducted at pH 5.0, the F/HN labeling ratio drops substantially relative to that at pH 7.4 (Table II), consistent with the role of HN in fusion at low pH. The decrease in the F/HN labeling ratio at pH 5.0 was similar during virus fusion with either vesicle type, also supporting a common fusion mechanism at low pH. Although F/HN ratios were similar during fusion with both vesicle types, the labeling of each protein was consistently higher by approximately 20% during fusion with CL vesicles

**Table 2**

Effect of pH, temperature, and target membrane composition on TID labeling of viral glycoproteins during Sendai virus fusion with CL and PS vesicles.

TID labeling of viral glycoproteins during fusion of Sendai virus with liposomes[a]

| | Cardiolipin | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 7.4 | | | pH 5.0 | | | % change[b] | | |
| | F | HN | F/HN | F | HN | F/HN | F | HN | F/HN |
| 37°C | .157 | .074 | 2.12 | .124 | .091 | 1.36 | -21 | +23 | -36 |
| 2°C | .129 | .090 | 1.44 | ND[c] | ND | | | | |
| % change[d] | +22 | -18 | +47 | | | | | | |

| | Phosphatidylserine | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 7.4 | | | pH 5.0 | | | % change[b] | | |
| | F | HN | F/HN | F | HN | F/HN | F | HN | F/HN |
| 37°C | .120 | .055 | 2.18 | .111 | .088 | 1.26 | -8 | +60 | -42 |
| 2°C | .104 | .101 | 1.04 | ND | ND | | | | |
| % change[d] | +15 | -84 | +110 | | | | | | |

[a] % of total sample activity (± .022).

[b] The percent change in labeling at pH 5.0 relative to that observed at pH 7.4.

[c] ND, not determined.

[d] The percent change in labeling at 37°C relative to that observed at 2°C.

than during fusion with PS vesicles, reflecting the higher extent of fusion occurring in the former case.

**Temperature dependence of labeling during RSVE-liposome fusion.** The fusion of RSVE, as well as intact virus, is dependent on the F protein (28). In order to determine whether the same types of hydrophobic interactions are operative during RSVE fusion, TID labeling during RSVE-liposome fusion was conducted under otherwise identical conditions as those in the experiments with intact virus. At pH 7.4, the ratio of F/HN labeling increased substantially going from 2°C to 37°C during RSVE fusion with both CL and PS vesicles (Table III; Figure 2), corresponding to the occurrence of F mediated fusion. At pH 5.0, the F/HN labeling ratio decreased dramatically at higher temperature during RSVE- CL vesicle fusion, due to a large amount of HN initiation of the fusion reaction. These results demonstrate that the hydrophobic interactions responsible for viral fusion are also operative in fusion of the reconstituted membrane.

In contrast, labeling during RSVE- PS vesicle fusion at pH 5.0 was not responsive to temperature, as both F and HN labeling were very small at 37°C, with the F/HN ratio remaining unchanged. This may reflect the absence of specific hydrophobic interactions of either glycoprotein in the RSVE with PS vesicles at pH 5.0, due to the lesser ability of PS vesicles to undergo fusion relative to CL vesicles as a result of greater membrane packing density.

**pH dependence of labeling during RSVE-liposome fusion.** At 37°C, the F/HN labeling ratio was substantially higher at pH 7.4 than at pH 5.0, reflecting the role of the F protein in initiating fusion at neutral pH. As expected, no change in F/HN ratios was observed at 2°C, in the absence of fusion. While the relative importance of hydrophobic interactions in RSVE-liposome fusion is similar to that observed in the fusion of intact virus with liposomes, it is apparent that differences

**Table 3**

Effect of pH, temperature, and target membrane composition on labeling of glycoproteins during RSVE-liposome fusion.

**TID labeling of glycoproteins during fusion of RSVE with liposomes[a]**

Cardiolipin

| | pH 7.4 | | | pH 5.0 | | | % change[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | F | HN | F/HN | F | HN | F/HN | F | HN | F/HN |
| 37°C | .360 | .227 | 1.58 | .140 | .455 | 0.31 | -61 | +100 | -80 |
| 2°C | .147 | .231 | 0.64 | .368 | .208 | 1.77 | +150 | -10 | +176 |
| % change[c] | +145 | -2 | +147 | -62 | +119 | -82 | | | |

Phosphatidylserine

| | pH 7.4 | | | pH 5.0 | | | % change[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | F | HN | F/HN | F | HN | F/HN | F | HN | F/HN |
| 37°C | .169 | .088 | 1.91 | .046 | .040 | 1.15 | -73 | -54 | -40 |
| 2°C | .150 | .126 | 1.19 | .152 | .130 | 1.17 | +1 | +3 | -2 |
| % change[c] | +13 | -30 | +60 | -70 | -69 | -2 | | | |

[a] % of total sample activity (± .022).

[b] The percent change in labeling at pH 5.0 relative to that observed at pH 7.4.

[c] The percent change in labeling at 37°C relative to that observed at 2°C.

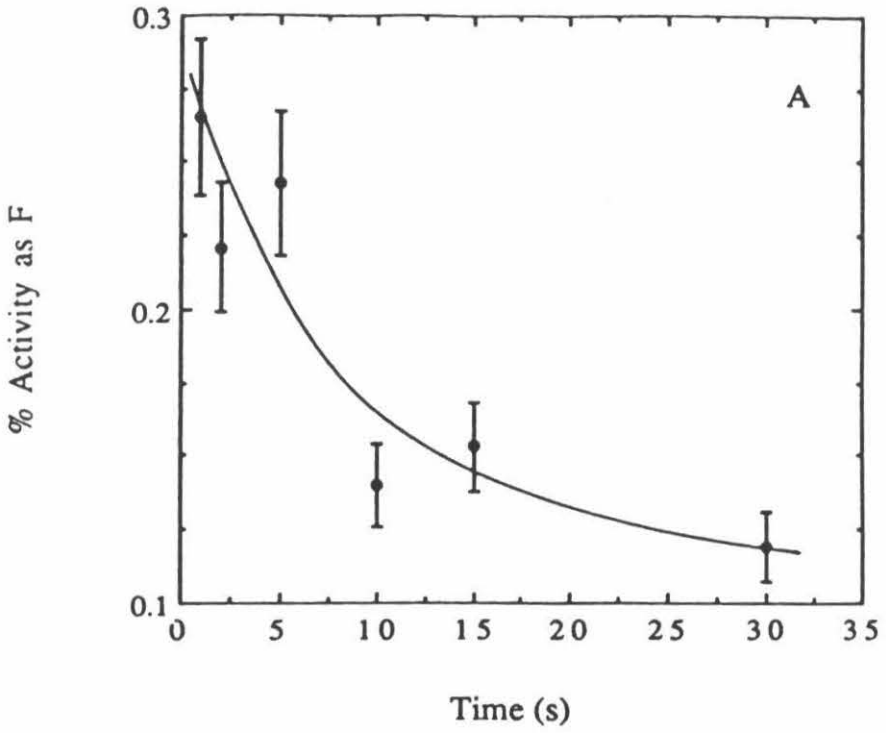
in the reconstituted and intact viral membranes contribute to differences in the function (and TID labeling) of the membrane glycoproteins.

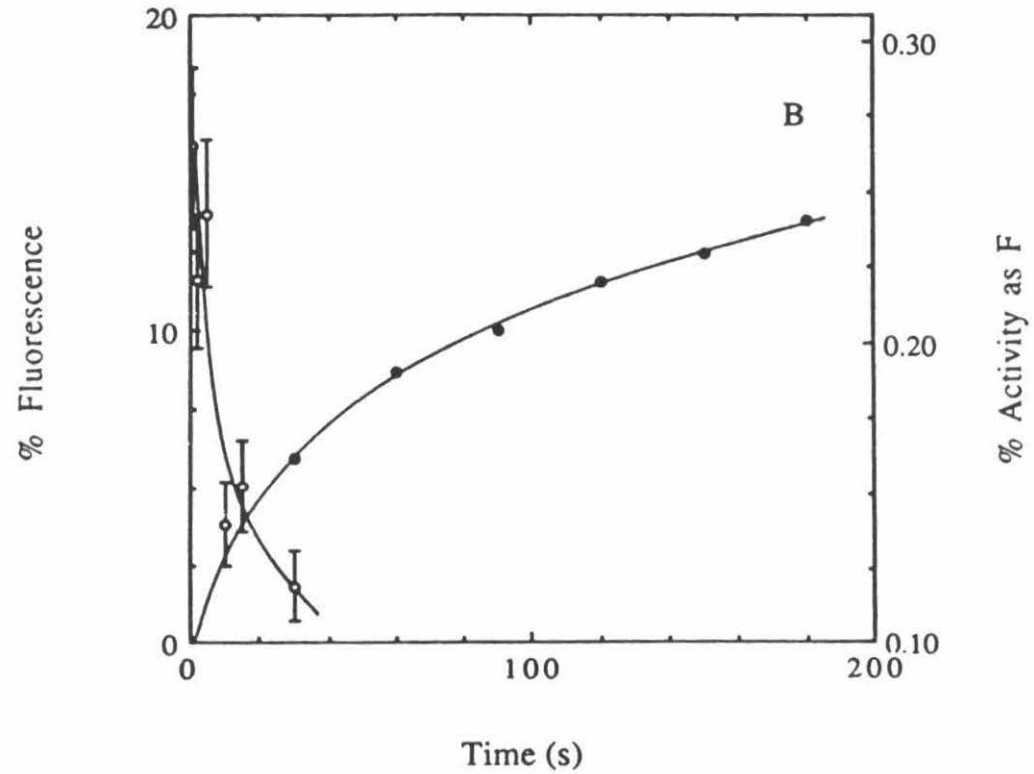
**Time dependence of F labeling with higher photon flux during photolysis.** Although the time dependence of preferential F protein labeling has been observed during virus-liposome fusion (21), the 30 s photolysis period limits the resolution with which kinetics can be examined. The correlation of F labeling with the kinetics of fusion showed the involvement of hydrophobic interaction between the F protein and the target membrane in the fusion event. The limited time resolution in the earlier studies also limited interpretation of the sequence of events just prior to and/or at the initiation of fusion. In order to examine the role of this hydrophobic interaction as a fusion trigger, labeling was conducted for shorter photolysis times (5 s) using a 1000 W light source.

Under these conditions, a strong time dependence of F labeling was also observed (Figure 4A). The magnitude of labeling decreases to half its maximal value within 10 s, nearly an order of magnitude faster than that seen using a longer photolysis period and lower photon flux (21). When compared to the kinetics of fusion between Sendai virus and liposomes as monitored with the $R_{18}$ assay (Figure 4B), the hydrophobic interaction of the F protein with the target membrane appears to precede the fusion event. This result strongly supports the notion that this interaction is the fusion trigger. In addition to the time dependence observed under these conditions, the F/HN labeling ratios showed the same trends as those seen with longer photolysis (Novick, S. L., Baldeschwieler, J. D., & Hoekstra, D., unpublished observation), also consistent with early F labeling prior to membrane mixing and subsequent labeling of other proteins.

**Figure 4**

Time dependence of F protein labeling during Sendai virus-CL vesicle fusion with high photon flux. (*A*) Fusion and photolabeling were conducted as described in METHODS using a 1000 W Xe arc lamp and a 5 s photolysis period. Samples were mixed for various times prior to photolysis (data points). F protein labeling was determined by gamma counting and was calculated as the percentage of sample activity. (*B*) Fusion kinetics were independently monitored (solid circles) under otherwise identical conditions using the $R_{18}$ lipid mixing assay, as described in METHODS. The time dependence of F protein labeling is superimposed (open circles) for comparison.


A
% Activity as F
0.3
0.2
0.1
0
5
10
15
20
25
30
35
Time (s)


B
% Fluorescence
% Activity as F
20
10
0
0.30
0.20
0.10
0
100
200
Time (s)

## DISCUSSION

The experiments described in this paper demonstrate the use of time dependent photolabeling within target liposomal membranes to examine hydrophobic protein-lipid interactions during fusion of Sendai virus with negatively charged liposomes. The results strongly support the hypothesis that the F protein triggers viral fusion via hydrophobic interaction with the target membrane.

The preferential labeling of F protein shows a strong time dependence at neutral pH (Figure 1). This provides chemical evidence that penetration of the F protein into the target membrane is an early event during fusion. Because of the high degree of conservation of hydrophobic residues in peptides associated with fusion among a wide variety of viruses, this type of initiation of fusion was proposed as the general mechanism of virus fusion (1,3).

The F protein labeling at pH 7.4 drops to half its maximal value in approximately 60 s, using the 100 W light source for photolysis. This preferential early labeling of the F protein is on a timescale consistent with the kinetics of fusion as assayed by lipid mixing (21,29). At pH 5.0, a somewhat lesser time dependence was observed, although the amounts of protein labeling were comparable in magnitude (Figure 1). The smaller early labeling of F at pH 5.0 is probably due to the hydrophobic interaction of HN with the target membrane, as it appears capable of mediating the low pH fusion event (Figure 3). This involvement of HN in fusion at low pH is consistent with the finding that fusion at pH 5.0 is not abolished by trypsinization of the virus, which specifically inactivates the F protein, prior to labeling (29). The greatly enhanced labeling of HN at pH 5.0 relative to that at pH 7.4 (Figure 3) demonstrates the high degree of hydrophobic interaction between HN and target membranes at low pH. Presumably, this type of fusion is induced by exposure of

hydrophobic regions in the HN protein at low pH, as a result of protonation of acidic amino acids in certain regions of the polypeptide chain (cf. ref. 29). The present results demonstrate that in this manner HN is converted into an "ordinary" fusion protein which displays this property by hydrophobic insertion into the core of a membrane. Penetration of hydrophobic peptides into membranes, at either neutral or acidic pH, may represent a common fusion trigger, as this behavior is also observed at low pH during fusion of liposomes induced by water soluble proteins such as α-lactalbumin (20), lysozyme (37), and clathrin (38).

Demonstration that labeling is due to a true protein contact with the hydrophobic core of the target liposomes is necessary when using a diffusible probe. Due to the inactivation of the F protein by carbene scavengers (23), the role of diffusional labeling was excluded by analyzing the labeling pattern observed upon addition of free TID to virus. Under these conditions, no preference in labeling is observed, regardless of sample or photolysis conditions (Table I). This is in stark contrast to the strong time, temperature, and pH dependence of both F and HN labeling during fusion, and rules out that any substantial amount of labeling is due to diffusion of TID out of the target bilayer. The very low amount of labeling attributable to diffusion during photolabeling using hydrophobic probes with high partition coefficients has been previously reported in studies employing TID (10) and [$^{125}$I]-5-iodonaphthalene-1-azide (11).

The preferential labeling of protein over lipid in the target membrane is bestowed by the zwitterionic nature of the singlet carbene (10,32), and allows examination of the relative amounts of interaction of both F and HN with the target membrane. The F/HN labeling ratio shows a strong temperature dependence during virus-liposome fusion (Table II), and is much higher at 37°C than at 2°C. This is consistent with the temperature dependence of the initial rates of fusion measured with the $R_{18}$ assay for lipid mixing (29). The temperature dependence of the fusion reaction

has been related to the rotational mobility of the F protein (36,39), and may reflect the protein's ability to undergo conformational changes. The ratios of F/HN labeling are similar during fusion with either CL or PS vesicles, indicating similar relative amounts of hydrophobic interaction of the proteins with the target bilayer, and a common mechanism for virus fusion with both liposome types. The range of F/HN values during fusion can sometimes be larger than those observed in the present study (not shown). This may be due to differences in virus activity with passage number (and differences in protein structures), or time between thawing and using frozen virus. In spite of these occasional differences, the temperature and pH dependent trends in labeling F and HN during virus-liposome fusion remain consistent.

At pH 5.0, the ratio of F/HN labeling is considerably lower than that observed at pH 7.4 (Table II), due to the involvement of HN in the fusion reaction at low pH. The drop in the F/HN ratio at low pH is mostly due to an increase in HN penetration into the target membrane, rather than to a decrease in F penetration. The ratios are again similar among both lipid types, consistent with common fusion mechanisms at both neutral and acidic pH.

Hydrophobic interactions of F and HN with target membranes are also important in initiating RSVE-CL vesicle fusion. At neutral pH, the high F/HN ratio at 37°C is consistent with the notion that F also mediates fusion of reconstituted viral membranes (Table III). At pH 5.0, the high amount of HN labeling at 37°C reflects the ability of HN to mediate the fusion reaction at low pH. This ability is consistent with the role of HN in fusion of intact virus with liposomes (Table II). The involvement of hydrophobic interactions of reconstituted F and HN with target membranes implies that RSVE fusion is initiated by the same mechanism as that observed for intact virus. During fusion with PS vesicles at pH 7.4, a similar increase in F/HN labeling was observed, although the temperature dependent increase in F labeling was not as

pronounced as that during RSVE fusion with CL vesicles (Table III). In contrast, RSVE-PS vesicle fusion at pH 5.0 was less sensitive to F and HN interactions. This may indicate a lack of specific hydrophobic interactions during RSVE-PS vesicle fusion at low pH, or simply a poor ability of the reconstituted viral proteins to interact hydrophobically with the more tightly packed PS membrane under these conditions.

The finding that hydrophobic interactions of F with target membranes occurs at neutral pH during both virus and RSVE fusion is consistent with the requirement of a functional F protein for reconstitution of viral fusogenic activity during RSVE fusion with erythrocyte membranes (28,40). The similar roles of F and HN in both virus and RSVE fusion suggest that functional reconstitution involves retention of the proteins' abilities to interact hydrophobically with target membranes. The differences observed between virus and RSVE labeling probably reflect differences in reconstituted membrane and protein structure, compared with the native virus. These differences may be due to residual detergent in the reconstituted membrane, inability of F and HN to insert and aggregate properly during dialysis, and the lack of interactions between the glycoproteins and the viral matrix and nucleocapsid proteins removed during reconstitution (28). Chemical crosslinking studies have shown close association between membrane and nucleocapsid proteins in the native virus (41), and these interactions may serve to stabilize the membrane protein structure and function.

The correlation of hydrophobic protein-lipid interaction with the kinetics of membrane fusion established the presence of such interactions in fusion (21). In order to gain greater insight into the time course of the protein-lipid interactions, photolysis was conducted for a shorter period (5 s) with a very intense light source (Figure 4A). The preferential time dependent labeling of the F protein shows that the interaction of F with the target bilayer core *precedes* the fusion event, as assayed by lipid mixing (Figure 4B). This sequence of early events during virus-membrane interaction can be

readily rationalized when the following is taken into account. The hydrophobic N-terminus is located at the tip of the $F_1$ polypeptide and possibly protrudes about 100 Å beyond the viral surface (16). As such, one may anticipate that this hydrophobic region can readily engage in an interaction with the target membrane. However, the distance remaining between the bilayer surfaces of virus and target membrane could still be as much as about 80 Å. As this distance must be overcome before viral and target membrane actually merge, in order to initiate membrane fusion, the fusion protein must first overcome the large steric barrier between the viral and target membranes imposed by the glycoproteins themselves (29,42,43). In addition, repulsive hydration forces due to phospholipid headgroup bound water must be overcome (44,45) for fusion to begin. It would therefore appear that the secondary step, after insertion, is time dependent as indicated by the slight delay (10-15 s) in the onset of fusion after penetration. It is also conceivable that the latter step imposes a considerable motional flexibility on the fusion protein. We conclude that this experiment provides evidence which strongly supports the hypothesis that the hydrophobic interaction of the F protein with the target membrane is the triggering event of virus membrane fusion.

Extrapolation of mechanistic studies of virus-liposome fusion to fusion with biological membranes must be done cautiously, since these events differ kinetically and, in all likelihood, mechanistically as well (29). Sendai virus fuses readily with negatively charged liposomes at neutral pH, which depends on the presence of the F protein. Yet, the fusion reaction is not dependent on viral recognition by specific sialic acid containing receptors (46), a phenomenon that has been reported before (47,48). With this precaution in mind, the use of liposomal models is very important in the isolation of structural elements involved in fusion, and in studying their function.

In summary, these studies show that the F protein interacts with the hydrophobic core of target liposomes, in an event preceding membrane mixing and fusion. The

labeling of the F and HN glycoproteins shows dependence on time, temperature, and pH consistent with regulation of both the kinetics and extent of fusion. These principles also appear to govern the fusion of reconstituted viral membranes. The use of time dependent photoaffinity labeling allows a mechanistic analysis of the fusion reaction. Continuing studies are in progress to analyze the specific peptide sequences labeled, to monitor hydrophobic interactions during virus fusion with biological membranes, and to examine the depth of peptide penetration into target membranes using amphiphilic probes incorporated into these membranes.

## REFERENCES


1. Choppin, P.W. and Scheid, A. (1980) *Rev. Infect. Dis.* **2**, 40-61.

2. Loyter, A. and Volsky, D.J. (1982) *Cell Surf. Rev.* **8**, 215-266.

3. White, J., Kielian, M. and Helenius, A. (1983) *Q. Rev. Biophys.* **16**, 151-195.

4. Haywood, A.M. (1974) *J. Mol. Biol.* **83**, 427-436.

5. Scheid, A. and Choppin, P.W. (1977) *Virology* **80**, 54-66.

6. Hsu, M.-C., Scheid, A. and Choppin, P.W. (1977) *J. Biol. Chem.* **256**, 3557-3563.

7. Brunner, J. (1981) *Trends Biochem. Sci.* **6**, 44-46.

8. Sigrist, H. and Zahler, P. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A.N., Ed.), Plenum: N.Y., 333-370.

9. Brunner, J. and Semenza, G. (1981) *Biochemistry* **20**, 7174-7182.

10. Richards, F.M. and Brunner, J. (1980) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 144-164.

11. Gitler, C. and Bercovici, T. (1980) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 199-211.

12. Chakrabarti, P. and Khorana, H.G. (1975) *Biochemistry* **14**, 5021-5033.

13. Radhakrishnan, R., Gupta, C.M., Erni, B., Robson, R.J., Curatolo, W., Majumdar, A., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1980) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 165-198.

14. Brunner, J., Spiess, M., Aggeler, R., Huber, P. and Semenza, G. (1983) *Biochemistry* **22**, 3812-3820.

15. Harter, C., Bächi, T., Semenza, G. and Brunner, J. (1988) *Biochemistry* **27**, 1856-1864.

16. Moscufo, N., Gallina, A., Schiavo, G., Montecucco, C. and Tomasi, M. (1987) *J. Biol. Chem.* **262**, 11490-11496.

17. Schroit, A.J., Madsen, J. and Ruoho, A. (1987) *Biochemistry* **26**, 1812-1819.

18. Zakowski, J.J. and Wagner, R.R. (1980) *J. Virol.* **36**, 93-102.

19. Boulay, F., Doms, R.W. and Helenius, A. (1986) in *Positive Strand RNA Viruses* (Brinton, M.A. and Rueckert, R.R., Eds.), Liss: N.Y., 103-112.

20. Kim, J. and Kim, H. (1986) *Biochemistry* **25**, 7867-7874.

21. Novick, S.L. and Hoekstra, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7433-7437.

22. Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) *Biochemistry* **23**, 5675-5681.

23. Hoekstra, D. and Klappe, K. (1986) *J. Virol.* **58**, 87-95.

24. Peterson, G.L. (1977) *Anal. Biochem.* **83**, 346-356.

25. Szoka, F.C. and Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4194-4198.

26. Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta* **557**, 9-23.

27. Böttcher, C.J.F., Van Gent, C.M. and Fries, C. (1961) *Anal. Chim. Acta* **24**, 203-204.

28. Harmsen, M.C., Wilschut, J., Scherphof, G., Hulstaert, C. and Hoekstra, D. (1985) *Eur. J. Biochem.* **149**, 591-599.

29. Klappe, K., Wilschut, J., Nir, S. and Hoekstra, D. (1986) *Biochemistry* **25**, 8252-8260.

30. Laemmli, U.K. (1970) *Nature* **227**, 680-685.

31. Doms, R.W., Helenius, A. and White, J. (1985) *J. Biol. Chem.* **260**, 2973-2981.

32. Turro, N.J. (1978) *Modern Molecular Photochemistry*, Benjamin/ Cummings: Menlo Park, CA, 551-557.

33. Liu, M.T.H. (1982) *Chem. Soc. Rev.* **11**, 127-140.

34. Kanakarajan, K., Goodrich, R., Young, M.J.T., Soundararajan, S. and Platz, M.S. (1988) *J. Am. Chem. Soc.* **110**, 6536-6541.

35. Bayley, H. and Knowles, J. (1978) *Biochemistry* **17**, 2414-2419.

36. Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1985) *Biochemistry* **24**, 4739-4745.

37. Arvinte, T., Hildenbrand, K., Wahl, P. and Nicolau, C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 962-966.

38. Hong, K., Yoshimura, T. and Papahadjopoulos, D. (1985) *FEBS Lett.* **191**, 17-23.

39. Lee, P.M., Cherry, R.J. and Bächi, T. (1983) *Virology* **128**, 65-76.

40. Osawa, M. and Asano, A. (1981) *J. Biol. Chem.* **256**, 5954-5956.

41. Markwell, M.A.K. and Fox, C.F. (1980) *J. Virol.* **33**, 152-166.

42. Hoekstra, D., Düzgünes, N. and Wilschut, J. (1985) *Biochemistry* **24**, 565-572.

43. Hoekstra, D. and Düzgünes, N. (1986) *Biochemistry* **25**, 1321-1330.

44. Rand, R.P. (1981) *Ann. Rev. Biophys. Bioeng.* **10**, 277-314.

45. Marra, J. and Israelachvili, J. (1985) *Biochemistry* **24**, 4608-4618.

46. Markwell, M.A.K., Svennerholm, L. and Paulson, J.C. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5406-5410.

47. Landsberger, F.R., Greenberg, N. and Altstiel, L.D. (1981) in *The Replication of Negative Strand Viruses* (Bishop, D.H.L. and Compans, R.W., Eds.), Elsevier/ North-Holland: Amsterdam, 519-522.

48. Haywood, A.M. and Boyer, B.P. (1984) *Biochemistry* **23**, 4061-4066.

# Chapter 5
# Hydrophobic Interactions Between Sendai Virus Glycoproteins and Target Membranes During Virus-Erythrocyte Membrane Fusion

The text of this chapter was co-authored with John D. Baldeschwieler and Dick Hoekstra and is being submitted for publication.

# Hydrophobic Interactions Between Sendai Virus Glycoproteins and Target Membranes During Virus-Erythrocyte Membrane Fusion

Steven L. Novick, John D. Baldeschwieler, and Dick Hoekstra,* Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, and *Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712KZ Groningen, The Netherlands

## ABSTRACT

Time-resolved hydrophobic photoaffinity labeling was used to examine interactions between Sendai virus glycoproteins and erythrocyte membranes during early stages of virus-erythrocyte ghost fusion. The lipid soluble photoaffinity label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine was incorporated into erythrocyte ghost membranes prior to fusion with virus particles, to label peptides inserting into the bilayer. Photolysis was conducted at various reaction times, and showed strong time-dependent membrane penetration by the $F_1$ subunit of the viral fusion (F) protein. The viral binding protein, the hemagglutinin/neuraminidase (HN), also exhibited time-dependent labeling, demonstrating that both viral binding to erythrocyte membranes as well as the fusion event involve hydrophobic contact of the viral proteins with the target cell membrane. The early penetration observed is in contrast to the relatively slow fusion kinetics, measured independently with the octadecyl Rhodamine B chloride membrane mixing assay, suggesting that after the penetration occurs, membrane components and/or the viral fusion protein may need to subsequently reorganize for fusion to be initiated. Labeling of erythrocyte membrane proteins in a separate

experiment showed close membrane contact of spectrin. A possible role in regulating membrane fusion is discussed.

## INTRODUCTION

Membrane fusion is a central event in many cellular processes such as endocytosis, protein processing, and fertilization, as well as viral infection. In spite of their diverse physiological roles, it is likely that some of the mechanistic details of all these events are common. However, little is presently understood regarding the molecular mechanism of fusion.

Enveloped viruses enter cells by fusion of viral and cellular membranes (1,2). Sendai virus, a paramyxovirus, enters cells by fusion at the plasma membrane, in an event involving the two viral glycoproteins (1,2). The viral hemagglutinin/neuraminidase (HN) binds to sialic acid-containing receptors on the cell surface, and the fusion (F) protein mediates the fusion event. The mature form of the F protein consists of two disulfide-linked subunits, $F_1$ and $F_2$ (3). $F_1$ is an integral protein in the viral membrane and is responsible for fusion, while $F_2$ is extrinsic and plays no apparent role in fusion (1,2,4-7). The finding of a hydrophobic $NH_2$ terminus in $F_1$ and the fusion proteins of many other viruses of about 20 amino acids in length has led to the hypothesis that insertion of the N-terminus into the membrane of a cell initiates fusion (1,2,4,8).

Hydrophobic photoaffinity labeling has proven a very useful technique for identifying transmembrane segments of proteins and membrane binding proteins (9,10). Recently, hydrophobic photolabeling evidence of hydrophobic interaction between the $F_1$ subunit and liposomal membranes during early stages of virus-liposome fusion has been reported (6,7), supporting the model of hydrophobic protein-target membrane interaction as an initiating event in viral fusion. Additional photolabeling

evidence of hydrophobic viral fusion proteins binding to liposomes at equilibrium supports this notion (11-13).

In spite of the evidence for hydrophobic interaction of the Sendai virus $F_1$ subunit with liposomes as a trigger of fusion, it is not clear that viral fusion with liposomes is entirely relevant to fusion with biological membranes. While many mechanistic similarities in the fusion behavior of both artificial and biological membranes exist, differences in binding kinetics and extent of fusion, and roles of viral proteins in fusion, have been reported (14,15). Since the principle of hydrophobic photoaffinity labeling has been established in the relatively simple liposomal system (6,7), it is appropriate to begin to probe the role of hydrophobic protein-lipid interactions in initiating virus fusion with complex biological membranes. Erythrocyte membranes have been shown to fuse with Sendai virus, and some of the kinetic and mechanistic aspects of the reaction have been characterized (16-19).

In the presently described study, the hydrophobic photoaffinity label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID) was incorporated into erythrocyte ghost membranes, as a probe of hydrophobic viral protein interaction with the ghost membrane during virus-ghost fusion. This experiment provides a test of the hypothesis that such interactions trigger membrane fusion in biological systems.

## METHODS

**Virus.** Sendai virus (Z strain) was grown for 72 h in the allantoic compartment of ten-day-old fertilized chicken eggs. Virus was purified by differential ultracentrifugation and stored in 150 mM NaCl/5 mM Hepes (Sigma), pH 7.4, at -70°C (17). Viral protein concentrations were determined with the Peterson modified Lowry assay (20).

**Erythrocyte ghosts.** Human erythrocytes (Type A+, American Red Cross, Los Angeles) were washed in 150 mM NaCl/5 mM $Na_2HPO_4$, pH 7.4 (PBS) and then were subjected to hypotonic lysis in 5 mM $Na_2HPO_4$, pH 8.0, at 4°C (21). After lysis was complete, the membranes were resealed in 120 mM KCl/30 mM NaCl/10 mM $Na_2HPO_4$, pH 7.4 (KNP buffer) containing 1 mM $MgCl_2$ at 37°C. Protein concentration was determined with the Peterson modified Lowry assay (20).

**Fusion and Photolabeling.** A 1 mCi/ml solution of TID (Amersham, 10 Ci/mmol, 90% radiochemical purity), in 10 µl KNP buffer containing 10% ethanol, was added to 200 µg (as protein) erythrocyte ghost membranes in approximately 960 µl buffer, in a polypropylene Eppendorf tube. The mixture was vortexed and incubated for 1 h on ice in the dark. Unincorporated TID is adsorbed by the Eppendorf tube (6,12) and does not require an additional separation step. Sendai virus (50 µg) was added and the mixture (1 ml total volume) was stirred continuously. The sample was thermostatted using a homemade sample holder, described elsewhere (7), connected to a circulating water bath. The sample was irradiated for 5 s by an Oriel (Stratford, CT) Model 6140 1000 W xenon arc lamp, with a 10 cm circulating cold water filter and a Schott Glass Technology WG-360 high-pass cutoff filter cooled in a water bath. The reaction was stopped by immersing the sample in ice in the dark.

Kinetics and extent of fusion were independently monitored with the probe octadecyl Rhodamine B chloride ($R_{18}$), as described earlier (22). Briefly, $R_{18}$ labeled virus was fused with unlabeled erythrocyte ghosts, and the relief of self-quenching of $R_{18}$ was monitored as the surface density of the probe was diluted during membrane mixing.

**Analysis of Labeled Proteins.** Protein was precipitated in the sample by adding 150 µl cold 72% trichloroacetic acid, incubated 1 h at 0°C after vortexing, and was pelleted by centrifugation at 12,000 x *g* for 15 min at 4°C. After denaturation in

reducing buffer (5% sodium dodecyl sulfate/5% β-mercaptoethanol/8M urea/62.5 mM Tris-HCl, pH 6.8/0.01% bromophenol blue), proteins were separated by polyacrylamide gel electrophoresis (23). Proteins were visualized with 0.2% Coomassie Brilliant Blue R-250 in 25% methanol/10% acetic acid. Erythrocyte glycoproteins were visualized with the periodic acid-Schiff reagent (24). Samples were run in duplicate and analyzed by either autoradiography or radioactive counting. Autoradiography was conducted at -70°C, with Kodak XAR-5 film and two Du Pont Cronex intensifying screens. Counting of gel slices was carried out in a Beckman Biogamma II gamma counter.

## RESULTS

**Time-Dependent Labeling of $F_1$ During Virus-Ghost Fusion.** Sendai virus fusion with erythrocyte membranes at neutral pH has been characterized kinetically (16-18), and more recently mechanistic studies have been conducted as well (19). The mechanistic studies indicated that the dominant effect in virus fusion with erythrocyte membranes is hydrophobic, which is consistent with the current observation that the viral $F_1$ peptide penetrates into erythrocyte membranes containing TID very early during the fusion reaction (Figure 1). The early maximum in $F_1$ labeling provides direct evidence supporting the hypothesis that the hydrophobic penetration of the fusion peptide into the target membrane is an initiating event of virus-erythrocyte membrane fusion.

Both $F_1$ labeling and fusion were smaller at pH 5.0 than at pH 7.4 (Figures 1,2), consistent with earlier observations of virus-erythrocyte membrane fusion (16,17), indicating only minimal virus-ghost fusion at low pH. $F_1$ labeling during the reaction at pH 5.0 was consistently below the background level of the later time points at pH 7.4 (cf. Figure 1). The small amount of fusion at low pH together with lowered

**Figure 1**

Time dependence of $F_1$ labeling during Sendai virus-erythrocyte ghost fusion. TID labeling was conducted as described in METHODS, with 5 s photolysis at the initiation of the fusion reaction. Fusion was conducted at either pH 7.4 (closed circles), or at pH 5.0 (open circles). $F_1$ labeling was calculated as the percentage of sample activity.

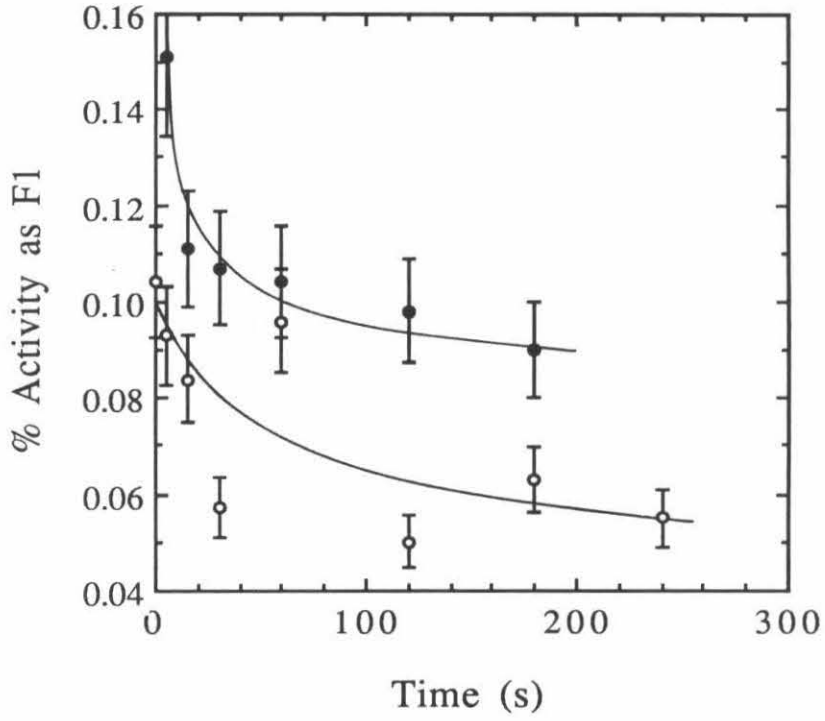
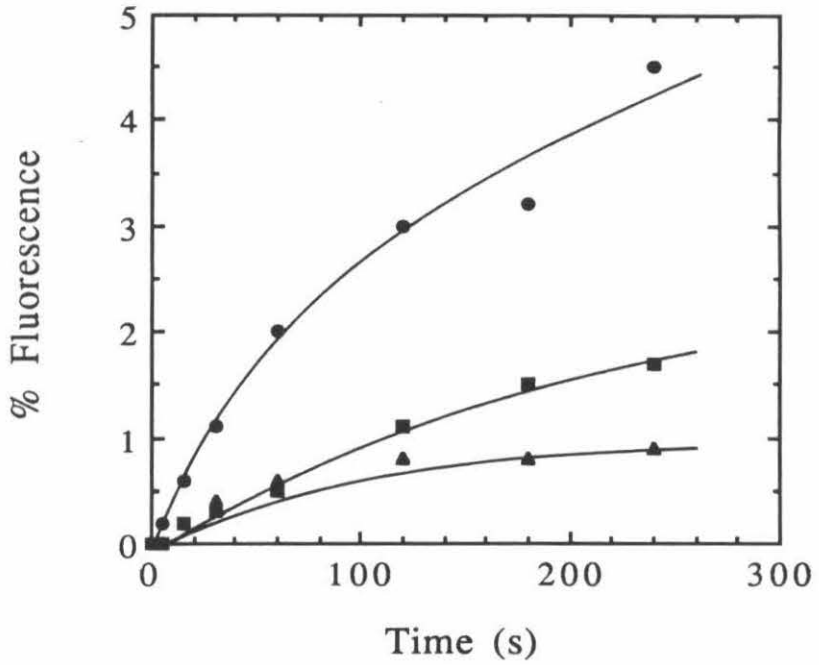
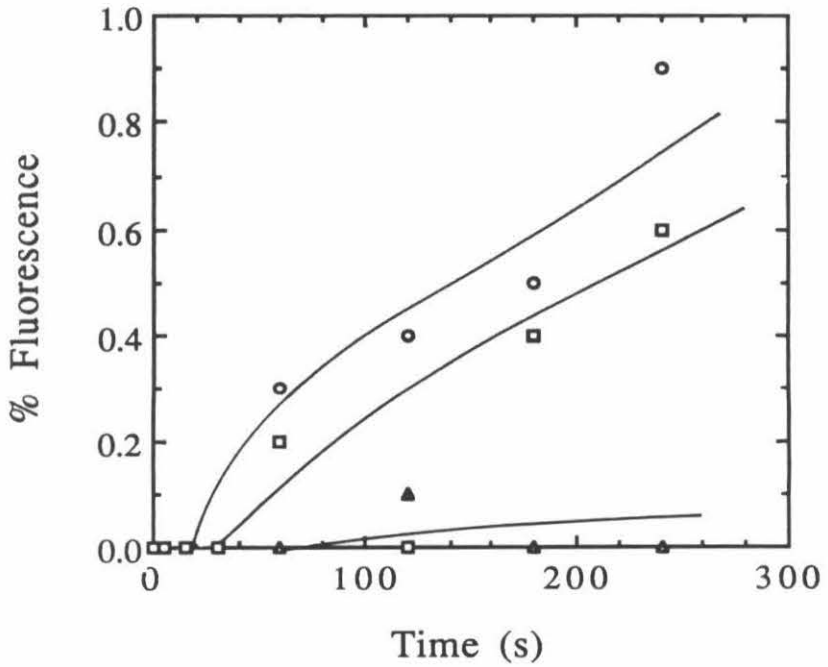

0.16
0.14
0.12
0.10
0.08
0.06
0.04
% Activity as F1
0
100
200
300
Time (s)

**Figure 2**

Kinetics of fusion of Sendai virus with erythrocyte membranes. Fusion was conducted as described in METHODS, with 25 μg virus (as protein) and either 25 μg (triangles), 50 μg (squares), or 100 μg (circles) erythrocyte membranes (as protein). Fusion was monitored at both pH 7.4 (upper graph) and pH 5.0 (lower graph), using the $R_{18}$ assay for membrane mixing. Zero % fusion corresponds to a self-quenched virus sample labeled with $R_{18}$, and 100% fusion corresponds to the signal upon addition of Triton X-100 to 1 % (infinite probe dilution), with correction for sample dilution.


% Fluorescence
0
1
2
3
4
5
0
100
200
300
Time (s)


% Fluorescence
0.0
0.2
0.4
0.6
0.8
1.0
0
100
200
300
Time (s)

$F_1$ labeling are also consistent with the hydrophobic penetration model of viral protein mediated fusion, i.e., less penetration and labeling should occur if less fusion is occurring. The kinetics and extent of virus-ghost fusion are illustrated in Figure 2, as measured by the $R_{18}$ assay for membrane mixing (22). Fusion is minimal at low pH (see ordinate scale), and mirrors the small amount of $F_1$ labeling (Figure 1).

**Labeling of HN.** The Sendai HN protein is responsible for binding to sialic acid-containing receptors on the surface of cells (25). Model studies have also shown that HN will mediate viral binding to negatively charged liposomes (14). In either case, the binding was viewed as a purely electrostatic interaction. As Figure 3 shows, however, the time-dependent HN labeling during virus-ghost fusion at pH 7.4 suggests that the binding event leading to fusion may also involve hydrophobic contact with the erythrocyte membrane. HN labeling at pH 5.0 shows a similar time dependence, suggesting that the degree of hydrophobic contact during virus binding to ghosts is retained. Labeling of both $F_1$ and HN were at or below the background levels under nonfusogenic conditions (2°C), at both pH 5.0 and pH 7.4, indicating that the labeling during fusion at early time points at 37°C was not due to diffusion of TID from the erythrocyte membrane to hydrophobic sites on the virus. We have also shown earlier that diffusional labeling is unresponsive to conditions of temperature and pH, and can be distinguished from labeling during fusion (6,7).

**Labeling of erythrocyte membrane proteins.** In order to determine whether erythrocyte membrane proteins labeled with TID in ghost membranes would comigrate with labeled viral proteins during electrophoretic analysis, erythrocyte membranes were labeled with TID in the absence of virus (Figure 4). The bands which were substantially labeled did not comigrate with the viral glycoproteins, so that additional separation steps between the termination of the reaction and analysis of the label's fate were not necessary. Somewhat surprisingly, the band labeled most heavily

**Figure 3**

TID labeling of HN during Sendai virus-erythrocyte membrane fusion. Fusion and photolabeling were conducted as described in METHODS. Labeling of the HN protein was determined by radioactive counting and calculated as percentage of sample activity, at pH 7.4 (closed circles) and pH 5.0 (open circles).

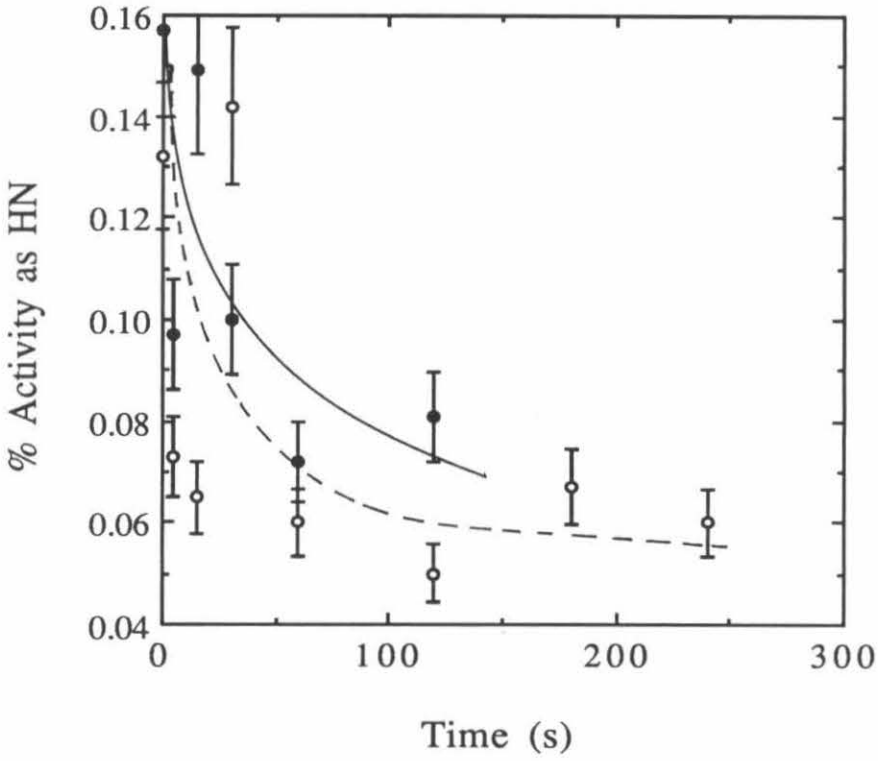
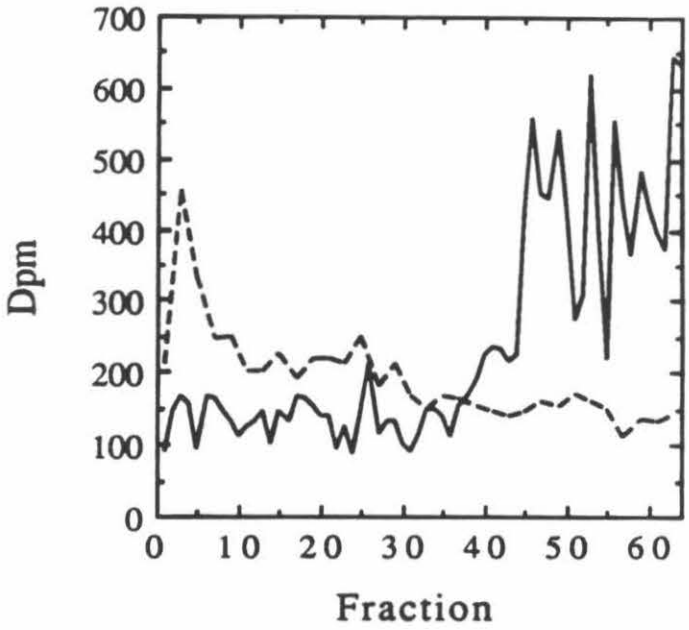

0.16
0.14
0.12
0.10
0.08
0.06
0.04
% Activity as HN
0
100
200
300
Time (s)

**Figure 4**

Labeling of erythrocyte membrane proteins. TID was added to erythrocyte membranes in the absence of virus to determine whether heavily labeled ghost proteins would comigrate with the viral proteins. Molecular weight of proteins was determined by comparison with molecular weight standards (not shown). Erythrocyte membrane proteins which were heavily labeled (dashed line) did not comigrate with the viral proteins (solid line). Erythrocyte membrane protein peaks correspond to spectrin (Fraction 2), band 3 (Fraction 15), and band 4.1 (Fraction 24).


700
600
500
400
300
200
100
0
Dpm
0
10
20
30
40
50
60
Fraction

corresponds to spectrin (26,27). This was unexpected because the popular model of erythrocyte cytoskeleton-membrane architecture views spectrin as connected to the membrane only via contacts with other proteins, e.g., bands 4.1 and 4.9. An earlier report showed heavily labeled integral membrane proteins (band 3 and glycophorin) and a lack of spectrin labeling (28), probably caused by the different labeling conditions in that experiment.

## DISCUSSION

The prevalence of hydrophobic amino acid sequences in viral fusion proteins led to the hypothesis that hydrophobic interactions of a viral fusion peptide with a target cell membrane trigger membrane fusion (1,2,4,8). Recent studies of Sendai virus fusion with erythrocyte membranes showed that hydrophobic interactions are determining events in fusion (19). The present work provides direct evidence that the hydrophobic penetration of the fusion peptide of an intact virus into the membrane is a triggering event in fusion, strongly supporting the hypothesis (Figure 1). The kinetics of penetration of the fusion peptide $F_1$ are substantially faster than the kinetics of membrane fusion, suggesting that additional steps, e.g., conformational change, may be required after penetration for membrane fusion to occur (cf. Figures 1,2). This is consistent with the fusion mechanism proposed on the basis of dehydration effects during fusion (19).

Labeling of the HN protein during fusion at pH 7.4 and at pH 5.0 indicates that hydrophobic interactions may play a role in virus binding, in addition to electrostatic effects (Figure 3). Aqueous carbene scavengers such as glutathione and dithiothreitol could not be used to control for labeling by TID diffusion out of the erythrocyte membrane, since they inactivate the viral fusion protein at low concentrations (17). Therefore, as a control for diffusional labeling, TID was added to virus in the absence

of ghosts, and the labeling occurring by diffusion through the aqueous phase was significantly different from labeling observed during fusion, and the diffusional labeling was independent of temperature and pH (cf. refs. 6,7). This indicates that diffusional labeling does not dominate the labeling observed during fusion. Furthermore, labeling under conditions at which virus-ghost fusion is inhibited, i.e., 2°C or pH 5.0, resulted in only background level labeling, which showed no time dependence. This lends further support to the conclusion that the majority of viral glycoprotein labeling during fusion is not due to TID diffusion.

The determination that viral glycoproteins did not comigrate during electrophoresis with heavily labeled erythrocyte proteins, native to the membrane containing TID (Figure 4), simplified the analysis of label distribution. A surprising observation was the predominant labeling of spectrin, and the relatively minor labeling of the integral membrane proteins band 3 and glycophorin (Figure 4). The lack of labeling of these proteins may be explained by the presence of an altered phase of tightly bound lipid around the integral proteins which excludes TID from labeling those proteins. This type of effect has been observed for lipid bound to glycophorin (29). The heavy labeling of spectrin suggests the possibility of some hydrophobic contact with the membrane. This is not accounted for in the currently popular model of erythrocyte membrane-cytoskeleton contact (26,27), but several investigators have reported close spectrin-membrane contact, binding of lipids, especially phosphatidylserine (PS), in the inner leaflet of erythrocyte membranes (30-35), and organizing membrane lipids in other cells (36). The implication for the fusion reaction is that the fusion peptide may interact with cytoskeletal components of the erythrocyte membrane, which could disrupt the phospholipid asymmetry, and hence promote fusion. The absence of PS and the large amount of phosphatidylcholine (PC) in the outer leaflet of the erythrocyte membrane would inhibit fusion, due to the greater

hydration of PC (14,37). Strong membrane hydration was shown to inhibit virus fusion with negatively charged liposomes in which PC was incorporated. The concept that a fusion peptide could possibly interact with cytoskeletal components is supported by the apparent association of complement proteins with cytoskeletal elements during complement attack (38).

The proposed model for the analogous series of events during virus fusion with an ordinary cell membrane, i.e., one not possessing the unique phospholipid asymmetry of the erythrocyte, is somewhat similar. The fusion peptide is proposed to interact with components of the cell membrane (lipid and/or protein) and cytoskeleton, to alter lipid packing and motional properties. This interaction would trigger additional conformational change in the fusion protein, and provide the driving force to overcome the repulsive hydration forces and bring the two bilayers close enough together to initiate fusion. The complex nature of the cell surface requires that additional studies be done to further clarify the details of this interaction.

In summary, the present work progresses toward a mechanistic understanding of fusion by showing that hydrophobic penetration of a viral fusion peptide into the membrane of a target cell is the initial triggering event in fusion. This interaction may serve to bring the two membranes into very close proximity, and to help overcome the large repulsive hydration forces at small interbilayer distances (39). Continuing experiments to identify the specific penetrating sequence, and to carry out labeling with a non-diffusible amphiphilic probe, are in progress.

## ACKNOWLEDGMENTS

The authors thank Karin Klappe and Roxanne Male for expert technical assistance. This work was supported by NIH grant RR07003 and ARO grant DAAG29-83-K-0128. S.L.N. was supported by an NSF Graduate Fellowship.

## REFERENCES


1. Choppin, P.W. and Scheid, A. (1980) *Rev. Infect. Dis.***2**, 40-61.

2. White, J., Kielian, M. and Helenius, A. (1983) *Q. Rev. Biophys.* **16**, 151-195.

3. Scheid, A. and Choppin, P.W. (1977) *Virology* **80**, 54-66.

4. Hsu, M.-C., Scheid, A. and Choppin, P.W. (1981) *J. Biol. Chem.* **256**, 3557-3563.

5. Moscufo, N., Gallina, A., Schiavo, G., Montecucco, C. and Tomasi, M. (1987) *J. Biol. Chem.* **262**, 11490-11496.

6. Novick, S.L. and Hoekstra, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7433-7437.

7. Novick, S.L., Baldeschwieler, J.D. and Hoekstra, D. (1989) submitted for publication.

8. Asano, A. and Asano, K. (1984) *Tumor Res.* **19**, 1-20.

9. Sigrist, H. and Zahler, P. (1985) in *The Enzymes of Biological Membranes* **10** (Martonosi, A.N., Ed.), Plenum: N.Y., 333-370.

10. Brunner, J. (1989) *Meth. Enzymol.* **172**, 628-687.

11. Boulay, F., Doms, R.W. and Helenius, A. (1986) in *Positive Strand RNA Viruses* (Brinton, M.A. and Rueckert, R.R., Eds.), Liss: N.Y., 103-112.

12. Harter, C., Bächi, T., Semenza, G. and Brunner, J. (1988) *Biochemistry* **27**, 1856-1864.

13. Harter, C., James, P., Bächi, T., Semenza, G. and Brunner, J. (1989) *J. Biol. Chem.* **264**, 6459-6464.

14. Klappe, K., Wilschut, J., Nir, S. and Hoekstra, D. (1986) *Biochemistry* **25**, 8252-8260.

15. Wilschut, J. and Hoekstra, D. (1986) *Chem. Phys. Lipids* **40**, 145-166.

16. Hoekstra, D., Klappe, K., De Boer, T. and Wilschut, J. (1985) *Biochemistry* **24**, 4739-4745.

17. Hoekstra, D. and Klappe, K. (1986) *J. Virol.* **58**, 87-95.

18. Nir, S., Klappe, K. and Hoekstra, D. (1986) *Biochemistry* **25**, 2155-2161.

19. Hoekstra, D., Klappe, K., Hoff, H. and Nir, S. (1989) *J. Biol. Chem.* **264**, 6786-6792.

20. Peterson, G.L. (1977) *Anal. Biochem.* **83**, 346-356.

21. Steck, T.L. and Kant, J.A. (1974) *Meth. Enzymol.* **31**, 172-180.

22. Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) *Biochemistry* **23**, 5675-5681.

23. Laemmli, U.K. (1970) *Nature* **227**, 680-685.

24. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) *Biochemistry* **10**, 2606-2617.

25. Markwell, M.A.K., Svennerholm, L. and Paulson, J.C. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5406-5410.

26. Bennett, V. (1985) *Ann. Rev. Biochem.* **54**, 273-304.

27. Cohen, C.M. (1988) *Adv. Cell Biol.* **2**, 1-24.

28. Brunner, J. and Semenza, G. (1981) *Biochemistry* **20**, 7174-7182.

29. Yeagle, P.L. and Kelsey, D. (1989) *Biochemistry* **28**, 2210-2215.

30. Juliano, R.L., Kimelberg, H.K. and Papahadjopoulos, D. (1971) *Biochim. Biophys. Acta* **241**, 894-905.

31. Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, G. (1978) *Biochim. Biophys. Acta* **509**, 21-32.

32. Bonnet, D. and Begard, E. (1984) *Biochim. Biophys. Acta* **120**, 344-350.

33. Williamson, P., Antia, R. and Schlegel, R.A. (1987) *FEBS Lett.* **219**, 316-320.

34. Connor, J., Bucana, C., Fidler, I.J. and Schroit, A.J. (1989) *Proc. Natl. Acad. Sci. USA* , in press.

35. Steiner, J.P. and Bennett, V. (1988) *J. Biol. Chem.* **263**, 14417-14425.

36. Del Buono, B.J., Williamson, P.L. and Schlegel, R.A. (1988) *J. Cell Biol.* **106**, 697-703.

37. Nir, S., Klappe, K. and Hoekstra, D. (1986) *Biochemistry* **25**, 8261-8266.

38. Liu, Z.-Y., Sanders, M.E. and Hu, V.W. (1989) *J. Immunol.* **142**, 2370-2376.

39. Rand, R.P. (1981) *Ann. Rev. Biophys. Bioeng.* **10**, 277-314.

# Chapter 6
# Significance of Hydrophobic Interactions in Membrane Fusion of Enveloped Viruses: A Comparison with Model Membranes

The text of this chapter was co-authored with Dick Hoekstra and has been accepted for publication in a book on biophysical aspects of membrane fusion, edited by Roland Glaser and David Gingell.

# Significance of Hydrophobic Interactions in Membrane Fusion of Enveloped Viruses: A Comparison With Model Membranes

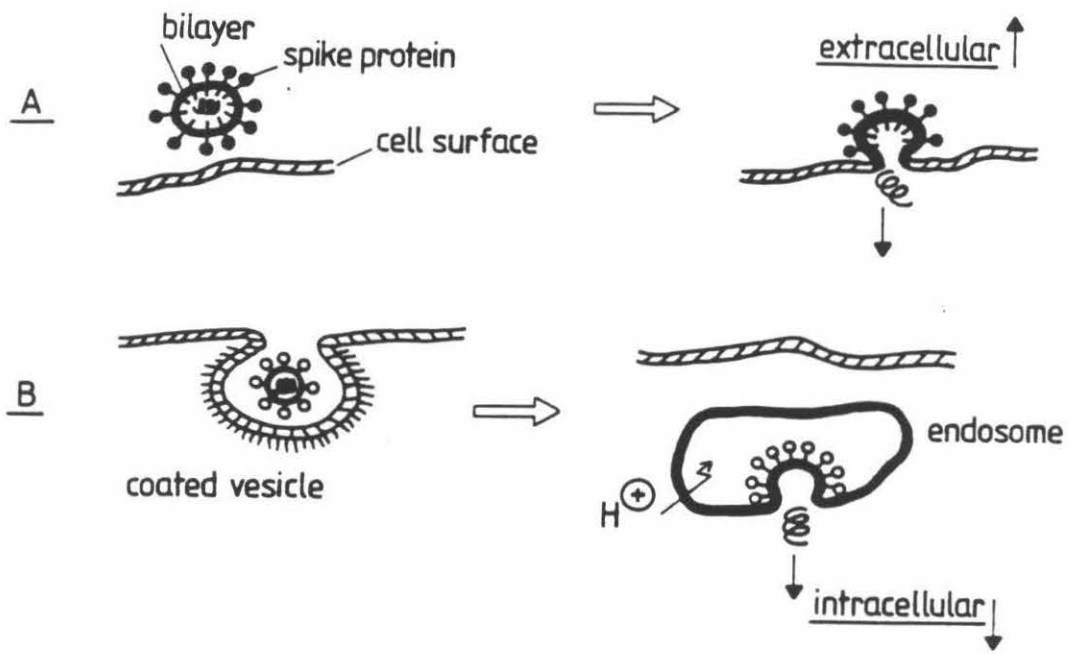
Steven L. Novick* and Dick Hoekstra, Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands, and *Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

## I. INTRODUCTION

An essential prerequisite for a virus to infect a cell is its ability to deposit the nucleocapsid into the cytoplasm of that "host"-cell. In the case of *enveloped* viruses, the nucleocapsid is surrounded by a membrane, consisting of a lipid bilayer that resembles the plasma membrane composition of the host-cell in which the virus was grown, and a unique set of virus-specific membrane proteins. To get access to the cytoplasm, the nucleocapsid must therefore traverse two membrane barriers. To accomplish this crossing, a virus uses a most efficient mechanism, namely membrane fusion. The cellular site where this fusion process occurs depends on the family to which the virus belongs. Some virions (for example, those belonging to the paramyxoviruses such as Sendai virus, Newcastle disease virus and measles virus) fuse directly with the plasma membrane at neutral pH. Most viruses, however, are first internalized by receptor-mediated endocytosis and subsequently fuse in the endosomal compartment "from within" (Figure 1). It has been shown that these viruses [such as

**Figure 1**

Entry pathways of enveloped viruses. The entry mechanism is family-dependent. Some viruses fuse directly with the plasma membrane at neutral pH (A). Others are first internalized by receptor-mediated endocytosis. Subsequently, fusion is triggered when the endosomal compartment acidifies (B).


A
bilayer
spike protein
cell surface
extracellular
B
coated vesicle
endosome
H
intracellular

orthomyxoviruses (influenza), togaviruses (Semliki Forest virus, SFV), and rhabdoviruses (vesicular stomatitis virus, VSV)] require a mild acidic pH in order to acquire fusogenic capacity (1).

Specific viral membrane proteins are intimately involved in the overall process that leads to fusion. For most viral membranes the protein composition is relatively simple, as they may contain as few as one or two types of proteins. In many instances, one of these proteins governs both binding to and fusion with a cellular membrane; in some cases, however, one protein contains only binding activity whereas a second protein contains the fusion activity. It is also possible, as might be the case for entry of Herpes simplex virus (2), that the overall fusion process is the result of a cooperative sequential chain of events requiring several (i.e., more than two) different viral envelope proteins.

Many of the aforementioned proteins have been identified; however, much of their structural and functional features remain to be elucidated. In this regard, the major influenza envelope protein, HA (hemagglutinin), represents an exception. To date, this protein is the best-characterized viral protein with respect to its synthesis, structure, and function (3,4). The HA protein possesses a homotrimeric structure, the assembly of which occurs in the endoplasmic reticulum. Post-translational modifications take place after transfer to the Golgi complex (carbohydrate addition) and during subsequent trafficking to the cell surface. The latter involves a proteolytic cleavage, at or near the cell surface, which is essential for converting the inactive precursor $HA_0$ into a fusion-active HA, consisting of two subunits $HA_1$ and $HA_2$. An analogous phenomenon is seen for the fusion protein, F, of paramyxoviruses. In this case the inactive precursor $F_0$ is converted into the active form F, which also consists of two polypeptides, $F_1$ and $F_2$ (5). Human immunodeficiency virus (HIV) displays a similar feature as the predominant envelope protein gp 160 also needs to be activated by endoproteolytic

cleavage in order to acquire fusion activity. As a result, a heterodimer consisting of two polypeptides, gp 120 and gp 41, is formed (6). However, not all viral proteins share this property. In the case of the fusion proteins of SFV ($E_1$) and VSV (G) no precursors have been identified (1).

Common to many viral fusion proteins appears to be the presence of a stretch of amino acids which is particularly hydrophobic, or acquires this property under appropriate conditions. This has led to the proposal that these regions are related to the ability of a virus to gain fusion activity. In the present paper, we will discuss the experimental evidence that supports this concept. In addition, guided by extensive work carried out with simple artificial membrane systems, we will briefly describe parameters that govern membrane fusion of liposomes and discuss whether similar parameters may govern virus fusion as well, emphasizing the role of hydrophobic interactions. In order to obtain insight into the role of various parameters in virus fusion, the development of sensitive techniques to monitor fusion continuously and directly, has proven to be of significant importance. We will therefore briefly outline the principles of these procedures, before discussing experimental details.

## II. TECHNIQUES FOR MEASURING VIRUS FUSION

Relevant to a mechanistic understanding of viral fusion is the quantitative detection and characterization of the fusion process. This entails that fusion should be monitored with direct techniques, i.e., procedures that monitor the fusion of a virus as such, as opposed to more indirect approaches such as virus-induced hemolysis or cell-cell fusion. Evidently, although quite useful to qualitatively determine the fusogenic potency of a virus preparation, the latter procedures register the consequences of a virus-target membrane interaction rather than the occurrence of virus membrane fusion *per se*. Moreover, the lack of hemolysis does not necessarily imply that the virus does

not fuse. Whether or not hemolysis occurs depends on the viral membrane structure, which in turn is affected by the age of the virus (7) or by the conditions of storage ("freezing and thawing") of the virus. For example, Sendai virus, harvested 24 h after infection, does fuse with erythrocytes without the induction of significant hemolysis. The extent of hemolysis greatly increases, however, when such virions undergo a freezing and thawing cycle. Similarly, when reconstituting the Sendai virus envelope, using the nonionic detergent octylglucoside, no detectable hemolysis is observed unless the reconstituted particles are subjected to freezing and thawing. Irrespective of such treatments, the fusion activity, monitored by a quantitative assay, is essentially the same (8). These examples thus emphasize the relevance of determining virus fusion in a direct manner.

The most common method of monitoring virus-membrane fusion is based on lipid mixing, using either resonance energy transfer (RET) between fluorescently labeled membrane components or relief of self-quenching of a fluorescent lipid-like derivative (Figure 2). In this way, fusion of complex biological membranes can be measured continuously and quantitatively. In conjunction with a kinetic mass action model, such an approach allows detailed kinetic analysis of the fusion reaction, and evaluation of the physical and chemical determinants of membrane fusion.

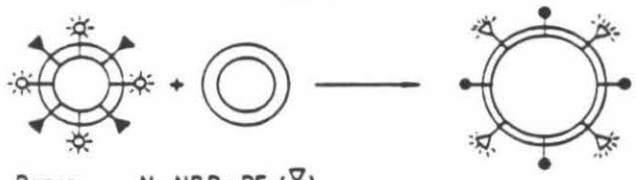
The fluorescent derivative octadecyl Rhodamine B chloride ($R_{18}$) can be readily inserted into intact membranes such as viral membrane envelopes (9). As the density of the probe increases, its fluorescence, being sensitive to intermolecular distance, becomes increasingly self-quenched. Thus when a population of labeled virus particles fuses with a population of unlabeled membranes, lipid intermixing between the two membranes allows lateral diffusion of the probe to greater intermolecular distances, with the observable result of a relief of self-quenching. This assay ("$R_{18}$-assay") overcomes some of the difficulties encountered in the addition of fluorescently labeled

**Figure 2**

Principle of fusion assays based on lipid mixing. (A) RET assay. The increase in donor fluorescence is usually monitored. This will occur when the energy transfer efficiency decreases as a result of probe dilution when a labeled membrane fuses with an unlabeled counterpart. (B) $R_{18}$ assay. In this assay the relief of fluorescence self-quenching is monitored, which occurs when one membrane population, labeled with a self-quenching concentration of the probe, fuses with unlabeled membranes. For details and references, see text.

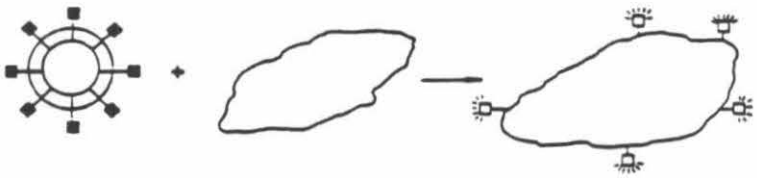
FUSION ASSAYS (LIPID MIXING)

A. Resonance Energy Transfer (RET assay)



Donor : N-NBD-PE (♈)
Acceptor: N- Rh -PE (♀)

B. Fluorescence Selfquenching ($R_{18}$ assay)



Probe: Octadecyl rhodamine B chloride ($R_{18}$)

phospholipids to intact membranes such as in the RET assay, using phosphatidylethanolamine molecules bearing headgroup fluorophores (N-NBD-PE and N-Rh-PE; 10). These lipid derivatives have been used successfully in measurement of fusion of viruses and their reconstituted envelopes with both liposomes and biological membranes, as the probes can be readily incorporated into the liposomal membranes or viral envelopes during their preparation (8; 11-13).

Mixing of aqueous contents is also a useful assay of fusion, since true fusion consists of both lipid and contents mixing. Contents mixing assays are based on interactions between aqueous fluorophores, such as Tb/DPA (terbium/dipicolinic acid; 14) and ANTS/DPX (aminonaphthalenetrisulfonic acid and *p*-xylylene bis(pyridinium) bromide; 15). Particularly in studies of fusion between artificial membranes, the use of both approaches, i.e., membrane and contents mixing, have been highly profitable in analyzing fusion events, as membrane mixing *per se* may accompanied by lipid exchange processes (16). Unfortunately, such a direct approach cannot be undertaken with intact virus particles, given the limitation on encapsulating aqueous content-markers into intact virions. In principle, encapsulation is possible when reconstituting viral envelopes, but thus far such efforts have been limited (17). It is important, therefore, to include extensive control experiments that exclude lipid exchange when monitoring virus fusion by membrane mixing alone.

Based upon kinetic measurements of Sendai virus fusion with both liposomes (18) and erythrocyte ghosts (19,20), the application of a mass action kinetic model has allowed distinction between the kinetics of binding and fusion and evaluation of the corresponding rate constants (21,22). It should be noted that with the fluorescence assays, the overall fusion rate (including adherence of virions and the fusion reaction itself), is measured when the virus particles are added to the target membrane at 37°C. By kinetic simulation, employing the mass action kinetic model, insight into the

parameters that govern binding and fusion *per se* can thus be obtained (21-23). The model views the overall fusion reaction as a sequence of a second order process of virus-target membrane adhesion, which is partially reversible, followed by the first-order fusion reaction itself.

Apart from fusion of Sendai virus, fusion of other viruses has also been characterized with the fluorescence assays described above, including influenza (24,25), vesicular stomatitis virus (VSV, 26,27) and Newcastle disease virus (28). Although the N-NBD-PE/N-Rh-PE energy transfer pair is most commonly used in cases in which lipid vesicles are fusion targets, other energy transfer donor/acceptor pairs (29,30) may also be used for monitoring virus fusion.

Other direct methods of measuring virus fusion activity are available such as those based on using radiolabeled virus (31) or virions labeled with paramagnetic probes (32-35). Although useful and more informative than indirect methods such as infectivity and polykaryon formation (1,36), these procedures are generally more time-consuming. Evidently, the spectroscopic assays have the advantage of rapid and sensitive data acquisition, as well as continuous measurement. It thus becomes possible to monitor the occurrence of membrane fusion under a wide variety of conditions, providing the possibility to correlate membrane and protein physical properties with their roles in viral entry and membrane fusion. In spite of the availability of quantitative assays for membrane fusion, many details of the mechanism of fusion remain to be elucidated. A major limitation stems from the fact that virus and cell membranes are complex systems, making it difficult to isolate structural membrane components and their associated physical contributions to fusion. Artificial membranes are therefore commonly used as models in membrane fusion (37,38). Although these model systems oversimplify the biological membranes involved in fusion, they provide an important and useful means of identifying basic structural elements involved in

fusion (16,39). Model studies may thus provide fundamental insight into the more complex fusion of biological membranes, although, as noted before (cf. 16,18,24), a direct extrapolation to pure biological systems is not always warranted.

## III. PARAMETERS AFFECTING MEMBRANE FUSION

In artificial systems, membrane fusion is dependent on disruption of the combination of hydration and hydrophobic stabilizing interactions, governing membrane self-assembly and stability (40-43). The close apposition of bilayers necessary for fusion requires overcoming the repulsive hydration layers surrounding each membrane. The repulsive regime of hydration forces is significant at interbilayer distances of less than 30 Å. Thus, disruption of the hydration shells is a prerequisite for fusion to occur. It should be noted that the work required for establishing fusion-susceptible contact between membranes is enormous. It has been estimated that an external pressure of $10^3$-$10^4$ atm is required to overcome the hydration barrier (44), thus illustrating the extreme potency of fusogenic agents. Since the product of fusion is a newly formed membrane, the hydrophobic interactions involved in stabilization of both the fusing and product membranes must also be involved in the thermodynamic driving force for fusion (16,45,46).

In addition to the repulsive forces between membranes at short distances, Rand et al. (47) have recently described an alternative model of intermembrane forces. In this model, the hydration and fusion behavior of membranes is related to a combination of attractive and repulsive forces, where the attractive forces result from intermembrane networks of hydrogen-bonded water. Although awaiting further experimental verification, this model is consistent with some observations of intermembrane interactions.

Artificial membranes have been especially useful in the analysis of membrane hydration and its role in fusion. The most prominent example in this respect is the cation-induced fusion of bilayers consisting of negatively charged phospholipids, such as phosphatidylserine (PS; for references, see 16). Upon addition of $Ca^{2+}$ to such vesicles, the gel/liquid-crystalline phase transition temperature of the lipid increases dramatically, consistent with removal of water from the bilayer interface. A variety of physical techniques (x-ray, NMR and Raman scattering) have provided evidence that $Ca^{2+}$/PS binding results in an essentially complete dehydration of intermembrane contact sites. By contrast, upon addition of $Mg^{2+}$, which, as opposed to $Ca^{2+}$, does not induce fusion of (large) PS vesicles, the $Mg^{2+}$/PS complexes formed still contain substantial amounts of water. Consequently, with $Mg^{2+}$ the apposed bilayers display larger interbilayer separations than with $Ca^{2+}$, and therefore, fusion will not take place. Synthetic non-phospholipid amphiphiles, which exhibit polymorphic phase behavior similar to that of phospholipids, have also been useful in relating membrane hydration to fusion (46,48-53).

In addition to the hydration barrier, there is also a hydrophobic barrier to fusion. The thermodynamic bilayer stabilization must be disrupted to induce formation of a fusion intermediate, regardless of whether this intermediate will display a defined structure. Synthetic phospholipid vesicles have also been used for investigation of the structure of fusion intermediates (54-56), leading to the notion that nonbilayer intermediates are formed during fusion of liposomes (54,57). Proposed intermediates in fusion include monolayer stalks (44,58) and "inverted micellar intermediates" (55). Recent efforts have been made to identify the latter by cryo-transmission electron microscopy (56).

The extensive work carried out with simple model systems has substantially contributed to an understanding of virus fusion, providing the current model of viral

glycoprotein function in fusion, namely to bring viral and target membranes into close proximity to overcome the repulsive hydration shells and to induce formation of a nonbilayer fusion intermediate in the target membrane via hydrophobic interactions (1,5,59,60). It should be noted, however, that viral protein-induced formation of any type of nonbilayer fusion intermediate has not been reported thus far.

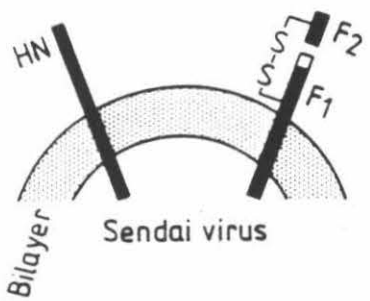
## IV. PROPERTIES OF VIRAL PROTEINS

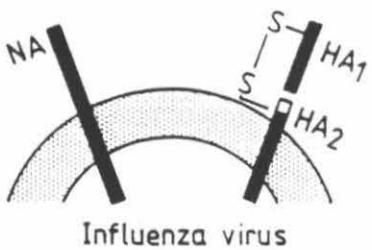
Virus entry into cells is accomplished by fusion of the viral lipid envelope with a membrane of the target cell. The viral glycoproteins (Figure 3) have the roles of binding to cell surface receptors and mediating the membrane fusion event (1,5,60). Many viruses have a glycoprotein which carries out both of these functions. Examples are the influenza hemagglutinin (HA) and the VSV G protein (1,61). Paramyxoviruses such as Sendai virus have separate binding (hemagglutinin/neuraminidase, HN) and fusion (F) proteins (5). Virus-cell binding takes place without any triggering event, but fusion activity requires some type of activation. In the case of influenza HA and VSV G proteins, a low pH activation is necessary to induce virus-cell fusion (1,61). In contrast, Sendai virus enters the cell by fusion with the plasma membrane at neutral pH (1,5,62).

A wide range of viruses have fusion proteins that contain a stretch of primarily hydrophobic amino acids in their primary sequence (1,5,59,63). These hydrophobic sequences are usually 20 or more amino acids in length, and often occur at the N-terminus of the proteins. The Sendai and influenza virus hydrophobic sequences are located at the N-termini of the $F_1$ and $HA_2$ subunits, respectively (3,63,64). X-ray crystallography of the bromelain-cleaved influenza $HA_2$ ectodomain ($BHA_2$) revealed that the fusion peptide is located near the viral membrane, hidden within the hemagglutinin trimer on the viral surface (65). Spectroscopic evidence has shown that

**Figure 3**

Schematic representation of some viral membrane proteins. The viral membrane glycoproteins comprise spike-like projections on the surface of the virus. For Sendai virus, HN is necessary for binding while fusion is mediated by the F protein. From the inactive precursor, $F_0$, the fusion active protein is generated, which consists of two polypeptides, $F_1$ and $F_2$, linked by a disulfide bridge. The hydrophobic segment resides in $F_1$ (open region). For influenza virus, only HA is relevant to binding (contained in $HA_1$) and fusion (contained in $HA_2$). The hydrophobic N-terminus of $HA_2$ (open segment) becomes exposed when the virus encounters a mild acid pH environment. The NA glycoprotein contains neuraminidase activity; its precise function is unknown, but this protein likely plays a role in viral assembly.


HN
S-S
F2
F1
Bilayer
Sendai virus


NA
S
S
HA1
HA2
Influenza virus

mild acidic pH induces a conformational change in HA, exposing the fusion peptide (65-68). The low pH induces the conformational change by titrating acidic amino acids and exposing hydrophobic residues (63,68). The titration of acidic amino acids has been demonstrated by studies of the pH-dependent fusion of liposomes induced by peptides similar to the $HA_2$ N-terminus (69). This mechanism is probably also active in fusion of other viruses, such as VSV, at low pH (61), as well as in fusion of liposomes induced by proteins such as lysozyme, clathrin, and α-lactalbumin (70-73). The ability of these proteins to induce fusion at low pH reflects the large negative free energy of insertion of hydrophobic peptides into liposomal membranes at low pH (63,74).

In addition to indirect evidence of hydrophobic interactions between fusion-inducing proteins and membranes, some attempts have been made to directly observe these protein-lipid interactions by photoaffinity labeling. In these experiments, a lipid-bound label or a hydrophobic label partitioning into the bilayer is photoactivated to covalently label proteins in contact with the hydrophobic core of the membrane. The label's fate is generally followed by using radioactive photoaffinity probes.

Hydrophobic photoaffinity labeling has proven a useful technique for identification of transmembrane segments of proteins, and protein subunits associated with membranes (75,76). Both lipid soluble probes (77-79) and lipid bound probes (80-84) have been successfully employed.

In hydrophobic photolabeling studies of influenza BHA, Harter et al. (84) demonstrated the specific low pH-induced labeling of the $BHA_2$ subunit after incubation with liposomes containing photoreactive lipids. Similar results were observed by Boulay et al. (85) using the membrane partitioning label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID).

Membrane organization of the Sendai virus F protein and the VSV G protein have been studied by photolabeling with lipid-bound and membrane soluble probes (86,87). Viral protein-protein interactions for these two viruses were also examined by chemical crosslinking (87,88). It is clear from these studies that protein-protein interaction is important in viral assembly and function. Supporting evidence suggesting the importance of protein-protein interaction in viral assembly was reported by Vaux et al. (89), demonstrating interactions between the cytoplasmic tail of SFV $E_2$ glycoprotein and the capsid protein, while trimerization of HA represents an essential feature in the biogenesis of influenza virus (90). In addition to a possible role in viral assembly, protein-protein interactions might also be important in stabilizing (fusion) active glycoprotein conformations.

## V. HYDROPHOBIC INTERACTION, A KEY EVENT IN VIRUS FUSION

Hydration and hydrophobic interactions are responsible for the stabilization of membrane bilayers (see Section III). As far as a virus is concerned, it is very likely therefore that its ability to undergo fusion is related to the physical properties of the target membranes, such as membrane packing, hydration, and phase. In this section several of these aspects will be discussed.

### *A. Penetration of viral proteins*

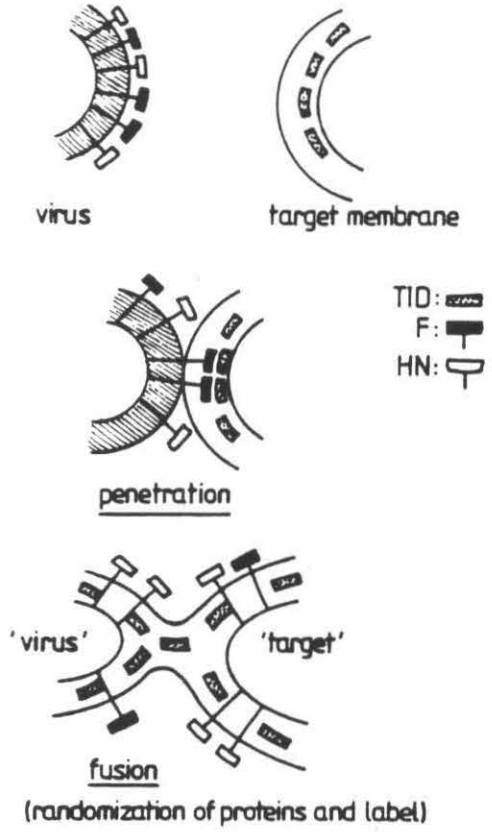
During the past several years, substantial evidence of the requirement for fusion proteins to initiate virus-membrane fusion has been reported (see Section IV). Based on the observation of hydrophobic amino acid sequences in a wide variety of fusion proteins, it was postulated that viral proteins initiate fusion by hydrophobic interactions with the target membrane (1,5,59,60). In this mechanism, insertion of the hydrophobic peptide into the target membrane might destabilize the bilayer structure and

lead to formation of a fusion intermediate. This type of mechanism is supported by reports of liposomal fusion induced by hydrophobic peptides at low pH (71-73). Titration of acidic amino acid side chains and exposure of hydrophobic surfaces for interaction with the membrane are necessary for promoting fusion.

Recently, several investigators have shown that the application of photoaffinity labels could be a promising tool in elucidating structural features of viral proteins (Section IV), including their interaction sites with target membranes. Photolabeling experiments are generally conducted by photolysis of a mixture of virus or viral protein with liposome or cell membranes containing a photoreactive label, after an incubation period for equilibration. These experiments provide important information about membrane organization of viral proteins, but do not necessarily give insight into the mechanism of protein-induced membrane fusion. The incubation period prior to photolysis may result in protein labeling after membrane fusion, i.e., under equilibration conditions. Hence, rather than reflecting the capacity of a putatively fusogenic peptide to induce fusion, it would reflect exposure of protein to the probe after reorganization of the membrane components accompanying fusion. In order to obtain mechanistic information about the role of hydrophobic interactions between a viral fusion peptide and a membrane involved in fusion, it is necessary to carry out the photoactivation *during* the early stages of the fusion reaction and before randomization of membrane components at stages secondary to fusion (Figure 4). This type of time-dependent photolabeling of a viral protein was recently reported (91), during Sendai virus fusion with liposomes. In these experiments, the hydrophobic photoaffinity label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID) was used. This molecule partitions effectively into membranes (82). Fusion between Sendai virus and reconstituted Sendai virus envelopes (RSVE) with both liposomes (91) and erythrocyte membranes (unpublished observations) was examined. The photoaffinity label was

**Figure 4**

Principle and problems of using photoaffinity labeling to detect protein penetration. Target membranes are labeled with the hydrophobic probe TID. A careful knowledge of the kinetics of fusion is essential in order to "catch" the penetration step and to avoid at random labeling that occurs when fusion proceeds to total membrane mixing. In the latter case transmembrane segments of all viral membrane proteins will become labeled. In addition, some probe may diffuse spontaneously from the target membrane, causing nonspecific labeling of the viral proteins. Appropriate controls are therefore required. The scheme depicts the fusion of Sendai virus, which requires HN for binding and F for fusion activity.


virus
target membrane
TID:
F:
HN:
penetration
'virus'
'target'
fusion
(randomization of proteins and label)

incorporated into the target membranes (large unilamellar vesicles composed of cardiolipin (CL) or PS, or erythrocyte membranes) by exogenous addition to the membrane preparations (for details, cf. 91). Virus and target membranes are mixed for various reaction times, and the samples are then photolyzed. After photolysis, the proteins are precipitated and separated by reducing SDS-PAGE and the fate of the TID probe is analyzed by gamma counting of slices of the polyacrylamide gel.

The TID probe, as a trifluoromethyldiazirine, preferentially generates a singlet carbene upon photolysis at temperatures in the range of 0 to 37°C (78,92). The singlet carbene shows zwitterionic reactivity and engages readily in "insertion" reactions. It displays a preference for addition to double bonds and insertion into polar single bonds, rather than a preference for formal C-H and C-C insertion that would be expected from a triplet (92,93). Phenyl azides commonly used in photolabeling usually generate a triplet nitrene. The result of the singlet carbene formation upon TID photolysis is that there is a preferential reaction with polar and double bonds present in proteins over the C-C and C-H bonds in the bilayer core. This effect permits substantial labeling of a transiently inserting protein in an excess of lipid, constituted by the membrane core. Normalization of the protein labeling is also possible as a result of the singlet reactivity, by comparison of the viral glycoprotein labeling (F/HN) under various conditions. Normalization of the data is important in viral work, due to the variability in viral activity with passage and handling.

The use of a lipid-soluble probe may result in some nonspecific labeling by diffusion out of the membrane prior to photolysis, causing background labeling of proteins not specifically involved in fusion. The extent to which such background labeling interferes with labeling during fusion can be readily evaluated by analyzing virus labeling with the free probe. When doing so for Sendai virus, it was excluded

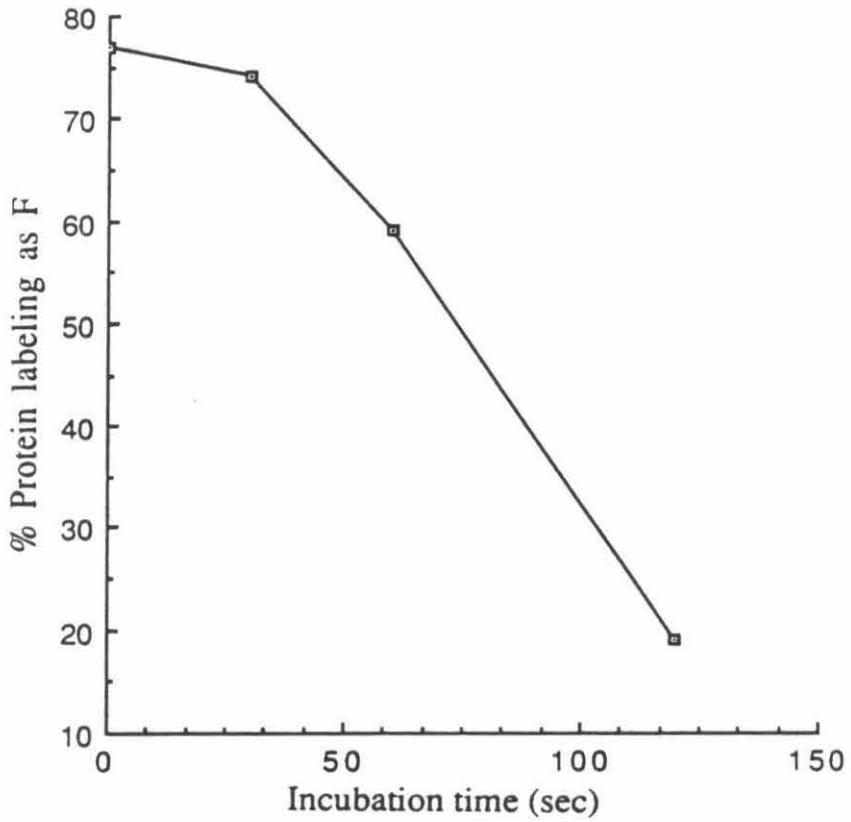
that diffusional labeling was responsive to conditions of the reaction, as labeling with the "free" probe was very dissimilar compared to labeling seen during fusion (91).

As described above, the fusion protein of Sendai virus (F) consists of two subunits, $F_1$ and $F_2$, which are generated upon proteolytic cleavage of the inactive precursor, $F_0$. As a result, a new N-terminal region is generated in $F_1$ and it is this strongly hydrophobic region (about 20 amino acids) which is thought to penetrate into the target membrane. Using TID-labeled liposomes, consisting of CL or PS, as target membranes and incubating such labeled membranes with Sendai virus, direct evidence was obtained, supporting this concept. During the early interaction events between virus and target membrane, a preferential labeling of $F_1$ is observed (Figure 5), consistent with the proposed hydrophobic insertion of this peptide into a target membrane. Furthermore, the extent of labeling increases substantially (about 2.5-3-fold) when the incubation temperature increases from 2 to 37°C. The increase in labeling parallels the increase in fusion activity of the virus, as monitored by a kinetic assay, as described in section II.

Fusion of viruses with CL liposomes does not in every respect follow the fusion characteristics seen when a biological membrane is used as the target (18,24). One peculiar aspect involves the ability of Sendai virus, normally fusing in the neutral pH region, to fuse with acidic phospholipid vesicles at acidic pH as well. This low pH-induced fusion has been proposed to result from an involvement of HN in fusion at those conditions (18,94), i.e., HN becomes an ordinary fusion protein, analogous to a variety of other, normally non-fusogenic proteins that acquire an increased hydrophobicity when the pH is lowered (for references, see 16). Indeed when incubating TID-labeled CL liposomes with Sendai virus at neutral pH and pH 5.0, respectively, the F/HN labeling ratio decreased strongly. More specifically, at pH 5.0, this ratio was approximately 5-fold lower than that observed at pH 7.4, which was due

**Figure 5**

Penetration of Sendai virus F protein during virus-liposome fusion. Sendai virus was incubated with TID labeled CL vesicles for various incubation times, followed by photolysis of the mixture for 30 s. Afterwards, the viral proteins were separated by PAGE, slices of the gel were counted and the labeling of $F_1$, as a percentage of total protein labeled, was calculated. Note that (i) $F_1$ and $F_2$ are separated on SDS gels (cf. Figure 3) and that (ii) $F_1$ is almost exclusively labeled when irradiation is started immediately upon mixing virus and liposomes (zero time). After longer incubation times, much of the label is associated with other viral proteins as well (cf. Figure 4). Data are from ref. 91.


80
70
60
50
40
30
20
10
% Protein labeling as F
0
50
100
150
Incubation time (sec)

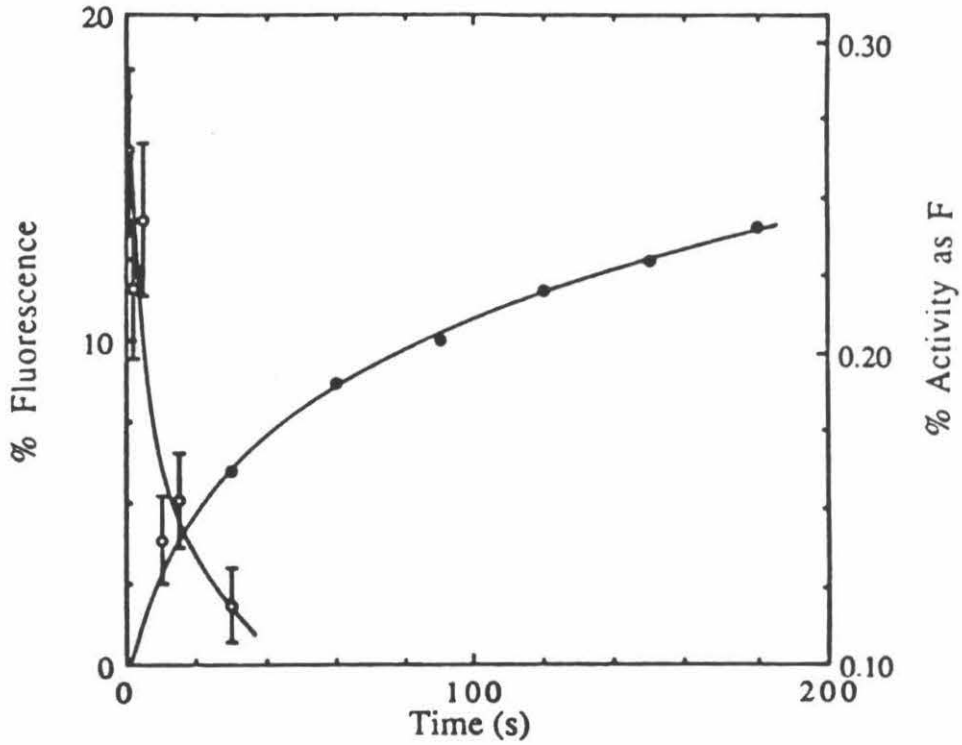
to a strong increase in HN-labeling at acidic pH. The same trends in F/HN interactions could be seen during fusion between reconstituted Sendai virus envelopes and liposomes, signifying that the same type of interactions were active in both the native and reconstituted viral fusion events.

In more recent experiments, in which a very high photon flux was used for photolysis, it was found that the hydrophobic insertion of the viral fusion protein ($F_1$) occurs on a substantially faster timescale than the kinetics of membrane fusion, assayed by lipid mixing (9; Figure 6). These results could imply a fusion mechanism involving more than one step. The first hydrophobic penetration step may serve simply to bring the apposed bilayers close enough together. The second step could involve additional conformational changes in the fusion protein which induce the bilayer destabilization necessary to initiate fusion (see also below).

Hydrophobic photolabeling with TID and TID-containing phospholipids has also been employed to show that the $HA_2$ subunit of influenza virus hemagglutinin mediates the interaction of the bromelain-cleaved HA ectodomain with liposomes at low pH (84). Using tritium labeled phosphatidylcholines with TID-derivatized *sn*-2 acyl chains, the authors demonstrated unambiguously that only the $HA_2$ subunit interacts hydrophobically with the membrane under fusogenic conditions (low pH). These data agree with the model of influenza virus fusion at low pH, in which a conformational change exposes the $HA_2$ hydrophobic N-terminus for interaction with the target membrane (66). In labeling experiments with lipid-soluble TID, some residual labeling of $BHA_1$ was observed. This is probably due to the presence of hydrophobic pockets on the protein in close proximity with, but not penetrating into, the membrane. Using water soluble proteins, the authors showed that TID labels hydrophobic pockets of proteins which are not in contact with the membrane. Labeling of these proteins by one

**Figure 6**

Kinetics of Sendai virus $F_1$ penetration vs. kinetics of fusion with phospholipid vesicles. Sendai virus was incubated with TID labeled CL vesicles and $F_1$ labeling was determined as a function of the incubation time. The amount of radioactivity associated with $F_1$ (open circles) is expressed as a percentage of the total liposome-associated activity. In this experiment photolysis was conducted with a high photon flux, using a 1000 W Xe arc lamp. In a parallel experiment, the kinetics of fusion were assayed, using the $R_{18}$ assay (closed circles). For details, see text.


20
10
0
0
100
200
Time (s)
% Fluorescence
0.30
0.20
0.10
% Activity as F

of the photoreactive lipids may indicate that water soluble proteins do interact hydrophobically with membranes at low pH.

Other investigators have also examined lipid interactions of viral fusion proteins using TID. Asano and Asano (59) reported the hydrophobic photolabeling of the intact Sendai virus F protein by TID, partitioned into the plasma membrane of cells. The F protein was labeled when virus was added to cells at 4°C, indicating that a close association of the protein with the target cell membrane occurs during the binding step, since fusion does not occur at temperatures below about 20°C.

The use of equilibrated systems for photolabeling must be avoided in mechanistic studies, however, in that the labeling under these conditions reflects interactions taking place after much of the fusion reaction has occurred. Since one would expect the hydrophobic interaction to occur at an early point during the fusion reaction, it will be necessary to photolyze the samples at early timepoints. This is often inconvenient and expensive, as the reaction must be carefully timed, and little probe will have accumulated in the fusion protein. This necessitates use of a larger amount of probe, which is an expensive proposition.

*B. Effect of target membrane packing*

Given the need for hydrophobic penetration of a viral fusion protein to trigger the fusion reaction, one could intuitively envision that penetration as such might be affected by the packing density of the target membrane. To investigate this parameter, liposomes are evidently a most convenient tool as biological membranes are not readily amenable to extensive changes in lipid composition. Various studies in which liposomes of different compositions have been used to investigate (viral) protein-induced fusion or protein-membrane interactions *per se* indicate that packing of the hydrophobic acyl chains can indeed affect fusion. In general, poorer packing promotes protein insertion (cf. 95) and fusion. The VSV G protein shows greater activity in the

presence of *cis*-unsaturated lipids (35), while clathrin induces fusion of DOPC vesicles, but not of egg PC vesicles (71). Furthermore, Sendai virus fuses more readily with CL liposomes than with PS liposomes (18). In fact, kinetic simulation of those data revealed that the fusion rate constant of virus-CL vesicle fusion was enhanced relative to that of virus-PS vesicle fusion, without a significant change in the aggregation rate constants (22). The amounts of TID labeling seen during virus fusion with CL vesicles, as discussed above, were, moreover, consistently higher than those during virus-PS vesicle fusion (about two- to three-fold), which is consistent with the higher extent and kinetics of fusion in the former case.

*C. Role of membrane hydration in virus fusion*

In studies of fusion of liposomes, it has become apparent that hydration forces govern to a major extent the ability of apposed membranes to fuse (16,37,38,42). As described above (section III), $Ca^{2+}$ appears to form a strongly dehydrated complex with PS, acting as a nucleation site in $Ca^{2+}$-induced fusion of PS vesicles. Inclusion of a strongly hydrated lipid such as PC in PS vesicles, inhibits $Ca^{2+}$-induced fusion whereas the fusion event is sustained when PE, a weakly hydrated phospholipid, is incorporated in negatively charged bilayers (37). In fusion studies involving the use of synthetic amphiphiles (48-50), the overall process of vesicle aggregation and fusion itself is triggered at different divalent cation concentrations. This control over both processes allows the detailed analysis of each step separately. In the case of the negatively charged amphiphile didodecylphosphate (DDP), it could be shown that fusion is triggered by formation of an anhydrous trans-$Ca^{2+}$/DDP complex (50,51), whose formation is dependent on membrane surface dehydration.

The dehydrating polymer poly(ethylene glycol) (PEG) has proven a useful tool in the study of hydration effects in membrane fusion. Binding of water molecules by PEG decreases the polarity of the solvent phase near the membrane surface,

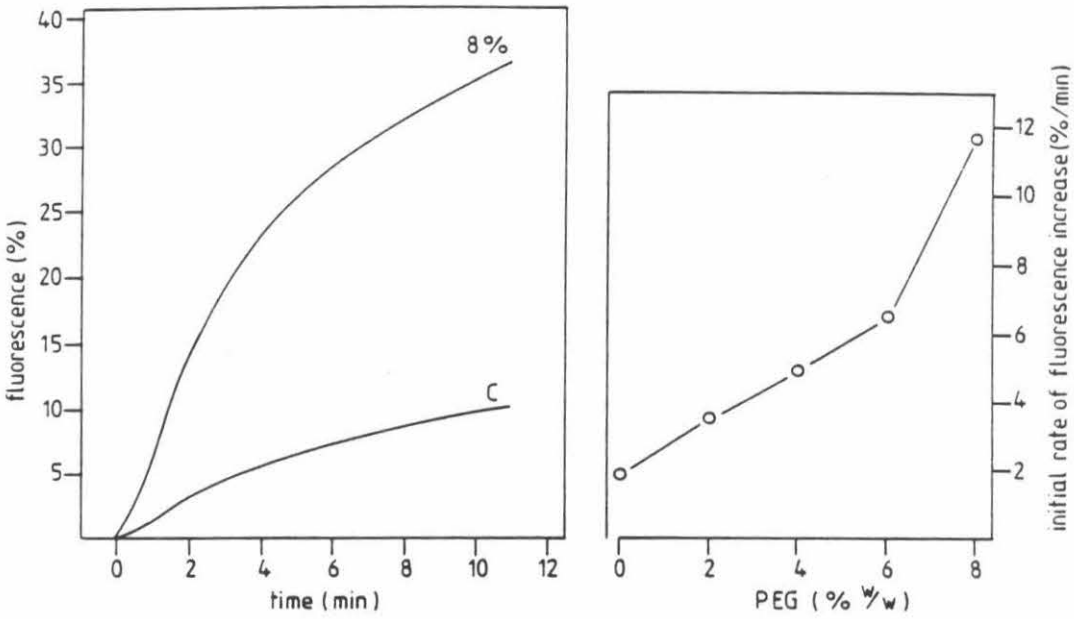
destabilizing the membrane (46,96). This dehydrating capacity limits water available for binding to lipid headgroups. By varying the state of surface hydration, low concentrations of PEG substantially increase the $Ca^{2+}$-dependent rate of fusion of PS vesicles (97). Thus, membrane dehydration can have a profound effect on fusion of artificial membranes.

To determine whether membrane hydration is also central to fusion of biological membranes, similar approaches have been used as those described for artificial systems. When studying Sendai virus-liposome interactions, it was observed that analogous to $Ca^{2+}$-induced fusion of liposomes, the presence of PC causes inhibition of fusion between the virus and negatively charged bilayers, with increasing mole ratios of PC. By contrast, the fusion reaction was sustained when similar concentrations of PE were included. Using a mass action kinetic model, it was shown that PC caused a two-fold decrease in the fusion rate constant, while barely affecting the rate constant for aggregation (21,22). In contrast, small amounts (0-8%) of PEG 8K caused the fusion rate constant to triple with little effect on the aggregation rate constant. Both of these results suggest that hydration of the membrane surface plays a critical role in regulating virus-liposome fusion.

As noted above, virus-liposome fusion does not necessarily reflect the fusion conditions as they occur in a physiological environment. That membrane hydration may be critical to biological membrane fusion as well, was suggested by experiments which demonstrated that low concentrations of PEG (which do not cause fusion by themselves) substantially increase the kinetics of virus-erythrocyte membrane fusion (Figure 7; 98). It was excluded that this increase was due to a nonspecific fusion process, since the limited number of the virus particles that fuse with one erythrocyte (approximately 100-200 out of 1000 active virus particles that bind) was the same, regardless of the presence of the polymer. Kinetic analysis showed that the aggregation

**Figure 7**

PEG facilitates Sendai virus-erythrocyte membrane fusion. Sendai virus was labeled with a self-quenching concentration of $R_{18}$, and fusion with erythrocyte ghosts was monitored as depicted in Figure 2. In the left panel, fluorescence tracings are shown for fusion taking place in the absence (C) or presence (8 wt %) of PEG 8K. The increase in the initial rate is dependent of the PEG concentration (right panel). The *final* extent of fusion, as determined after long-term incubations, does not increase.


40
35
30
25
20
15
10
5
fluorescence (%)
8%
C
0
2
4
6
8
10
12
time (min)
12
10
8
6
4
2
initial rate of fluorescence increase (%/min)
0
2
4
6
8
PEG (% w/w)

rate constant doubled and the fusion rate constant increased by nearly an order of magnitude in the presence of PEG. These results indicate that viral membrane fusion with a purely biological target is also strongly dependent on membrane hydration. The details of the effect of PEG have been further examined by determining the extent to which a separate preincubation of viruses or target membranes in PEG-containing media modulated the fusion reaction. It was observed (98) that the rate of fusion doubled when the virus was preincubated in a PEG-containing medium. By contrast, no change was seen when the target membranes were preincubated. This implies that the virus itself is highly sensitive towards the polarity of its environment and becomes more fusogenic in a more hydrophobic environment.

The observation that PEG also slightly increases the aggregation rate constant implies that the interaction of the binding protein of Sendai virus, HN, is not solely governed by electrostatic interactions with sialic acid-containing receptors on the cell surface. Indeed, previous results (20) indicated that structural requirements have to be met as well for the binding protein to interact properly with the receptor. It could thus be suggested that structural integrity of HN is necessary to allow for hydrophobic interactions, accompanying the initial electrostatic interactions. It should be noted in this regard, that at this initial attachment the interbilayer distance between virus and target membrane can be on the order of about 100 Å, a distance at which hydration forces are of minor significance. With liposomes, changes in the aggregation rate constants were less pronounced than those observed with erythrocyte membranes (22). This suggests differences in the mechanism of attachment of virions in the process of fusion with liposomes as compared to that during virus-erythrocyte membrane fusion. Indeed, except in the case of PS liposomes, PEG induced an enhancement of the final levels of fusion between Sendai virus and cardiolipin and cardiolipin/phosphatidylcholine vesicles (22). It would appear, therefore, that fusion of

a virus with a biological target membrane is more strictly controlled than the fusion of a virus with a liposomal membrane.

The effect of PEG on virus-erythrocyte membrane fusion suggests that the hydrophobic environment can modulate the fusion efficiency of the virus. After penetration of $F_1$ into the target membrane, an interbilayer distance (between virus and target membrane) of about 100 Å still exists and this gap obviously has to be overcome before merging of both bilayers occurs. One would anticipate that additional structural alterations have to take place in the F protein to "bridge" this gap. As a preincubation of virus alone in PEG causes a doubling of the virus fusion rate, relative to that seen for fusion (in a PEG-containing medium) without preincubation, it is possible that hydrophobic dehydration may trigger such a conformational change. If this scenario were true, the *overall* activation of the fusion protein of viruses fusing at neutral pH would entail a two step activation mechanism. The first step would involve proteolytic cleavage of the inactive fusion protein precursor, occurring during virus biogenesis, while the second step would represent a structural change, which accompanies the penetration step. This sequence of events would bear some similarity to that seen for viruses fusing at acidic pH. For viruses penetrating via receptor-mediated endocytosis, mild acidic pH induces a major conformational change in the protein structure, responsible for fusion, before the (buried) hydrophobic sequence becomes exposed (3,66).

In summary, it is evident that many details of the mechanism of virus fusion remain to be elucidated. It is also clear that differences exist in virus fusion mechanisms when comparing artificial and biological target membranes. However, in developing basic strategies to unravel the details relevant for fusion at physiological conditions, liposomes can provide a valuable tool.

## ACKNOWLEDGMENTS

Personal work cited in this paper was supported by grants from the National Institutes of Health (AI 25534 and RR07003), Monsanto and Amersham. S.L.N. is the recipient of an NSF Graduate Fellowship. The expert secretarial assistance of Mrs. Rinske Kuperus and Mrs. Lineke Klap is gratefully acknowledged.

## REFERENCES

1. White, J., Kielian, M. and Helenius, A. (1983) *Q. Rev. Biophys.* **16**, 151-195.

2. Spear, P.G., Wittles, M., Fuller, A.D., Wudunn, D. and Johnson, R. (1989) in *Cell Biology of Virus Entry, Replication and Pathogenesis* (Compans, R., Helenius, A. and Oldstone, M.B.A., Eds.), Liss: N.Y., 163-175.

3. Wiley, D.C. and Skehel, J.J. (1987) *Ann. Rev. Biochem.* **56**, 365-394.

4. Doms, R.W. and Helenius, A. (1988) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W. and Mayhew, E., Eds.), Plenum: N.Y., 385-398.

5. Choppin, P.W. and Scheid, A. (1980) *Rev. Infect. Dis.* **2**, 40-61.

6. McCune, J.M., Rabin, L.B., Feinberg, M.B., Lieberman, M., Kosek, J.C., Reyes, G.R. and Weissman, J.L. (1988) *Cell* **53**, 55-67.

7. Homma, M., Shimizu, Y.K. and Ishida, D. (1976) *Virology* **71**, 41-47.

8. Harmsen, M.C., Wilschut, J., Scherphof, G., Hulstaert, C. and Hoekstra, D. (1985) *Eur. J. Biochem.* **149**, 591-599.

9. Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) *Biochemistry* **23**, 5675-5681.

10. Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) *Biochemistry* **20**, 4093-4099.

11. Eidelman, O., Schlegel, R., Tralka, T.S. and Blumenthal, R. (1984) *J. Biol. Chem.* **259**, 4622-4628.

12. Stegmann, T., Hoekstra, D., Scherphof, G. and Wilschut, J. (1985) *Biochemistry* **24**, 3107-3113.

13. Chejanovsky, N. and Loyter, A. (1985) *J. Biol. Chem.* **260**, 7911-7918.

14. Wilschut, J. and Papahadjopoulos, D. (1979) *Nature* **281**, 690-692.

15. Ellens, H., Bentz, J. and Szoka, F.C. (1985) *Biochemistry* **24**, 3099-3106.

16. Hoekstra, D. and Wilschut, J. (1989) in *Water Transport in Biological Membranes* (Benga, G., Ed.), CRC Press: Boca Raton, FL, 143-176.

17. Loyter, A., Citovsky, V. and Blumenthal, R. (1988) *Meth. Biochem. Anal.* **33**, 129-164.

18. Klappe, K., Wilschut, J., Nir, S. and Hoekstra, D. (1986) *Biochemistry* **25**, 8252-8260.

19. Hoekstra, D., Klappe, K., De Boer, T. and Wilschut, J. (1985) *Biochemistry* **24**, 4739-4745.

20. Hoekstra, D. and Klappe, K. (1986) *J. Virol.* **58**, 87-95.

21. Nir, S., Klappe, K. and Hoekstra, D. (1986) *Biochemistry* **25**, 2155-2161.

22. Nir, S., Klappe, K. and Hoekstra, D. (1986) *Biochemistry* **25**, 8261-8266.

23. Nir, S., Stegmann, T. and Wilschut, J. (1986) *Biochemistry* **25**, 257-266.

24. Stegmann, T., Hoekstra, D., Scherphof, G. and Wilschut, J. (1986) *J. Biol. Chem.* **261**, 10966-10969.

25. Bundo-Morita, K., Gibson, S. and Lenard, J. (1987) *Biochemistry* **26**, 6223-6227.

26. Blumenthal, R., Bali-Puri, A., Walter, A., Covell, D. and Eidelman, O. (1987) *J. Biol. Chem.* **262**, 13614-13619.

27. Puri, A., Winick, J., Lowy, R.J., Covell, D., Eidelman, O., Walter, A. and Blumenthal, R. (1988) *J. Biol. Chem.* **263**, 4749-4753.

28. Lorge, P., Cabiaux, V., Long, L. and Ruysschaert, J.M. (1986) *Biochim. Biophys. Acta* **858**, 312-316.

29. Vanderwerf, P. and Ullman, E.F. (1980) *Biochim. Biophys. Acta* **596**, 302-314.

30. Uster, P.S. and Deamer, D.W. (1981) *Arch. Biochem. Biophys.***209**, 385-395.

31. Haywood, A.M. and Boyer, B.P. (1982) *Biochemistry* **21**, 6041-6046.

32. Maeda, T., Asano, A., Ohki, Y. and Ohnishi, S. (1975) *Biochemistry* **14**, 3736-3741.

33. Maeda, T., Kawasaki, K. and Ohnishi, S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4133-4137.

34. Maeda, T., Kuroda, K., Toyama, S. and Ohnishi, S. (1981) *Biochemistry* **20**, 5340-5345.

35. Yamada, S. and Ohnishi, S. (1986) *Biochemistry* **25**, 3703-3708.

36. Stein, B.S., Gowda, S.D., Lifson, J.D., Penhallow, R.C., Bensch, K.G. and Engleman, E.G. (1987) *Cell* **49**, 659-668.

37. Düzgünes, N. (1985) *Subcell. Biochem.* **11**, 195-286.

38. Wilschut, J. and Hoekstra, D. (1986) *Chem. Phys. Lipids* **40**, 145-166.

39. Hoekstra, D. (1988) *Ind. J. Biochem. Biophys.* **25**, 76-84.

40. Israelachvili, J.N., Marcelja, S. and Horn, R.G. (1980) *Q. Rev. Biophys.* **13**, 121-200.

41. Tanford, C. (1980) *The Hydrophobic Effect*, Wiley-Interscience: N.Y.

42. Rand, R.P. (1981) *Ann. Rev. Biophys. Bioeng.* **10**, 277-314.

43. Rand, R.P. and Parsegian, V.A. (1984) *Can. J. Biochem. Cell Biol.* **62**, 752-759.

44. Leikin, S.L., Kozlov, M.M., Chernomordik, L.V., Markin, V.S. and Chizmadzhev, Y.A. (1987) *J. Theor. Biol.* **129**, 411-425.

45. Ohki, S. (1985) *Studia Biophysica* **110**, 95-104.

46. Hoekstra, D., Rupert, L.A.M., Engberts, J.B.F.N., Nir, S., Hoff, H., Klappe, K. and Novick, S.L. (1988) *Studia Biophysica* **127**, 105-112.

47. Rand, R.P., Fuller, N., Parsegian, V.A. and Rau, D.C. (1988) *Biochemistry* **27**, 7711-7722.

48. Rupert, L.A.M., Hoekstra, D. and Engberts, J.B.F.N. (1985) *J. Am. Chem. Soc.* **107**, 2628-2631.

49. Rupert, L.A.M., Engberts, J.B.F.N. and Hoekstra, D. (1986) *J. Am. Chem. Soc.* **108**, 3920-3925.

50. Rupert, L.A.M., Van Breemen, J.F.C., Van Bruggen, E.F.J., Engberts, J.B.F.N. and Hoekstra, D. (1987) *J. Membr. Biol.* **95**, 255-263.

51. Rupert, L.A.M., Hoekstra, D. and Engberts, J.B.F.N. (1987) *J. Colloid Interface Sci.* **120**, 125-134.

52. Rupert, L.A.M., Engberts, J.B.F.N. and Hoekstra, D. (1988) *Biochemistry* **27**, 8232-8239.

53. Rupert, L.A.M., Van Breemen, J.F.C., Hoekstra, D. and Engberts, J.B.F.N. (1988) *J. Phys. Chem.* **92**, 4416-4420.

54. Prestegard, J.H. and O'Brien, M.P. (1987) *Ann. Rev. Phys. Chem.* **38**, 383-411.

55. Siegel, D.P. (1986) *Biophys. J.* **49**, 1171-1183.

56. Siegel, D.P., Burns, J.L., Chestnut, M.H. and Talmon, Y. (1989) *Biophys. J.* **55**, 342a.

57. Verkleij, A.J., Leunissen-Bijvelt, J., De Kruijff, B., Hope, M. and Cullis, P.R. (1984) in *Cell Fusion* (Evered, D. and Whelan, J., Eds.), Pitman: London, 45-59.

58. Markin, V.S., Kozlov, M.M. and Borovjagin, V.L. (1984) *Gen. Physiol. Biophys.* **2**, 361-377.

59. Asano, A. and Asano, K. (1984) *Tumor Res.* **19**, 1-20.

60. Loyter, A. and Volsky, D.J. (1982) *Cell Surf. Rev.* **8**, 215-266.

61. Pal, R., Barenholz, Y. and Wagner, R.R. (1987) *Biochim. Biophys. Acta* **906**, 175-193.

62. Scheid, A. and Choppin, P.W. (1977) *Virology* **80**, 54-66.

63. Ohnishi, S. (1988) *Curr. Top. Membr. Transp.* **32**, 257-296.

64. Blumberg, B.H., Giorgi, C., Rose, K. and Kolakofsky, D. (1985) *J. Gen. Virol.* **66**, 317-331.

65. Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A. and Wiley, D.C. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 968-972.

66. Doms, R.W., Helenius, A. and White, J.M. (1985) *J. Biol. Chem.* **260**, 2973-2981.

67. Wharton, S.A., Skehel, J.J. and Wiley, D.C. (1986) *Virology* **149**, 27-35.

68. Wharton, S.A., Ruigrok, R.W.H., Martin, S.R., Skehel, J.J., Bayley, P.M., Weis, W. and Wiley, D.C. (1988) *J. Biol. Chem.* **263**, 4474-4480.

69. Lear, J.D. and De Grado, W.F. (1987) *J. Biol. Chem.* **262**, 6500-6505.

70. Arvinte, T., Hildenbrand, K., Wahl, P. and Nicolau, C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 962-966.

71. Hong, K., Yoshimura, T. and Papahadjopoulos, D. (1985) *FEBS Lett.* **191**, 17-23.

72. Kim, J. and Kim, H. (1986) *Biochemistry* **25**, 7867-7874.

73. Maezawa, S., Yoshimura, T., Hong, K., Düzgünes, N. and Papahadjopoulos, D. (1989) *Biochemistry* **28**, 1422-1428.

74. Von Heijne, G. (1981) *Eur. J. Biochem.* **116**, 419-422.

75. Brunner, J. (1981) *Trends Biochem. Sci.* **6**, 44-46.

76. Sigrist, H. and Zahler, P. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A.N., Ed.), Plenum: N.Y., 333-370.

77. Brunner, J. and Semenza, G. (1982) *Biochemistry* **20**, 7174-7182.

78. Richards, F.M. and Brunner, J. (1980) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 144-164.

79. Gitler, C. and Bercovici, T. (1980) in *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 199-211.

80. Chakrabarti, P. and Khorana, H.G (1975) *Biochemistry* **14**, 5021-5033.

81. Radhakrishnan, R., Gupta, C.M., Erni, B., Robson, R.J., Curatolo, W., Majumdar, A., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1980) IN *Applications of Photochemistry in Probing Biological Targets* (Tometsko, A.M. and Richards, F.M., Eds.), N.Y. Acad. Sci.: N.Y., 165-198.

82. Brunner, J., Spiess, M., Aggeler, R., Huber, P. and Semenza, G. (1983) *Biochemistry* **22**, 3812-3820.

83. Schroit, A.J., Madsen, J. and Ruoho, A. (1987) *Biochemistry* **26**, 1812-1819.

84. Harter, C., Bächi, T., Semenza, G. and Brunner, J. (1988) *Biochemistry* **27**, 1856-1864.

85. Boulay, F., Doms, R.W. and Helenius, A. (1986) in *Positive Strand RNA Viruses* (Brinton, M.A. and Rueckert, R.R., Eds.), Liss: N.Y., 103-112.

86. Moscufo, N., Gallina, A., Schiavo, G., Montecucco, C. and Tomasi, M. (1987) *J. Biol. Chem.* **262**, 11490-11496.

87. Zakowski, J.J. and Wagner, R.R. (1980) *J. Virol.* **36**, 93-102.

88. Markwell, M.A.K. and Fox, C.F. (1980) *J. Virol.* **33**, 152-166.

89. Vaux, D.J.T., Helenius, A. and Mellman, I. (1988) *Nature* **336**, 36-42.

90. Copeland, C.S., Zimmer, K.P., Wagner, K.R., Healy, G.A., Mellman, I. and Helenius, A. (1988) *Cell* **53**, 197-209.

91. Novick, S.L. and Hoekstra, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7433-7437.

92. Turro, N.J. (1978) *Modern Molecular Photochemistry*, Benjamin/Cummings: Menlo Park, CA, 551-557.

93. Kanakarajan, K., Goodrich, R., Young, M.J.T., Soundararajan, S. and Platz, M.S. (1988) *J. Am. Chem. Soc.* **110**, 6536-6541.

94. Amselem, S., Barenholz, Y., Loyter, A., Nir, S. and Lichtenberg, D. (1986) *Biochim. Biophys. Acta* **860**, 301-313.

95. Boggs, J.M., Stollery, J.G. and Moscarello, M.A. (1980) *Biochemistry* **19**, 1226-1234.

96. Arnold, K., Hermann, A., Gawrisch, K. and Pratsch, L. (1988) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W. and Mayhew, E., Eds.), Plenum: N.Y., 255-272.

97. Hoekstra, D. (1982) *Biochemistry* **21**, 2833-2840.

98. Hoekstra, D., Klappe, K., Hoff, H. and Nir, S. (1989) *J. Biol. Chem.*, in press.

# Part II:
# Kinetics of Bacteriophage λ DNA Injection

# Chapter 7
# Introduction

## IMPORTANCE AND USES OF BACTERIOPHAGE λ

Bacteriophage λ has become the predominant tool used by molecular biologists and biochemists for cloning in bacteria, and has had a major impact on both science and medicine. The bacteriophage contains a double-stranded 48.5 kb genome, which includes cohesive ("cos") sites in which exogenous DNA can be incorporated. The plasmid to which the exogenous DNA is added ("cosmid") can be packaged in the bacteriophage *in vitro*. When the phage infects bacteria, the inserted gene is carried along and reproduces with the bacteriophage. The cosmid carries a gene for antibiotic resistance, so bacteria containing the gene of interest can be selected (Figure 1).

The *in vitro* packaging capacity of λ is specific and efficient (2-5). Commercial packaging kits generally contain two separate phage solutions, with complementary mutations in the phage assembly genes. Mixing the two solutions together creates infectious phage particles with packaging efficiencies of $10^8$-$10^9$ pfu/μg DNA (6; Figure 2).

## CHARACTERISTICS OF THE CELLULAR RECEPTOR

Bacteriophage λ infects Gram-negative bacteria by attaching to the LamB protein in the outer membrane (7). LamB is an integral membrane protein, consisting of three 47 kD subunits (8), and functions in the cell as a pore for maltose transport (9). The LamB protein is exceptionally stable to laboratory chemical treatment (10), and when reconstituted into liposomal membranes, has been shown indirectly to bind phage and trigger DNA injection (11). This data was obtained using the plaque inactivation assay, which measures receptor capacity to inactivate the bacteriophage's ability to infect bacteria and form plaques on a plate (Figure 3). These observations led to the hypothesis that the phage might be capable of injecting DNA into eukaryotic cells, if the receptor could be implanted into a cell membrane (12).

**Figure 1**

Method of cloning in cosmids in bacteriophage λ.

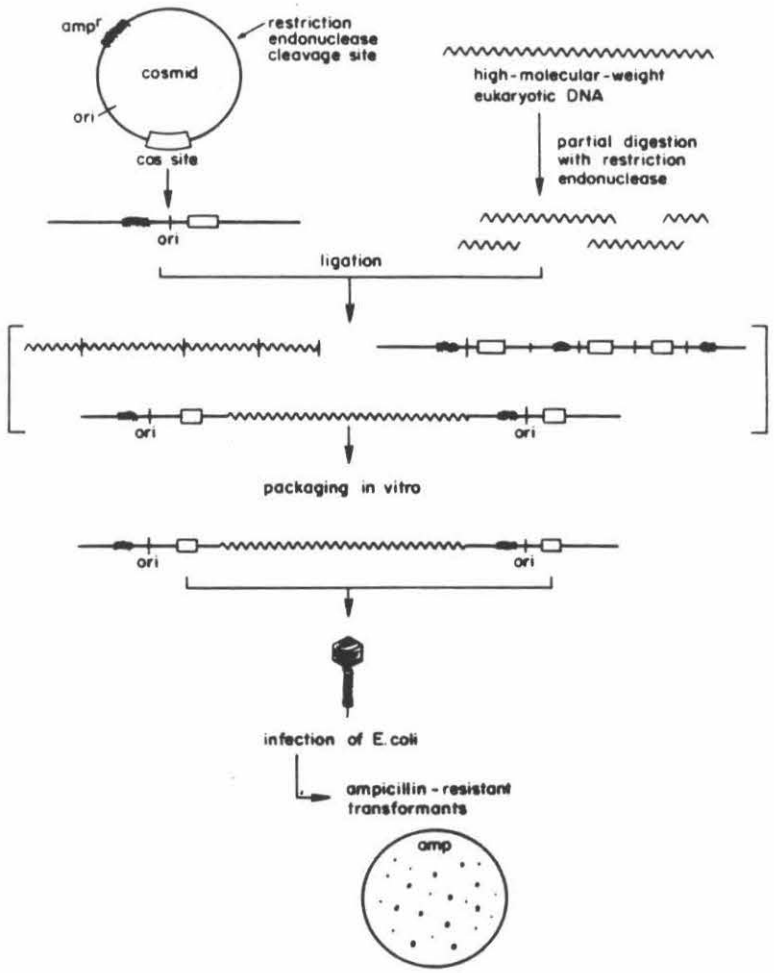

amp^r
restriction endonuclease cleavage site
cosmid
ori
cos site
high-molecular-weight eukaryotic DNA
partial digestion with restriction endonuclease
ligation
packaging in vitro
infection of E.coli
ampicillin-resistant transformants
amp

**Figure 2**

Pathway of λ assembly. Many *in vitro* packaging kits contain mutations of the D and E gene products.

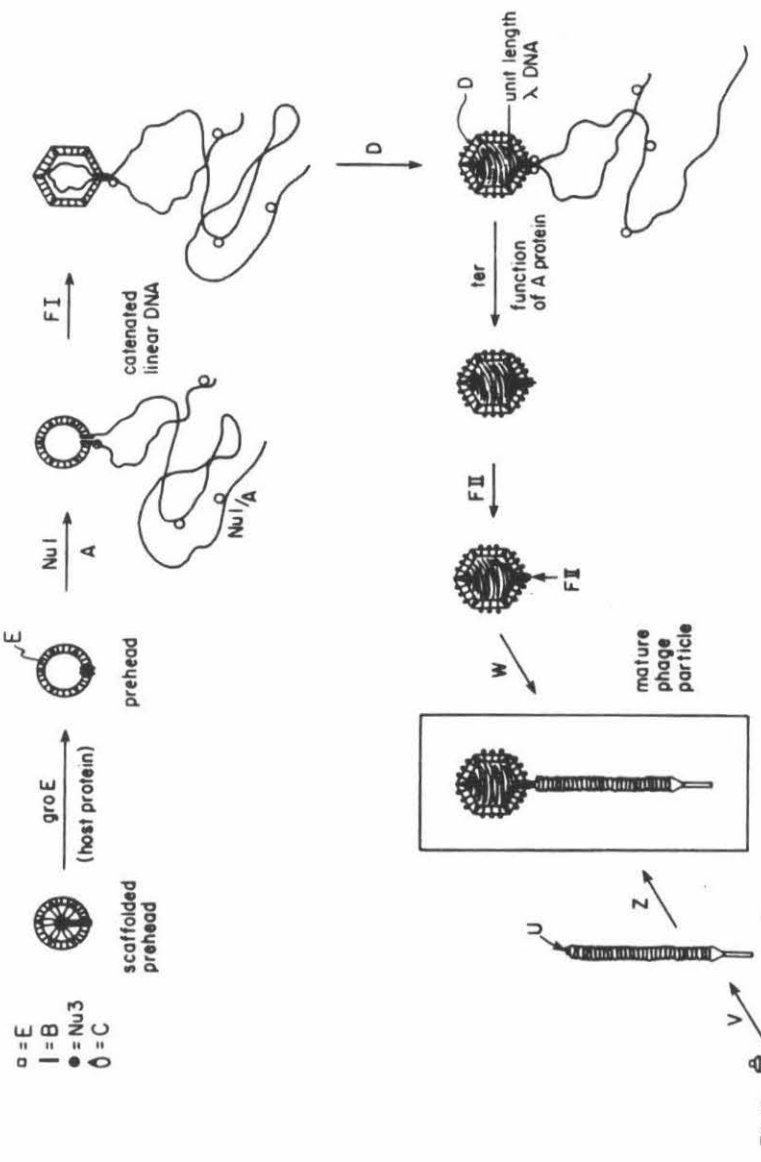
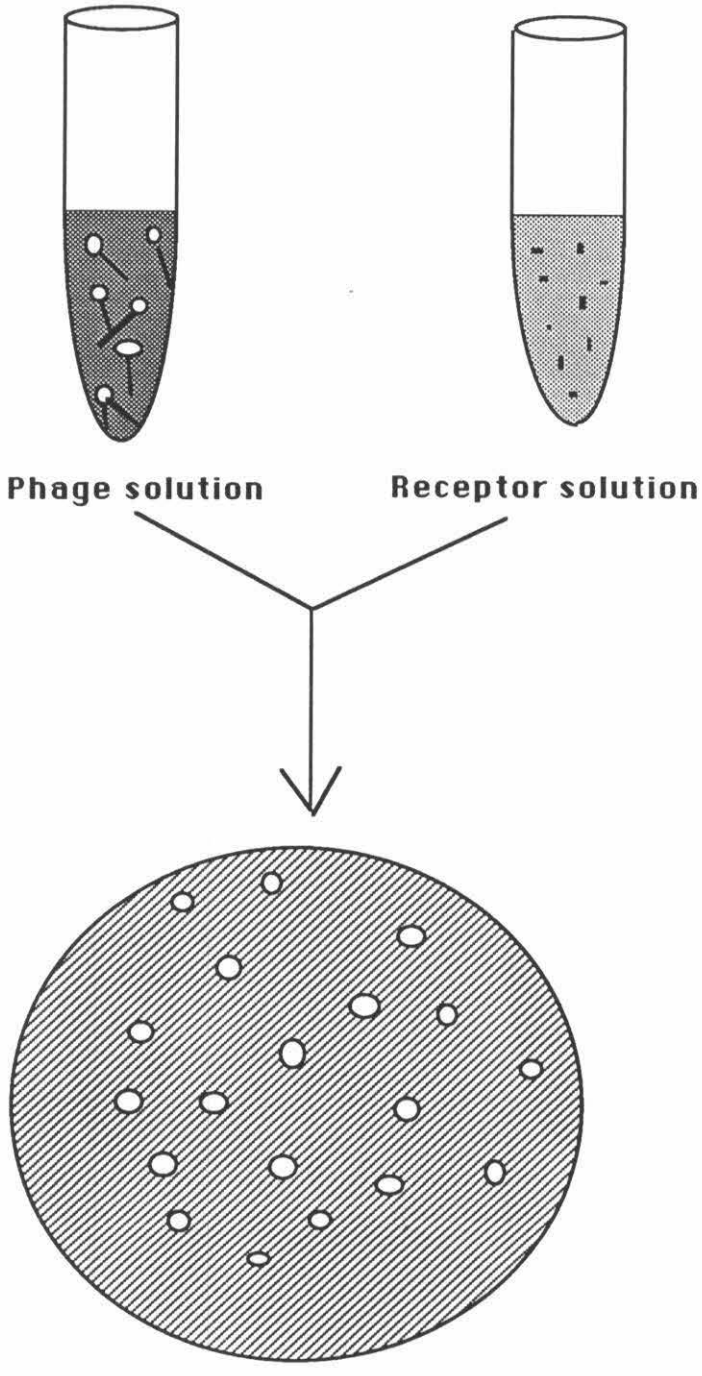

□ = E
I = B
● = Nu3
◊ = C
scaffolded prehead
gro E
(host protein)
prehead
E
Nu I
A
Nu I/A
catenated linear DNA
FI
D
unit length λ DNA
D
ter
function of A protein
FII
FII
W
mature phage particle
U
Z
V
I L K
G H M
J

**Figure 3**

The plaque inhibition assay measures receptor capacity to inactivate bacteriophage particles, preventing them from infecting and causing plaque formation on a plate of bacteria.


Phage solution
Receptor solution

## IMPLICATIONS AND EXPERIMENTAL APPROACH

Current efforts at gene delivery to mammalian cells are limited by various technical difficulties. Calcium phosphate/DNA mediated gene transfer, electroporation, and microinjection are limited by variable or low efficiency transfer in many cell types and the inability to transfect large numbers of cells (13-15). Virus mediated gene transfer is usually limited by small gene capacity (16). Retroviral vectors frequently delete key genes during replication, causing loss of the gene of interest (17). Liposomal gene transfer is restricted by low efficiency encapsulation of macromolecules (18,19). Animal viruses using existing host cell receptors are limited by natural host range. By artificially implanting LamB into target cells, host range restrictions for λ mediated gene transfer may possibly be overcome.

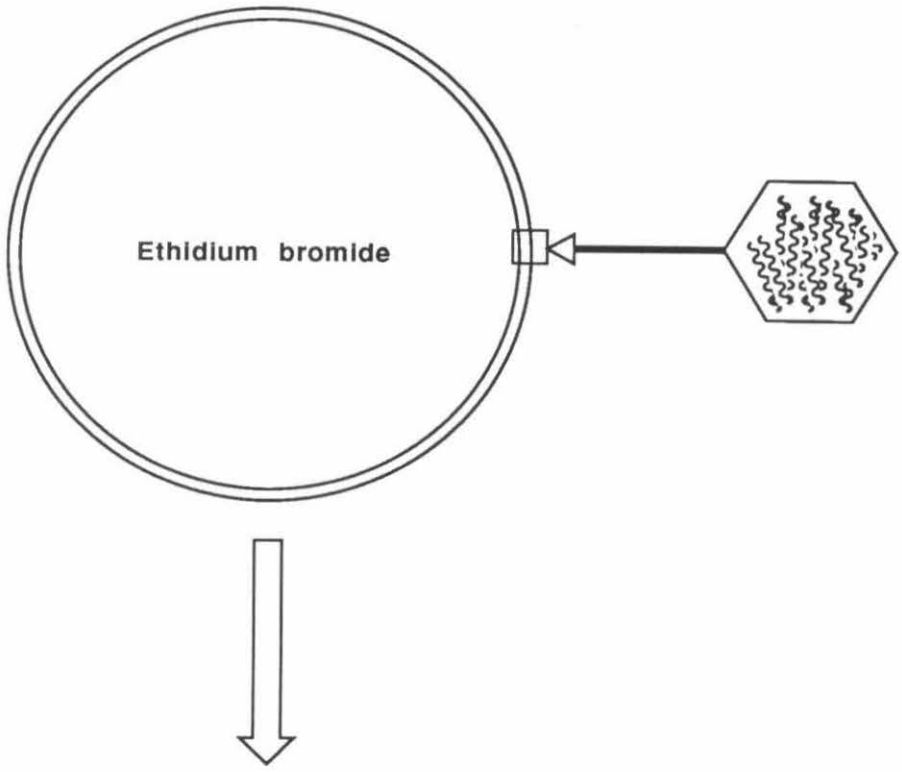
The strategy for implanting the receptor into the cell membrane is by fusion of the cell membrane with a membrane containing LamB (cf. Figure 6, Chapter 1). A fusogenic membrane in which LamB can be incorporated is a reconstituted viral envelope (20-22). Infection of cells by viruses attaching to receptors which have been implanted by reconstituted Sendai virus envelope (RSVE) fusion with cells, after reconstituting the receptor in the RSVE, has been previously demonstrated (23).

The following study represents the first direct examination of the λ-LamB interaction in model membranes. Using spectroscopic techniques, it is possible to assay DNA injection efficiency and kinetics in a reconstituted membrane. The scheme of the experiment is shown in Figure 4. DNA injection by the phage into the liposome should cause a change in the spectroscopic signal of the encapsulated reporter molecules.

The current study confirms the single-step injection of DNA *in vitro* of bacteriophages with noncontractile tails, as reported previously for both bacteriophages λ and T5 (24,25). The kinetics of the injection are very rapid (see following chapter),

**Figure 4**

Scheme for spectroscopic detection of DNA injection by bacteriophage λ into liposomes bearing reconstituted LamB, based on fluorescence of encapsulated ethidium bromide.




PHYSICAL OBSERVATION OF DNA INJECTION
(Enhanced ethidium bromide fluorescence)

and the driving force appears to be largely entropic. The existence of a "guidance" mechanism to lead the DNA through the bacteriophage tail is suggested by the observation that a phage protein is injected with DNA into the host cell (26). It is currently difficult, however, to ascertain the interactions triggered by LamB binding which lead to injection, since studies of the LamB membrane topology have yielded conflicting results (27-29), and the portions of the protein which bind λ remain unclear.

It is hoped that this study will help lay the groundwork for future applications of the type described earlier. The potential for gene therapy and the study of eukaryotic gene expression makes an efficient, laboratory constructed gene delivery vehicle a valuable tool.

## REFERENCES


1. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1983) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y.), 17-49.

2. Hohn, B. and Murray, K. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3259-3262.

3. Sternberg, N., Tiemeier, D. and Enquist, L. (1977) *Gene* **1**, 255-280.

4. Hohn, B. (1979) *Meth. Enzymol.* **68**, 299-309.

5. Enquist, L. and Sternberg, N. (1979) *Meth. Enzymol.* **68**, 281-298.

6. Amersham technical report, λ DNA *in vitro* packaging kit.

7. Szmelcman, S. and Hofnung, M. (1975) *J. Bacteriol.* **124**, 112-118.

8. Schwartz, M. (1975) *J. Mol. Biol.* **99**, 185-201.

9. Luckey, M. and Nikaido, H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 165-171.

10. Kühl, P.W. (1980) *Biochim. Biophys. Acta* **622**, 63-70.

11. Roessner, C.A., Struck, D.K. and Ihler, G.M. (1983) *J. Biol. Chem.* **258**, 643-648.

12. Roessner, C.A., Struck, D.K. and Ihler, G.M. (1984) *BioTechniques* **2**, 140-146.

13. Corsaro, C.M. and Pearson, M.L. (1981) *Som. Cell Genet.* **7**, 603-616.

14. Anderson, W.F., Killos, L., Sanders-Haigh, L., Kretschmer, P.J. and Diacumakos, E.G. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5399-5403.

15. Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) *EMBO J.* **1**, 841-845.

16. Volsky, D.J., Gross, T., Sinangil, F., Kuszynski, C., Bartzatt, R., Dambaugh, T. and Kieff, E. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5926-5930.

17. Emerman, M. and Temin, H.M. (1984) *J. Virol.* **50**, 42-49.

18. Lichtenberg, D. and Barenholz, Y. (1988) *Meth. Biochem. Anal.* **33**, 337-462.

19. Novick, S.L., Di Simone, C., Amatruda, T. and Baldeschwieler, J.D., unpublished data.

20. Loyter, A. and Volsky, D.J. (1982) in *Membrane Reconstitution* (Poste, G. and Nicolson, G.L., Eds.), Elsevier: Amsterdam, 215-266.

21. Novick, S.L. and Baldeschwieler, J.D. (1988) *Biochemistry* **27**, 7919-7924.

22. Wang, W.-C. and Baldeschwieler, J.D., unpublished data.

23. Shapiro, I.M., Klein, G. and Volsky, D.J. (1981) *Biochim. Biophys. Acta* **676**, 19-24.

24. Mackay, D.J. and Bode, V.C. (1976) *Virology* **72**, 167-181.

25. Zarybnicky, V., Zarybnicka, A. and Frank, H. (1973) *Virology* **54**, 318-329.

26. Roa, M. (1981) *FEMS Microbiol. Lett.* **11**, 257-262.

27. Clement, J.M. and Hofnung, M. (1981) *Cell* **27**, 507-514.

28. Charbit, A., Clement, J.M. and Hofnung, M. (1984) *J. Mol. Biol.* **175**, 395-401.

29. Desaymard, C., Debarbouille, M., Jolit, M. and Schwartz, M. (1986) *EMBO J.* **5**, 1383-1388.

# Chapter 8
# Fluorescence Measurement of the Kinetics of DNA Injection by Bacteriophage λ into Liposomes

The text of this chapter was co-authored with John D. Baldeschwieler and published in *Biochemistry*, Volume 27, pages 7919-7924, October 1988.

# Fluorescence Measurement of the Kinetics of DNA Injection by Bacteriophage λ into Liposomes†

Steven L. Novick‡ and John D. Baldeschwieler*, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

†This investigation was supported by ARO grant DAAG-29-83-K-0128, by NIH Biomedical Research Support Grant RR07003 awarded by the Division of Research Resources, and by gifts from Amersham and Monsanto.

*To whom correspondence should be addressed.

‡Recipient of an NSF predoctoral research fellowship and a CIT institute fellowship.

## ABSTRACT

Bacteriophage λ attaches to Gram-negative bacteria using the outer membrane protein LamB as its receptor. Subsequently, DNA is injected by the bacteriophage into the host cell for replication and expression. The mechanism of DNA injection, however, is poorly understood. In order to begin to characterize DNA injection, a quantitative kinetic assay to detect injection into reconstituted LamB liposomes is described. The technique involves monitoring the increase in fluorescence of liposome-encapsulated ethidium bromide, which occurs as DNA enters the aqueous compartment of the vesicles. The data indicate that injection is several times faster than indicated by

earlier studies and is complete within one min. Such assays which allow direct observation of this process are necessary first steps toward a mechanistic understanding.

## INTRODUCTION

Bacteriophage λ is an important tool in molecular biology and biochemistry due to its ease of laboratory manipulation. Little is known, however, about the molecular mechanisms by which it functions. Bacteriophage λ is a temperate, double-stranded DNA-containing phage which infects Gram-negative bacteria, using the outer membrane maltose porin LamB as its receptor (1). LamB exists in the membrane as an integral trimer of 47-kDa subunits (2,3). In addition to its role in bacteriophage attachment, LamB is also the channel through which phage DNA passes as it enters the host cell (4).

Some studies have been conducted to investigate the phage-receptor interaction *in vitro* and in well-characterized model membranes such as phospholipid vesicles. These studies focused on phage λ interactions with LamB extracted from *Escherichia coli* K-12. The complexes are formed reversibly (2) and require addition of chloroform to trigger irreversible binding and DNA injection (2,5,6). λ host range mutants (λh) which bind LamB from *E. coli* K-12 irreversibly and inject their DNA in the absence of chloroform have been identified (7). In addition, the *Shigella* receptor binds wild-type phage λ irreversibly (8). *E. coli* pop154 expresses the LamB-encoding region of *Shigella sonnei* 3070 (9), and the extracted receptor causes DNA injection by the phage (8-10).

Previous kinetic studies of λ DNA injection *in vitro* have employed indirect assays for injection (2,4-6). These include a plaque inhibition assay, analysis of nuclease sensitivity of free DNA, and separation of labeled solutes leaking from LamB-

containing liposomes upon DNA injection. Studies of wild-type λ interactions with LamB from strain K-12 required chloroform addition to trigger DNA injection, a condition which would seem to greatly affect the measurement being made. Kinetic measurements in these systems have indicated that injection takes minutes to hours to occur. The plaque inhibition assay assumes that none of the steps between phage-receptor binding and DNA injection are rate limiting. Phage may also bind irreversibly without subsequent injection. These limitations restrict the conclusions which can be drawn from data collected using indirect assays.

In the present study, we report a direct method for measurement of the kinetics of bacteriophage λ DNA injection into LamB-containing liposomes. Ethidium bromide is entrapped inside the liposomes, and the fluorescence enhancement is monitored continuously as the fluorophore binds to injected DNA (11). Fluorescence spectroscopy combines the advantages of fast data acquisition and high sensitivity and is well-suited to kinetic studies. The injection step is much slower than either phage-receptor attachment or ethidium bromide-DNA binding (2,12,13), so that the apparent rate reflects that of DNA injection. Use of this direct assay in the wild-type λ-*Shigella* LamB system shows that DNA injection is complete within one min, much faster than has been shown thus far with indirect assays.

## MATERIALS AND METHODS

**Reagents.** Octyl β-D-glucopyranoside and Triton X-100 were purchased from Aldrich. λ DNA and sodium [$^{125}$I]iodide were purchased from Amersham. Egg yolk phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL) as the chloroform solution and was stored at -20°C. Bio-Beads SM-2 and Enzymobeads radioiodination reagent were obtained from Bio-Rad. 5(6)-Carboxyfluorescein was purchased from Eastman (Rochester, NY). Cholesterol, ethidium bromide, Sephadex

G25-50, and Sepharose 4B were all purchased from Sigma. Spectrapor-2 dialysis tubing was obtained from Spectrum Medical Industries (Los Angeles, CA). Cholic acid was obtained from Sigma and was recrystallized from ethanol/water prior to use. *E. coli* strain C600(λcI857Sam7) was used as the source of bacteriophage; strain pop154 was used for production of LamB; strain Ymel was used as the lysable indicator for plaque assays.

**Phage Preparation.** The procedure for phage growth and purification is essentially the same as described previously (10,14) with minor modifications in the phage precipitation step (15,16). A 2-l culture of *E. coli* C600(λcI857Sam7) was grown to approximately 5 x $10^8$ cells/ml, and phage growth was induced by heating at 43°C for 20 min, with an additional 3 h of growth at 37°C. The cells were centrifuged at 5000 rpm for 5 min and were resuspended in 20 ml of λ dil buffer [10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5)/2 mM $MgSO_4$]. Cells were lysed by adding 2 ml of $CHCl_3$. The mixture was vortexed and allowed to stand for 30 min at room temperature. To reduce the viscosity due to cellular nucleic acids, 10 mg of crystalline pancreatic DNase I and 30 mg of crystalline RNase A were added, and the mixture was allowed to stand 10 min at room temperature. After centrifugation at 10,000 rpm for 10 min to remove cell debris, the phage-containing supernatant was decanted. Solid NaCl and PEG6000 were added to 1 M and 10%, respectively, to precipitate phage. The solutes were dissolved by slow stirring on a magnetic stirrer. The solution was cooled in ice water and allowed to stand for at least 1 h on ice. Phage were pelleted by centrifuging at 10,000 rpm for 10 min at 4°C, resuspended in 0.7 g/ml CsCl, and allowed to stand overnight at 4°C. The solution was centrifuged at 30,000 rpm for 16 h at 4°C, and the phage band was removed in 1-1.5 ml and stored at 4°C. λ ghosts were prepared according to the method of Roessner and Ihler (4). Phage particles were dialyzed against 10 mM Tris (pH 7.5)/5 mM EDTA and then heated to

50°C for 30 min. $MgSO_4$ was added to 10 mM, and the mixture was incubated at 37°C for 30 min with 10 mg/ml DNase I, followed by dialysis against 10 mM Tris (pH 7.5)/10 mM $MgSO_4$. Phage particles were titered with the plaque assay (7), plating on *E. coli* Ymel. Typically, a titer of $1.4 \times 10^{12}$ pfu/ml (± 50%) was obtained from a starting 2-l culture of the C600 lysogen.

**LamB Preparation.** A 1-l culture of *E. coli* pop154 was grown in tryptone broth containing 0.4% maltose to $O.D._{600\ nm} = 1.0$. Cells were pelleted by centrifugation at 5,000 rpm for 10 min and resuspended in 60 ml of 10 mM EDTA/2% sodium cholate/10 mM Tris (pH 7.5). The solution was shaken for 30 min at 37°C and then probe sonicated for 3 min with at Heat Systems-Ultrasonics W-225R sonicator operated at 50% duty cycle and an output setting of 5. Insoluble material was removed by centrifugation at 10,000 rpm for 10 min. The supernatant was further clarified by centrifugation at 50,000 rpm for 1 h. A ten-fold volume of butanol was added and the mixture was allowed to stand for 30 min in ice. The precipitate was collected by centrifugation at 10,000 rpm for 10 min. Residual butanol was removed by lyophilization overnight. The powder was solubilized in 1 M NaCl/2% sodium cholate/10 mM EDTA/10 mM Tris (pH 7.5) by probe sonicating for 30 s. After being cooled to room temperature, insoluble material was pelleted by centrifuging at 10,500 rpm for 5 min. The supernatant, containing LamB, was lyophilized and stored at 4°C. Receptor activity was determined by using the plaque inhibition assay (10).

**Liposome Preparation.** Egg PC (10 mg) and 4.9 mg of cholesterol in chloroform solution were dried under a stream of nitrogen, and residual chloroform was removed by vacuum. The dry lipid film was hydrated in 1 ml of PBS (pH 7.4) and vortexed to solubilize the lipid. The aqueous dispersion was sonicated to clear opalescence in a Laboratory Supplies (Hicksville, NY) bath sonicator. Octyl glucoside was then added in a 10:1 detergent to lipid molar ratio to form mixed micelles and the

mixture clarified immediately. LamB (40 μg) in 10 mM Tris (pH 7.5)/2% sodium cholate was added, and the mixture was dialyzed for 24 h in Spectrapor-2 dialysis tubing against a 100-fold volume of PBS (pH 7.4) containing 1 mM ethidium bromide, to which prewashed Bio-Beads had been added (17-19), for 24 h. The Bio-Bead:detergent ratio used was 1 mg of beads/0.1 μmol of octyl glucoside. Unincorporated solute was removed by gel filtration on a Sephadex G25-50 spin column. Lipid concentration was determined with the Böttcher modification of the Bartlett assay (20). Protein concentration was determined with the Peterson modification of the Lowry assay (21).

**Efficiency of LamB Reconstitution.** A solution containing approximately 75 ng of LamB was radioiodinated using Enzymobead reagent (Bio-Rad) and 20 μl of 100 mCi/ml sodium [$^{125}$I]iodide solution, yielding 2.8 Ci/μmol labeled protein. Reconstitution of LamB was conducted as described above. Reconstitution efficiency was determined by gel filtration of the dialysate on Sepharose 4B followed by counting of eluted fractions in a Beckman Biogamma II counter. Column packing was also dried, sliced into 1-cm increments, and counted to check for sticking of unincorporated protein to the column.

**Carboxyfluorescein Release Studies.** The relief of self-quenching of the fluorophore carboxyfluorescein is a common method of measurement of liposome integrity and has been reviewed by Weinstein et al. (22). Vesicles with and without LamB were prepared as described previously by dialysis against PBS (pH 7.4) containing 100 mM carboxyfluorescein and Bio-Beads SM-2. Carboxyfluorescein was purified prior to use by the method of Weinstein et al. (22). Leakage was monitored as a function of time and phage binding, with excitation and emission wavelengths of 492 and 520 nm, respectively. Triton X-100 was added to 1% as a control to rupture the

vesicles. Fluorescence measurements were made with an SLM 4800 spectrofluorometer.

**Perturbed Angular Correlation Measurements of Solute Leakage.** The perturbed angular correlation (PAC) technique allows quantitation of liposomal integrity by monitoring the tumbling rate of $^{111}In^{3+}$ (23), which is entrapped in the liposomes with the chelator nitrilotriacetic acid (NTA). Measurements of the angular correlations of the two $\gamma$ rays emitted in a cascade from the nucleus allow calculation of rotational correlation times [for a review, see (24)]. Inside intact vesicles, $^{111}In^{3+}$ tumbles rapidly in the chelated complex. When vesicles leak or rupture, $^{111}In^{3+}$ binds to large serum proteins and has a slow tumbling rate. According to this technique, the effect of incorporation of LamB into liposomes on solute leakage was assayed in 50% serum.

The NTA was encapsulated by performing the LamB reconstitution as described earlier, in buffer containing 1 mM NTA. Unencapsulated NTA was removed over a Sephadex G25-50 spin column. The $^{111}In^{3+}$ was loaded into liposomes using the ionophore acetylacetone (ACAC) (25). $^{111}InCl_3$ was evaporated to dryness under a heat lamp and redissolved in a minimum volume of 3 mM HCl. The solution was diluted 10-fold in 30 mM ACAC/10 mM Tris (pH 7.4). This loading buffer was then added to a 5-fold volume of liposomes, in three aliquots while vortexing. After incubation at room temperature for 1 h, unencapsulated $^{111}In(ACAC)_3$ was separated from liposomes on a Sephadex G25-50 spin column. Liposome stability was measured as a function of time in 50% fetal calf serum.

**Fluorescent Measurement of DNA Injection.** Ethidium bromide was encapsulated in liposomes by dialyzing against Bio-Beads SM-2 in buffer containing 1 mM ethidium bromide. Unencapsulated solute was removed on a Sephadex G25-50 spin column. Fluorescence was measured in an SLM 4800 spectrofluorometer with a

Schott Glass Technologies 450-nm high-pass cutoff filter in the emission channel (Model GG-495). Excitation and emission wavelengths used were 295 and 598 nm, respectively. Zero fluorescence was taken as the background fluorescence due to ethidium bromide in the liposomes. Bacteriophage was added to the sample in a quartz cuvette in a thermostatted 37°C sample chamber equipped with a magnetic stirrer, and fluorescence was monitored continuously as a function of time.

## RESULTS

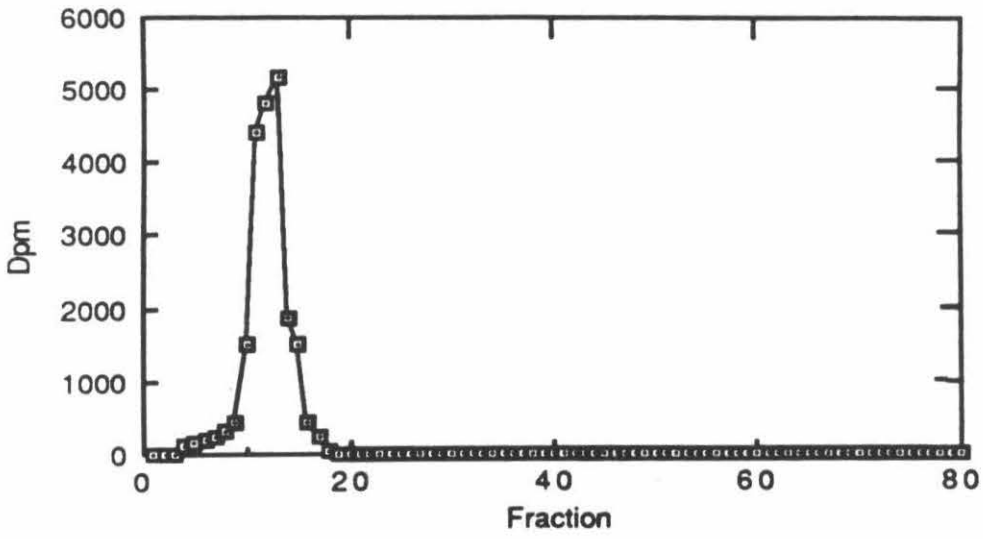
**Vesicle Size.** Vesicles with and without LamB were sized by negative-stain electron microscopy on a Philips EM201 electron microscope operated at 80 kV, using 1% phosphotungstic acid. Mean vesicle diameters were 159 ± 44 nm without LamB and 118 ± 52 nm with LamB, from populations of 300 vesicles each.

**Efficiency of LamB Reconstitution.** Radioiodinated LamB was reconstituted as described under MATERIALS AND METHODS. The dialysate was subjected to gel filtration chromatography on Sepharose 4B and was eluted with PBS (pH 7.4). The elution profile is shown in Figure 1. All of the labeled protein was associated with the liposomal fraction eluting at the void volume. Free protein, which normally elutes at the included volume (not shown), was not present, indicating essentially quantitative reconstitution efficiency. This finding is consistent with that of Roessner et al. (10), who observed 98% retention of phage-inactivating capacity in reconstituted LamB liposomes using the plaque inactivation assay for LamB receptor activity.

**Solute Leakage from Reconstituted LamB Liposomes.** It was necessary to determine the effect of LamB on membrane permeability to solutes, since the fluorescence assay depends on solute retention in the liposomes. Liposome stability in both buffer and serum was measured by using entrapped carboxyfluorescein and

**Figure 1**

Gel filtration chromatographic profile of $^{125}$I-LamB liposomes. Radioiodinated LamB was reconstituted into vesicles composed of egg PC/cholesterol, as described under MATERIALS AND METHODS. Liposomes and free protein were separated on Sepharose 4B equilibrated in PBS, pH 7.4. The same buffer was used as eluant. Fractions were counted in a Beckman Biogamma II counter. The column packing was sliced into 1-cm increments and counted after elution.


6000
5000
4000
3000
2000
1000
0
Dpm
0
20
40
60
80
Fraction

$[^{111}In(NTA)_2]^{3-}$. In PBS at 37°C, no leakage of CF was observed either before or after addition of bacteriophage (Figure 2). After 1000 s, Triton X-100 was added to rupture the vesicles as a control. This result is consistent with the finding that no ion leakage accompanies DNA injection by bacteriophage T5, a phage also having a noncontractile tail (26). The lack of solute leakage upon phage binding is not consistent, however, with observation of ATP release by Roessner et al. (10) or the conductance measurements of Benz et al. (27), both indicating that the DNA pore may remain open after injection.

Solute leakage in 50% serum was assayed by the PAC assay (see MATERIALS AND METHODS). Figure 3 shows that approximately 95% of the vesicles composed of only lipid and 85% of the reconstituted LamB vesicles remained intact after 120 h. The large initial drop in the stability of LamB-containing vesicles is probably due to the early rupture of vesicles containing many receptors (an average of one receptor per vesicle was used).

**Fluorescent Measurement of DNA Injection Kinetics.** Kinetics of DNA injection by bacteriophage λ into reconstituted LamB liposomes containing ethidium bromide are shown in Figure 4. The most striking feature of these data is the rapid kinetic behavior. Injection is essentially complete within 1 min. This is several times faster than the kinetics observed in studies in which indirect methods of detecting DNA injection have been employed (2,5,6). Using the method of initial rates, the second-order rate constant measured for injection was $4.9 \times 10^3$ $M^{-1}$ $s^{-1}$ (± 50%). The reaction was first order with respect to both phage and receptor, consistent with earlier findings (2,5,12). The observed rate law was $R_0 = k\ [\lambda]^{0.84\pm0.20} \times [\text{LamB}]^{1.05\pm0.26}$, where $R_0$ is the measured initial rate, [λ] and [LamB] are the molar concentrations of phage particles and LamB-containing liposomes, respectively, and $k$ is the measured rate constant. The effect of bacteriophage concentration is shown in curves B and C,

**Figure 2**

Carboxyfluorescein assay of solute retention by LamB-containing liposomes. Carboxyfluorescein (100 mM) was entrapped by dialysis (see MATERIALS AND METHODS) in buffer containing the same concentration of solute. After removal of unentrapped solute on Sephadex G-25, NaCl was added to the liposomes, so that the buffer and liposome contents would be isotonic. Carboxyfluorescein release was monitored continuously at 37°C. Excitation and emission wavelengths used were 492 and 520 nm, respectively. Bacteriophage (12 pM) was added at 100 s. Triton X-100 was added to 1 % after 1000 s as a control to rupture the liposomes. All measurements were made on an SLM 4800 spectrofluorometer.

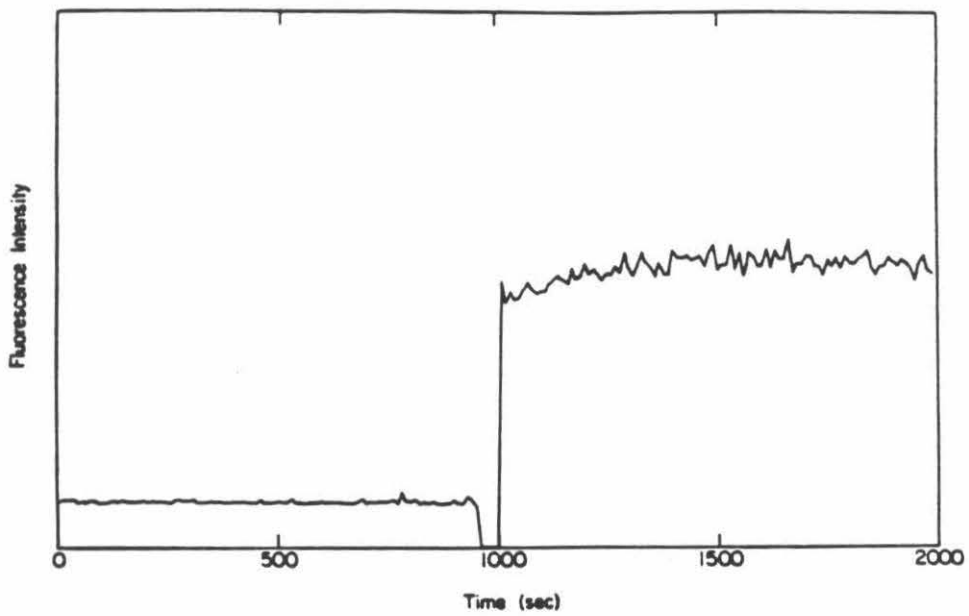

Fluorescence Intensity
0
500
1000
1500
2000
Time (sec)

**Figure 3**

Perturbed angular correlation assay of serum stability of LamB-containing liposomes. NTA (1 mM) was entrapped in liposomes by dialysis in buffer containing the same concentration of solute. Unentrapped NTA was removed by gel filtration chromatography on a Sephadex G25-50 spin column, and $^{111}In^{3+}$ was loaded into liposomes using the ionophore acetylacetone (ACAC; see MATERIALS AND METHODS). Free $^{111}In(ACAC)_3$ was also removed by gel filtration. Liposomal retention of $[^{111}In(NTA)_2]^{3-}$ was monitored by correlation counting in the PAC spectrometer at 37°C in 50 % fetal calf serum. The percentages of solute retention by vesicles composed of only lipid PC (upper curve) and reconstituted LamB vesicles at an average of one receptor per vesicle (lower curve) were determined and plotted as a function of time.

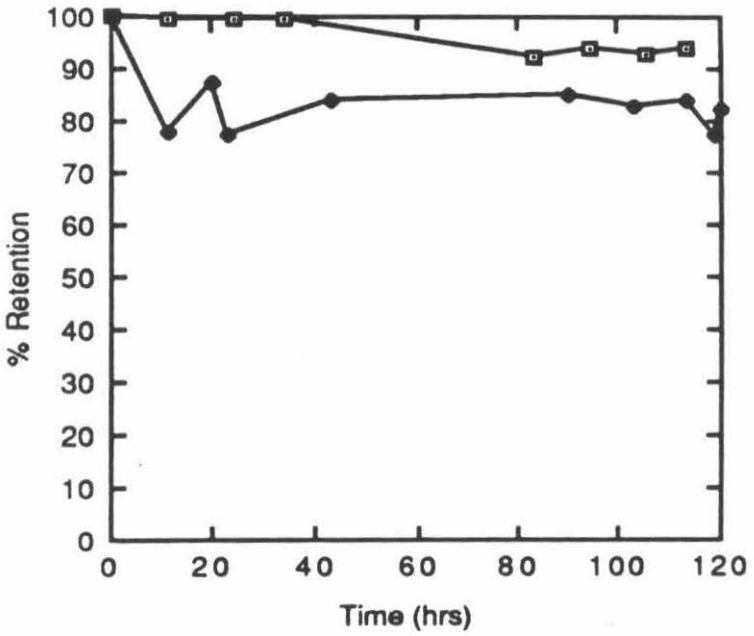
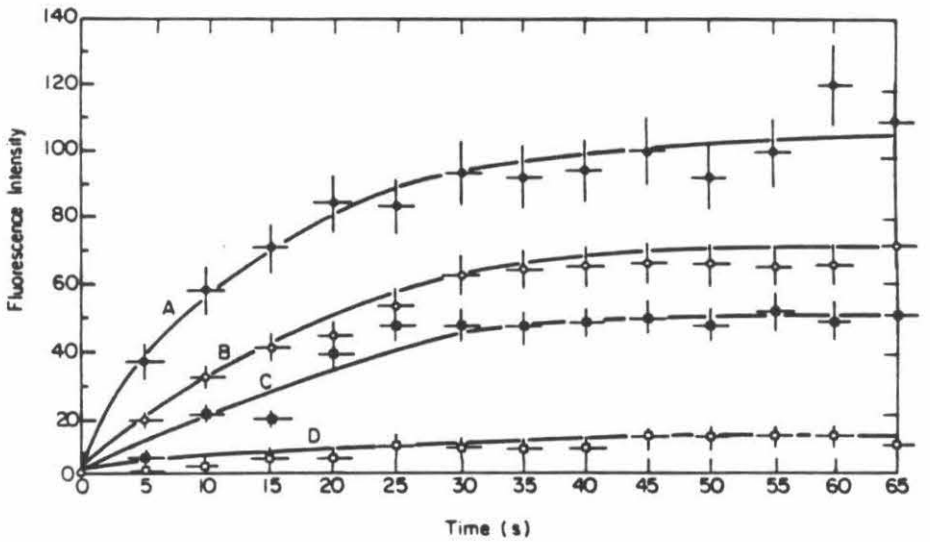

100
90
80
70
60
50
40
30
20
10
0
% Retention
0
20
40
60
80
100
120
Time (hrs)

**Figure 4**

Fluorescence measurement of DNA injection kinetics into LamB-containing liposomes. Ethidium bromide (1 mM) was entrapped in liposomes by dialysis in buffer containing the same concentration of solute. Unentrapped fluorophore was removed by gel filtration on Sephadex G25-50. Fluorescence was monitored continuously, with the preinjection fluorescence taken as zero. Excitation and emission wavelengths used were 295 and 598 nm, respectively, and a 450-nm high-pass cutoff filter was used in the emission channel. Phage concentration was (A) 12 pM; (B) 110 pM; (C) 61 pM; and (D) 12 pM. Concentration of receptor (at an average of one receptor per liposome) was 840 nM in curves A,B, an C and 85 nM in curve D. The rate law was calculated by the method of initial rates.


Fluorescence Intensity
140
120
100
80
60
40
20
0
A
B
C
D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (s)

where the 50 % higher concentration in curve B results in a corresponding increase in the final level of fluorescence, compared with curve C. Similarly, the ten-fold higher concentration of receptor carrying liposomes in curve A compared with curve D causes an approximately ten-fold increase in the measured extent of injection. The level of fluorescence in curve A corresponds to injection by approximately 20% of the phage in the sample, consistent with the finding that only a fraction of the particles in a given sample inject their DNA (28). The technique showed sensitivity to approximately 800 μg of injected DNA in a sample.

However, in spite of increasing phage concentrations from curve A to curve C to curve B, the extent of fluorescence does not continue to increase linearly, due to increasing amounts of light scattering by the dense phage particles at higher concentrations, causing an inner filter effect. Such behavior is a common interference in optical spectroscopy, in which scattering of both exciting and emitted light within the sample greatly reduces the observed fluorescence (29). Thus, while clearly a useful kinetic technique, the overall extent of injection can be measured by this method over only a limited phage concentration range.

As a control to verify that fluorescence was due to DNA injection into the liposomes and not due to ethidium bromide diffusion into the bacteriophage, phage ghosts were incubated with LamB-carrying liposomes containing ethidium bromide. DNA was added to the external aqueous medium. Ethidium bromide diffuses easily through the phage capsid (30). If leakage into the phage was occurring, the ethidium would have bound the exogenous DNA and fluoresced. No fluorescence was observed, indicating that ethidium bromide does not diffuse out of the liposome upon binding by λ. Upon Triton X-100 addition, the vesicles ruptured, and fluorescence due to binding of the ethidium bromide to exogenous DNA was observed. If phage were added to vesicles at 4°C, at which temperature injection does not occur (10), no

fluorescence occurred on addition of exogenous DNA to the sample after allowing binding to occur for 30 min (Novick, S.L. and Baldeschwieler, unpublished observation). This also supports the notion that phage-receptor binding does not induce solute leakage.

## DISCUSSION

A direct assay was used to measure the kinetics of DNA injection of bacteriophage λ into reconstituted LamB liposomes, based on fluorescence of liposome-entrapped ethidium bromide. The data presented show that DNA injection is a rapid one-step process, occurring much faster than had been previously shown (2,5,6). An advantage of this technique is that it allows direct observation of the phenomenon of interest. With the proper choice of phage and bacteria, the need for chloroform addition is obviated (10). Its use in earlier studies (2,5,6) probably was responsible for a great deal of conformational change, perturbing both the system and the measurements.

The high efficiency of LamB reconstitution (Figure 1) is consistent within experimental error with the finding of Roessner et al. (10), who reported 98% retention of plaque inhibition activity in LamB reconstituted into egg phosphatidylcholine liposomes. The binding of phage may be independent of membrane fluidity, then, since the liposomes in the present study contained equimolar egg PC and cholesterol, in contrast to the egg PC membrane used in the former study.

Solute leakage from the reconstituted vesicles was negligible (Figure 2). No carboxyfluorescein leakage was observed either before or after addition of bacteriophage. The lack of solute leakage upon phage binding is consistent with a similar finding for phage T5 (26). The difference in leakage results of Roessner and Ihler (4) and Benz et al. (27) may be due to the large hydrophobic character of the

solutes employed in the present work relative to ATP or ions involved in single-channel conductance used in the earlier studies, or to a different source of small ion leakage in those studies. In the control experiment for ethidium leakage in the present study, binding of phage ghosts did not induce leakage, lending further support to the notion that the transmembrane channel does not remain open after injection, although Roessner and Ihler (4) did not observe ATP leakage upon ghost binding. If the pore were to remain open, the lack of fluorescence due to ethidium bromide binding to exogenous DNA after phage-receptor binding at 4°C suggests that it is not open between the binding and injection steps.

Vesicle stability in 50% serum was quite high, with 85-95% of the entrapped $^{111}$In complex remaining entrapped after 120 h (Figure 3). Such stability is desirable in applications of a reconstituted DNA injection system to liposomal delivery of DNA to cells, as suggested by Roessner et al. (31).

The rapid kinetics of DNA injection shown in Figure 4 are much faster than those obtained with indirect methods. The absence of a lag phase before injection is consistent with *in vitro* observations of λ-wild-type receptor interactions (5,6) and bacteriophage T5-receptor interactions (32). The reaction was first order with respect to both reactants, also consistent with earlier findings (2,5,12). The bimolecular rate law seen suggests that injection may be a rate limiting step in plaque inactivation assays used in earlier kinetic studies, although this type of reaction would still apply to binding even if injection had not been rate limiting. The fact that only about 20% of the phage inject their DNA is consistent with earlier observations that 15-30% of phage inject their entire DNA complement immediately (28), as shown by nuclease sensitivity of the DNA. This result does not indicate that injection into liposomes is substantially less efficient than injection into bacteria. The efficiencies of injection into bacteria have been characterized by plaque inhibition assays, which measure injection efficiency only in

the lysable indicator strain. Binding and injection are measured as an aggregate in the target bacteria, as an irreversible binding event inactivates the phage, and the assay is a measure only of inactivation. In one study, in which cellular incorporation of radiolabeled DNA was used to measure *in vivo* injection efficiency, only 50% of bound phage particles injected their DNA at 37°C (33). It therefore remains unclear whether injection *in vitro* is markedly less efficient than the *in vivo* process.

The observation that DNA injection both by λ (33) and by T5 (32) into Gram-negative bacteria occurs in two steps suggests an additional transport step *in vivo*, during which DNA injected into the periplasmic space is carried into the cytoplasm. The mechanism by which this occurs is unclear, however (34), and remains the subject of continuing study. Spectroscopic assays based on the conformational dynamics of bacteriophage DNA during injection are also currently under investigation.

The direct fluorescence method described here will allow much greater insight into the process of DNA transfer across membranes in a wide range of systems. The ability to monitor this process directly provides the exciting opportunity to begin to obtain a molecular understanding of DNA injection, in both artificial and biological systems. Such an understanding could allow extensions of the use of bacteriophage in molecular biology and biochemistry.

## ACKNOWLEDGMENTS

We thank Drs. C.A. Roessner and G.M. Ihler (Texas A & M University) for their generous gift of bacterial stocks and Drs. Manfred H. Fleschar and Sean M. Sullivan (California Institute of Technology) and Dr. Ian A. McKay (Amersham International, Amersham, U.K.) for their many helpful suggestions and comments.

## REFERENCES


1. Szmelcman, S. and Hofnung, M. (1975) *J. Bacteriol.* **124**, 112-118.

2. Schwartz, M. (1975) *J. Mol. Biol.* **99**, 185-201.

3. Schwartz, M. (1983) *Meth. Enzymol.* **97**, 100-112.

4. Roessner, C.A. and Ihler, G.M. (1986) *J. Biol. Chem.* **258**, 386-390.

5. Mackay, D.J. and Bode, V.C. (1976) *Virology* **72**, 167-181.

6. Zgaga, V., Medic, M., Salaj-Smik, E. and Novak, D. (1973) *J. Mol. Biol.* **79**, 697-708.

7. Randall-Hazelbauer, L. and Schwartz, M. (1973) *J. Bacteriol.* **116**, 1436-1446.

8. Schwartz, M. and Le Minor, L. (1975) *J. Virol.* **15**, 679-685.

9. Roa, M. and Scandella, D. (1976) *Virology* **72**, 182-194.

10. Roessner, C.A., Struck, D.K. and Ihler, G.M. (1983) *J. Biol. Chem.* **258**, 643-648.

11. Le Pecq, J.-B. and Paoletti, C. (1966) *Anal. Biochem.* **17**, 100-107.

12. Delbrück, M. (1940) *J. Gen. Physiol.* **23**, 631-642.

13. Bresloff, J.L. and Crothers, D.M. (1975) *J. Mol. Biol.* **95**, 103-123.

14. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 76-84.

15. Kaslow, D.C. (1986) *Nucleic Acids Res.* **14**, 6767.

16. Latchman, D.S. and Brickell, P.M. (1986) *Nucleic Acids Res.* **14**, 9220.

17. Holloway, P.W. (1973) *Anal. Biochem.* **53**, 304-308.

18. Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J. (1981) *Biochemistry* **20**, 833-840.

19. Philippot, J., Mutaftschiev, S. and Liautard, J.P. (1983) *Biochim. Biophys. Acta* **734**, 137-143.

20. Böttcher, C.J.F., Van Gent, C.M. and Fries, C. (1961) *Anal. Chim. Acta* **24**, 203-204.

21. Peterson, G.L. (1977) *Anal. Biochem.* **83**, 346-356.

22. Weinstein, J.N., Ralston, E., Leserman, L.D., Klausner, R.D., Dragsten, P., Henkart, P. and Blumenthal, R. (1984) in *Liposome Technology* **3** (Gregoriadis, G., Ed.), CRC Press: Boca Raton, FL, 183-204.

23. Hwang, K.J. and Mauk, M.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4991-4995.

24. Hwang, K.J. (1984) in *Liposome Technology* **3** (Gregoriadis, G., Ed.), CRC Press: Boca Raton, FL, 247-262.

25. Beaumier, P.L. and Hwang, K.J. (1982) *J. Nucl. Med.* **23**, 810-815.

26. Filali Maltouf, A. and Labedan, B. (1983) *J. Bacteriol.* **153**, 124-133.

27. Benz, R., Schmid, A., Nakae, T. and Vos-Scheperkeuter, G. (1986) *J. Bacteriol.* **165**, 978-986.

28. Roa, M. (1981) *FEMS Microbiol. Lett.* **11**, 257-262.

29. Lakowicz, J.R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum: N.Y., 44-47.

30. Hall, S.B. and Schellman, J.A. (1982) *Biopolymers* **21**, 2011-2031.

31. Roessner, C.A., Struck, D.K. and Ihler, G.M. (1984) *BioTechniques* **2**, 140-146.

32. Zarybnicky, V., Zarybnicka, A. and Frank, H. (1973) *Virology* **54**, 318-329.

33. Mackay, D.J. and Bode, V.C. (1976) *Virology* **72**, 154-166.

34. Labedan, B. and Letellier, B. (1984) *J. Bioenerg. Biomembr.* **16**, 1-9.

# Appendix
## On the Role of Hydrophobic Interactions in Membrane Fusion: Effects of Poly(Ethylene Glycol)

The text of the Appendix was co-authored with Dick Hoekstra, Leo Rupert, Jan Engberts, Shlomo Nir, Hettie Hoff, and Karin Klappe and has been published in *Studia Biophysica*, Volume 127, pages 105-112, 1988.

# On the Role of Hydrophobic Interactions in Membrane Fusion: Effects of Poly(Ethylene Glycol)

Dick Hoekstra, Leo A.M. Rupert[1], Jan B.F.N. Engberts[1], Shlomo Nir[2], Hettie Hoff, Karin Klappe, and Steven L. Novick[3]

Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands,

[1]Laboratory of Organic Chemistry, University of Groningen, Groningen, The Netherlands,

[2]Hebrew University of Jerusalem, Department of Agriculture, Rehovot, Israel, and

[3]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

## INTRODUCTION

Membrane fusion is a crucial event in numerous intra- and intercellular processes (1,2). For example, the biosynthetic delivery of membrane constituents, after synthesis in the endoplasmic reticulum, to their sites of destination as well as exocytosis and endocytosis involve a host of intermediate steps in which the merging of membranes is essential.

Although a wealth of knowledge has accumulated in the past decade on the widespread occurrence, frequency and significance of membrane fusion in biological systems, progress on elucidating the underlying mechanism of fusion has been relatively slow, not in the least because of the complex experimental accessibility of the aforementioned systems.

It is therefore that artificial membranes are frequently used as model systems (3,4). Obviously, such membranes are by far too simple a model for clarifying the

molecular details relevant to biological membrane fusion, as specific membrane and/or cytoplasmic proteins must be implicated as well (5,6). However, it is equally clear that such models may provide a valuable tool to examine *fundamental* aspects of membrane-membrane interaction in general which *may* bear relevance to fusion of biological membranes as well.

In particular, studies carried out with model systems have shown that membrane hydration provides a critical structural stability to the integrity of membranes (7,8). Repulsive hydration forces will keep apposing membranes separated and thus prevent fusion. It is reasonable to assume, therefore, that for fusion to occur, a perturbation of the water structure is essential. The ensuing hydrophobic interactions conceivably play the ultimate key role as a driving force for the merging event (6,9).

To further examine these aspects of membrane fusion, we have investigated the effect of the dehydrating agent poly(ethylene glycol) on the fusion of artificial and biological membranes. In addition, using a hydrophobic photoaffinity label that was incorporated in the hydrophobic core of liposomal membranes, we demonstrate that fusion of an enveloped virus (Sendai virus) is triggered by a hydrophobic interaction involving penetration of the viral fusion protein into the core of the target membrane. Preincubation experiments with PEG suggest that the hydrophobic dehydration, triggered by the penetration mechanism, may further modulate the conformation of the fusion protein. Such changes would allow the establishment of a *direct* interbilayer contact, an obvious prerequisite for fusion.

## EFFECT OF PEG ON THE FUSION OF ARTIFICIAL MEMBRANE VESICLES

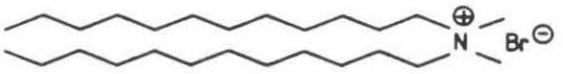
The strong dehydrating capacity of PEG (10) results from its ability to bind water molecules via hydrogen bond interactions. About three water molecules are

bound per monomer unit. It has been calculated that at PEG concentrations above about 13 wt %, all water molecules experience the presence of the polymer, while at about 40 wt % essentially all water molecules are bound. When added to liposomal suspensions, PEG reduces the amount of water bound to the lipid headgroups. Consistent with the lowering of the free water content, the interbilayer separation of apposed membranes is drastically reduced (10,11). Modulation of the state of surface hydration has dramatic effects on the $Ca^{2+}$-induced fusion of liposomes, consisting of phosphatidylserine (PS) (12). At relatively low PEG concentrations (5-10 wt %, MW 8K) a drastic enhancement in the initial fusion rate is seen while the threshold cation concentration required for fusion decreases about 5-fold. At these conditions PEG does not induce fusion by itself, nor does it affect the phase behavior of the bilayer. Furthermore, the combined action of $Ca^{2+}$ and PEG neither results in lipid phase separations (12). Taken together, these observations emphasize the significance of membrane dehydration and the creation of local "point defects" as dominant parameters in bringing about membrane fusion of *liposomal* membranes.

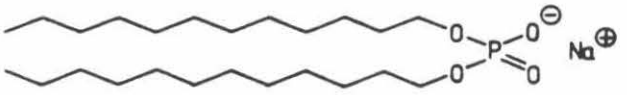
More recently, we have introduced a novel membrane system to study the dynamic properties of membranes, including membrane fusion (13-15). This system involves the use of vesicles, prepared from simple synthetic amphiphiles such as didodecyldimethylammonium bromide (DDAB) or didodecylphosphate (DDP, Figure 1). The vesicles readily fuse in the presence of divalent anions and cations, respectively. Most interestingly, in both systems the two distinct steps in the overall fusion reaction, i.e., vesicle aggregation and the actual fusion event, can be strictly controlled. This is in contrast to aggregation and fusion of phospholipid vesicles as in such systems, aggregation, triggered by adding divalent cations, is immediately followed by fusion. In the case of DDP vesicles, $Ca^{2+}$-induced aggregation occurs at a

**Figure 1**

Structural formulas of didodecyldimethylammonium bromide (DDAB) and didodecylphosphate (DDP).




DDAB




DDP

cation threshold concentration of 1.4 mM while fusion requires a concentration of at least 1.75 mM (14).

This difference allowed us to examine in detail the molecular and physical alterations occurring in the bilayer and at the bilayer/water interface when the vesicles were in the aggregated state and about to fuse. The results demonstrated that fusion of the vesicles is triggered by a perturbation of the bilayer/water interface, involving a local dehydration of the amphiphile headgroups, concomitant with the formation of a high affinity, anhydrous *trans* $Ca^{2+}$/DDP complex, which triggers the actual fusion process (15,16).

To corroborate these observations, we subsequently investigated how manipulation of the hydration of the headgroups of the amphiphiles affected fusion. To this end, the effect of PEG on DDP bilayers as such and on the fusion activity was examined as a function of the molecular weight of PEG (17). Analogous to the fusion of phospholipid vesicles (see above), PEG 8K (10 wt %) stimulated the initial rate of $Ca^{2+}$-induced fusion of DDP vesicles while the $Ca^{2+}$ threshold concentration was lowered from 1.7 to 1.0 mM. PEG-induced fusion, in the absence of $Ca^{2+}$, was not observed.

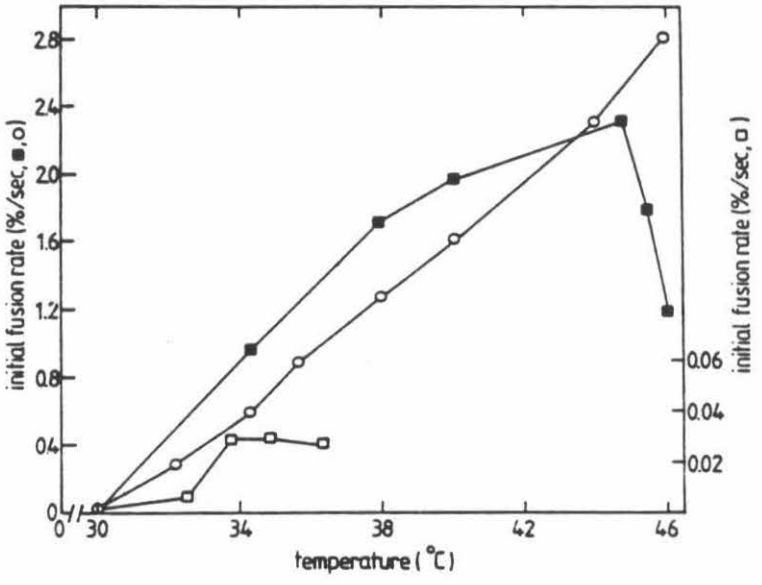
A striking difference was seen when similar experiments were carried out in the presence of PEG 20K. With only 0.1 wt % PEG 20K and 2.9 mM $Ca^{2+}$, the initial fusion rates were about 20- and 40-fold *lower* than those observed in the absence of PEG or in the presence of 10 wt % PEG 8K, respectively. The final extents of fusion at these various conditions differ only slightly (33 vs. 40 vs. 31 %, respectively), indicating that PEG 20K interferes with close approach of the vesicles. Thus $Ca^{2+}$ is not able to induce aggregation above a polymer concentration of about $1.5 \times 10^{-3}$ wt %. This is consistent with the observation that $Ca^{2+}$-induced fusion is restored when the PEG 20K is less than $1.0 \times 10^{-3}$ wt %. The inhibitory effect of PEG 20K on $Ca^{2+}$-

induced fusion of DDP was no longer apparent at polymer concentrations as low as 5 x $10^{-4}$ wt %. $^{31}P$ NMR measurements revealed that PEG 8K and 20 K exert different effects on the DDP bilayers. In short, PEG 20K causes a significant dehydration of the headgroups and reduces the mobility of the DDP molecules in the lateral plane of the bilayer. Based on previous work (18), the diminished mobility could not account for the decrease in fusion activity. Furthermore, PEG 20K does not significantly interfere with the binding of $Ca^{2+}$ to the DDP bilayers, i.e., the *cis* complexation constant is not affected.

Taken together, the above results can be reconciled best with the view that PEG 20K, in contrast to PEG 8K, binds to the vesicle surface, thereby acting as a steric barrier for fusion. The efficiency by which PEG 20K causes this effect is much more pronounced than could be reasonably expected from the difference in molecular weight. Therefore, an additional factor most likely governs the difference in binding efficiency when comparing PEG 20K vs. 8K. In water, PEG displays a lower critical solution temperature ("clouding temperature"). Above this temperature, a phase separation takes place, resulting in a concentrated (e.g., 20-40 %) PEG and a dilute (< 1 % PEG) polymer phase. The clouding temperature for PEG 8K and 20K are 116 and 103°C, respectively (19). Certain salt solutions, in particular $H_2PO_4^-$ and $Na^+$, effectively lower the clouding temperature (20), and in 1 M $NaH_2PO_4$ this temperature is lowered from 103 to 42°C for PEG 20K. Taking into account that the DDP bilayer/water interface is comparable to a concentrated $(RO_2)PO_2Na$ electrolyte solution with a concentration of about 1.0-1.5 M and that the fusion experiments were carried out at 40°C, it appears likely that the inhibitory action of PEG 20K can be attributed to a clouding phenomenon. Indeed, in the presence of PEG 20K the fusion activity becomes impaired when raising the temperature above about 35°C (Figure 2). In the presence of PEG 8K, the fusion activity drops around 45°C, which is 12°C higher than

**Figure 2**

Effect of PEG on $Ca^{2+}$-induced fusion of DDP vesicles as a function of temperature. DDP vesicles (60 μM) were suspended in media containing 0.3 wt % PEG 20 K (open squares) or 10 wt % PEG 8K (solid squares) at the indicated temperatures. Fusion, induced by adding 2.9 mM $Ca^{2+}$ (final concentration), was monitored with the resonance energy transfer assay (cf. ref. 14). The initial fusion rates were plotted vs. temperature. Open circles: fusion in the absence of PEG.


initial fusion rate (%/sec, ■, o)
initial fusion rate (%/sec, □)
temperature (°C)
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
0.02
0.04
0.06
30
34
38
42
46

that for PEG 20K which correlates well with the difference in clouding temperatures in aqueous solution.

## MEMBRANE HYDRATION AND FUSION OF BIOLOGICAL MEMBRANES

From studies such as described above, much insight has been obtained as to the relevance of repulsive hydration forces in fusion of artificial membranes. To evaluate the significance of these observations for fusion of biological membranes we have investigated the interaction of Sendai virus with both artificial and biological membranes.

At physiological conditions this virus fuses with membranes at neutral pH, a process that is thought to be triggered by the viral membrane glycoprotein ("spike"), F (2,5,21). In earlier work (22), it was observed that the virus rapidly fuses with acidic phospholipid vesicles. Inclusion of phosphatidylcholine (PC) strongly inhibited the fusion reaction. By contrast, inclusion of phosphatidylethanolamine (PE) sustained the merging process. Since PE is much less hydrated than PC, these results would be consistent with the notion that the virus "senses" the repulsive hydration force.

A more detailed insight could be obtained when a kinetic model was developed that allowed us to analyze the distinct steps involved in the overall fusion reaction, i.e., the aggregation step and the fusion reaction itself (23,24). It could thus be shown that inclusion of PC in a negatively charged bilayer led to a two-fold increase in the fusion rate constant, whereas the aggregation rate constant was barely affected. Furthermore, inclusion of small amounts (up to 8 wt %) of PEG 8K in the medium stimulated the overall fusion reaction substantially as reflected by a three-fold increase in the initial fusion rate (0 vs. 8 wt % PEG). Simulation of the fusion process by the kinetic model revealed that the fusion rate constant similarly increased while very little effect was seen

on the aggregation rate constant. Taken together, these observations would support the notion that with *liposomes* as target membranes, the state of hydration of the membrane surface represents a fundamental parameter that modulates the viral fusion activity. It was of interest therefore, to further examine this parameter in a pure biological system.

To this end, the fusogenic properties of Sendai virus were studied, using erythrocyte membranes as targets. To modify the state of membrane hydration, virus, erythrocytes or both were treated with PEG 8K, and the kinetics of fusion were monitored. As shown in Figure 3, when fusion took place in a medium supplemented with small amounts of PEG, the overall rate of fusion increased dramatically.

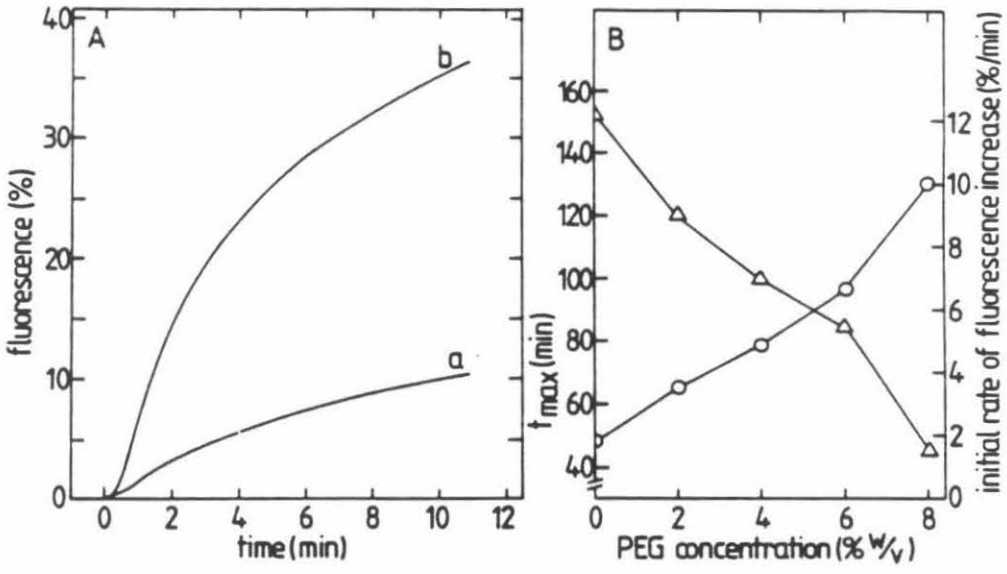
Previously (25), we showed that only a limited number (100-200) of virus particles fuse per erythrocyte, in spite of the fact that more than 1000 (fusion-active) viruses bind per cell. The presence of PEG does not alter the limited fusion capacity, implying that these concentrations of PEG do not trigger a massive non-specific fusion event. Furthermore, trypsinization of the virus, prior to its incubation with erythrocyte membranes, destroys the fusion activity, due to cleavage of the viral fusion protein. Hence, these experiments indicate that PEG by itself does not induce fusion, nor does the lipid probe, reporting the fusion reaction (26), spontaneously transfer between viral and target membrane.

The kinetic model, described above, revealed that, in the presence of PEG, the aggregation constant increased about two-fold (from about $1 \times 10^9$ to $2 \times 10^9$ $M^{-1}$ $s^{-1}$). The fusion rate constant increased by almost an order of magnitude, however (from 0.06 to 0.5 $s^{-1}$). It would appear, therefore, that membrane dehydration promotes the interaction of a virus with a biological target membrane, particularly at the level of the actual fusion reaction.

Fusion of Sendai virus is mediated by the fusion protein, F, which consists of two disulfide-bound subunits, $F_1$ and $F_2$. The N-terminus of $F_1$ contains a stretch of

**Figure 3**

PEG facilitates Sendai virus-erythrocyte membrane fusion. Fusion between virus (25 μg) and erythrocyte ghosts (76μg protein) was monitored at 37°C, using the $R_{18}$ fusion assay. Curves (left panel) represent fluorescence tracings obtained in the absence (a) and presence (b) of 8 % PEG 8K. The initial rates of fluorescence increase (open circles) were plotted (right panel) as a function of the PEG concentration. $t_{max}$ (open triangles) indicates the time necessary to reach the maximal level of fluorescence.

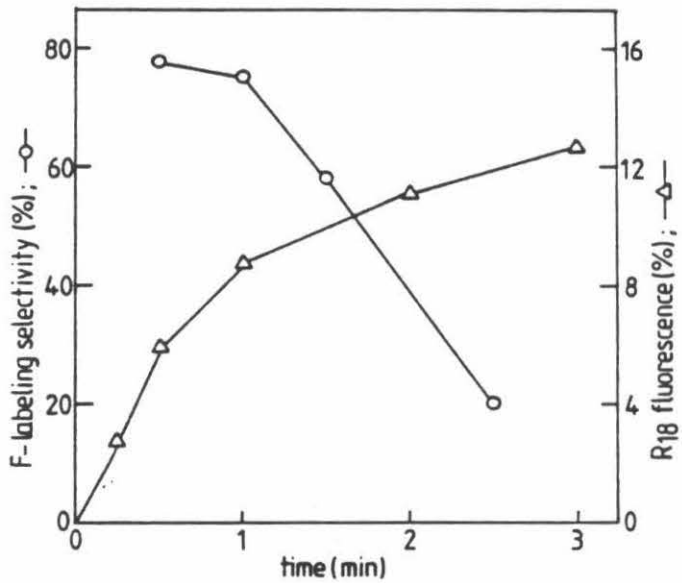

A
B
b
a
fluorescence (%)
time (min)
$t_{max}$ (min)
initial rate of fluorescence increase (%/min)
PEG concentration (% w/v)

approximately 20 hydrophobic amino acids and it has been proposed that this segment is directly involved in triggering viral fusion (27 and references therein) by its ability to penetrate into the target membrane. It could be suggested therefore that PEG facilitates penetration as a result of its capacity to dehydrate the local contact area between viral and target membrane. Also, it is possible that PEG, by virtue of modulating the polarity of the viral protein environment, affects the entire extraviral structure of the $F_1$ protein. Given the size of the $F_1$ polypeptide, the interbilayer distance could still amount to as much as about 80 Å after penetration of the hydrophobic terminus. Obviously, this distance has to be overcome before a direct contact between viral and target membrane is established. Possibly, hydrophobic dehydration may trigger such a structural change. The observation that viruses, but not ghosts, when preincubated in PEG containing media for extended time intervals (up to 15 min), display an enhanced fusion activity (two-fold) when compared to the activity seen without a preincubation in PEG, may provide support for this hypothesis. Further work will be necessary to corroborate this possibility.

From the work described above, it appears that hydrophobic interactions govern to a major extent the fusion of a virus, and presumably, this interaction involves the ability of the hydrophobic N-terminus to penetrate into the (hydrophobic) core of the target membrane. To obtain direct evidence for the hydrophobic penetration concept, we used the hydrophobic photoaffinity label 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine (TID) (28). The probe was incorporated into the hydrophobic core of liposomal bilayers and the labeled vesicles were subsequently incubated with Sendai virus at neutral pH. This approach allowed us to covalently label (upon photolysis) the viral protein interacting with the hydrophobic core of the target membrane at the onset of membrane fusion (29). As shown in Figure 4, the hydrophobic interaction of the F protein with cardiolipin (CL) vesicles during the early

**Figure 4**

Preferential association of $F_1$ with lipid vesicles during initial stages of membrane fusion between Sendai virus and liposomes. Sendai virus was mixed with TID-containing CL vesicles at 37°C, pH 7.4. After various time intervals samples were photolyzed (30 s). Protein label incorporation was determined by liquid scintillation counting of gel slices after SDS-PAGE (open circles). The kinetics of fusion were determined in a parallel experiment (open triangles). For details, see ref. 29.


80
60
40
20
0
16
12
8
4
0
0
1
2
3
time (min)
F-labeling selectivity (%); —o—
R18 fluorescence (%); —Δ—

stages of fusion, i.e., during the penetration step, is preferential. About 80 % of all labeling immediately after the addition of the virus is associated with the F protein. Moreover, gel analysis revealed that, more specifically, the $F_1$ polypeptide was labeled, i.e., the peptide in which the hydrophobic segment is contained. After initial insertion, the membranes merge, accompanied by total randomization and protein reorientation. As a result, other proteins become labeled as well, with a concomitant decrease in F labeling (Figure 4). Critical to the approach is therefore to "catch" the initial event during which only protein penetration occurs.

The primary purpose of the above experiments was to set up the principle of the approach. With CL vesicles, fusion of Sendai virus at neutral pH is mediated by the F protein. Trypsinization of the virus, which specifically removes the F protein, inhibits fusion by more than 80 % (10). However, since Sendai virus can also fuse with such liposomes at low pH (10), but not at those conditions with biological target membranes (8), it appears that the fusion between a virus and a liposome may not resemble in every respect the biological fusion conditions. Hence, the following challenge will be to evaluate this approach in a pure biological system. These experiments are currently in progress.

## ACKNOWLEDGMENTS

Mrs. Lineke Klap is gratefully acknowledged for expert secretarial assistance. This work was supported by grants from NIH (AI 25534), the US-Israel Binational Science Foundation (86-00010), ARO (DAAG29-83-K-0128) and Monsanto. Part of the work was carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

## REFERENCES


1. Sowers, A.E., Ed. (1987) *Cell Fusion*, Plenum, N.Y.

2. Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W. and Mayhew, E., Eds. (1988) *Molecular Mechanisms of Membrane Fusion*, Plenum, N.Y.

3. Düzgünes, N. (1985) *Subcell. Biochem.* **11**, 195-286.

4. Wilschut, J. and Hoekstra, D. (1986) *Chem. Phys. Lipids* **40**, 145-166.

5. Hoekstra, D. (1988) *Ind. J. Biochem. Biophys.* **25**, 76-84.

6. Hoekstra, D. and Wilschut, J. (1988) in *Water Transport in Biological Membranes* (Benga, G., Ed.), CRC Press: Boca Raton, FL, in press.

7. Rand, R.P. (1981) *Ann. Rev. Biophys. Bioeng.* **10**, 277-314.

8. Rand, R.P. and Parsegian, V.A. (1984) *Can. J. Biochem. Cell Biol.* **62**, 752-759.

9. Ohki, S. (1985) *Studia Biophysica* **110**, 95-104.

10. Arnold, K., Herrmann, A., Gawrisch, K. and Pratsch, L. (1988) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W. and Mayhew, E., Eds.), Plenum: N.Y., 255-272.

11. Tilcock, C.P.S. and Fisher, D. (1979) *Biochim. Biophys. Acta* **577**, 53-61.

12. Hoekstra, D. (1982) *Biochemistry* **21**, 2833-2840.

13. Rupert, L.A.M., Hoekstra, D. and Engberts, J.B.F.N. (1985) *J. Am. Chem. Soc.* **107**, 2628-2631.

14. Rupert, L.A.M., Engberts, J.B.F.N. and Hoekstra, D. (1986) *J. Am. Chem. Soc.* **108**, 3920-3925.

15. Rupert, L.A.M., Van Breemen, J.F.C., Van Bruggen, E.F.J., Engberts, J.B.F.N. and Hoekstra, D. (1987) *J. Membr. Biol.* **95**, 255-263.

16. Rupert, L.A.M., Hoekstra, D. and Engberts, J.B.F.N. (1987) *J. Colloid Interface Sci.* **120**, 125-134.

17. Rupert, L.A.M., Engberts, J.B.F.N. and Hoekstra, D. (1988) *Biochemistry* **27**, in press.

18. Rupert, L.A.M., Van Breemen, J.F.C., Hoekstra, D. and Engberts, J.B.F.N. (1988) *J. Phys. Chem.* **92**, 4416-4420.

19. Saeki, S., Kuwakara, N., Nakata, M. and Kaneko, M. (1976) *Polymer* **17**, 685-689.

20. Atamar, M. (1987) *Colloid Polymer Sci.* **265**, 19-25.

21. Hoekstra, D., Wilschut, J., Klappe, K., Stegmann, T. and Harmsen, M. (1985) *Studia Biophysica* **110**, 127-134.

22. Klappe, K., Wilschut, J., Nir, S. and Hoekstra, D. (1986) *Biochemistry* **25**, 8252-8260.

23. Nir, S., Klappe, K. and Hoekstra, D. (1986) *Biochemistry* **25**, 2155-2161.

24. Nir, S., Klappe, K. and Hoekstra, D. (1986) *Biochemistry* **25**, 8261-8266.

25. Hoekstra, D. and Klappe, K. (1986) *J. Virol.* **58**, 87-95.

26. Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) *Biochemistry* **23**, 5675-5681.

27. Hoekstra, D., Klappe, K., Stegmann, T. and Nir, S. (1988) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W. and Mayhew, E., Eds.), Plenum: N.Y., 399-412.

28. Brunner, J. and Semenza, G. (1982) *Biochemistry* **20**, 7174-7182.

29. Novick, S.L. and Hoekstra, D. (1988) *Proc. Natl. Acad. Sci. USA*, in press.

# Chapter 9
# Summary and Concluding Remarks

## SUMMARY

The finding that hydrophobic interaction of the viral fusion peptide with the target membrane core is the initial triggering event during Sendai virus fusion with both artificial and biological membranes confirms a central hypothesis about how viruses (and probably other membranes) undergo membrane fusion. The observation that retention of the capability of viral glycoproteins to carry out hydrophobic interactions is necessary for functional reconstitution of viral envelopes may be of significance for designing reconstitution methods for drug delivery. In a more general sense, the correlation of hydrophobic interactions with membrane hydration, packing, and other physical parameters, provides a fuller description of the course of events during membrane fusion.

The first direct measurement of bacteriophage DNA injection kinetics showed a one-step process occurring much faster than previously thought. The quantitative measurement of the process is a necessary first step toward obtaining a mechanistic understanding of injection. The rapidity of injection may have further implications for the structure of DNA packaged in the bacteriophage capsid as well.

## CONCLUDING REMARKS

The initial goal of this thesis project was to develop a gene delivery system based on injection of *in vitro* packaged DNA by bacteriophage λ into cells via an implanted receptor. The receptor, LamB, was to be co-reconstituted into Sendai virus envelopes and implanted into cells by membrane fusion.

We quickly realized the complexity of the viral systems, however, and the need to improve our basic understanding of them. Initial attempts at the applications were carried out with some encouraging results (not shown), but too many unknowns remained for development of a mature delivery system at that time.

It is hoped that the basic studies in this thesis improve our understanding of viral infectious processes, will lay the groundwork for successful development of virus-mimetic delivery vehicles, and will also set a precedent for future mechanistic studies.