HAMDAR **BILAYER STABILIZATION AND VIRAL FUSION** **M.Sc.**

THE EFFECTS OF BILAYER SIDEDNESS AND FLIP-FLOP OF LYSOPHOSPHATIDYLCHOLINE ON VIRAL FUSION WITH MODEL AND BIOLOGICAL SYSTEMS

BY

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1999) McMaster University
(Biochemistry) Hamilton, Ontario

TITLE: THE EFFECTS OF BILAYER SIDEDNESS AND FLIP-FLOP OF LYSOPHOSPHATIDYLCHOLINE ON VIRAL FUSION WITH MODEL AND BIOLOGICAL SYSTEMS

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NUMBER OF PAGES: xiii, 91

ABSTRACT

Intermediate lipid structures such as inverted micelles and interlamellar attachment are thought to play a crucial role in different biological processes like viral infection. Lysophosphatidylcholine has been shown to inhibit membrane fusion at stabilizing concentrations (between 1 and 10% with respect to membrane lipids).

Studies in this thesis looked at the effects of Lysophosphatidylcholine (LPC) properties on the inhibition of Sendai viral fusion. The effects of bilayer sidedness preference as well as flip-flop of Lysophosphatidylcholine (lyso PC) were examined. Octadecylrhodamine (R_{18}) lipid mixing assays were used to measure the fusion of Sendai virus with different biological, erythrocyte ghosts, and artificial systems consisting of different lipids and different viral receptor compositions. The data showed that external addition of LPC exhibits a dependency between the incubation time of the lysolipids and the inhibition of viral fusion. The results also demonstrate a relationship between the location of LPC in the bilayer and its ability to inhibit lipid mixing. LPC present only on the outer monolayer plays a role in the inhibition of viral fusion. Reorientation of LPC was also measured for the same incubation periods. A method using Bovine Serum Albumin (BSA) and radioactivity labelled LPC, was applied to measure

the flip-flop. Significant transbilayer reorientation of Lyso PC in the bilayer was shown to take place. The rate of flip-flop was measured at 0.32 ± 0.08 % LPC /min. Such reorientation can explain the time dependency observed earlier.

The conclusions of this thesis lend support to stalk intermediate mechanism of viral membrane fusion. The ability of LPC to inhibit only when present on one side of the bilayer supports the idea of a negatively curved stalk intermediate. Moreover, it showed that the shape and curvature tendencies of the bilayer stabilizer determine its effects on viral fusion.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Richard Epand for his guidance, direction, patience and inspiring discussion. I would also like to thank members of the Epand lab (past and present) for stimulating discussion, helpful suggestions and making the lab such an enjoyable place to work. These include Dr. Raquel Epand, Joses Jones, Jennifer Giorgione, and all the post-doctoral fellows and project students.

I would like also to thank my supervisory committee members; Dr. John Capone, Dr. Albert Berghuis, Dr. Hara Ghosh and Dr. Richard Epand for their comments and criticism. I acknowledge the many other friends I have made throughout this experience for making it a much more fulfilling and a memorable one.

To my family, I thank God for blessing me with your love and unwavering support throughout all my efforts. I couldn't have done it without your help. Thank you.

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LIST OF ABBREVIATIONS

BCA	Bichinconinic acid
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonion]-1-propanesulfonate
c.m.c.	Critical micellar concentration
ddH2O	Double distilled water
EDTA	Ethylenediaminetetraacetate (disodium salt)
Egg PC	Egg phosphatidylcholine
Egg PE	Egg phosphatidylethanolamine
F	Fusion protein
GD1a	Disialoganglioside
GQ1b	Tetrasialoganglioside
GT1b	Trisialoganglioside
HEPES	4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid
HN	Sendai virus hemagglutinin/neuraminidase protein
LIS	Lithium diiodosalicylate
LPC	Lyso phosphatidylcholine
LUV	Large unilamellar vesicles
M	Molar
MES	2-[N-morpholino]ethanesulfonic acid
min	Minutes
MLV	Multilamellar vesicles
PBS	Phosphate buffered saline
PEG	Polyethyleneglycol

R_{18}	Octadecylrhodamine-B-chloride
SDS	Sodium dodecyl sulfate
SUV	Small unilamellar vesicles
t	Time
TMC	Trans monolayer contact
λex	Excitation wavelength
λem	Emission

1.0 INTRODUCTION

1.1 Lipid Morphology

Membrane lipids have mostly an amphipathic structure. One end has a polar group, such as phosphate esterified to an amino alcohol. The rest of the molecule is mostly hydrophobic [Yeagle, 1993 (a)]. There are three classes of lipids present in biological membranes. These are the glycerophospholipids, sterols and finally sphingolipids [Yeagle, 1993 (a)].

Sphingolipids contain a ceramide moiety that has a hydrocarbon tail, a fatty acid linked to the 2-amino position of sphingosine in addition to a polar head group. On the other hand, glycerophospholipids contain a glycerol backbone with a phosphate group esterified to one of the hydroxyl groups and fatty acids esterified to the other two [Yeagle, 1993 (a)].

1.1.1 Structure of Phospholipids

The name is derived from the phosphate group located at the polar head of the molecule. In addition to the head group, two hydrocarbon chains are esterified to a glycerol which is esterified to the phosphate at the 3' position [Yeagle, 1993 (a)]. The phosphate is esterified, in turn to an alcohol. The naming of specific phospholipids lends itself to the alcohol. Phosphatidylcholines , for example , are named after the choline group { $HO\ CH_2\ CH_2\ N(CH_3)^+_3$ }.

1.1.2 Lipid Polymorphism

Many biological lipids can self-assemble in a variety of different phases and thus exhibit polymorphic behaviour [Gruner, 1992; Epand, 1997]. This ability has been theorised to be important in many physiological processes performed by biological membranes such as membrane fusion [Ellen *et al.*, 1989; Cheetham *et al.*, 1994]. The self-organization of phospholipids is an effect of their amphiphilic nature. Geometric factors related to the chemical structure of the individual lipid molecules and how they pack together determines the morphological structure formed by the lipids when suspended in water. The ability of lipids to act differently in their pure form, compared to biological membranes, indicates the presence of limitations on morphological rearrangements in native membranes [Gruner, 1992]. The polymorphic behaviour of lipids has been theorized to be a result many factors.

The ability of biological lipids to exist in various phases has been explained by a lipid shape theory [Cullis *et al.*, 1979; Israelachvili *et al.*, 1980]. According to the theory, lipids have a dimensionless critical packing parameter which determines the structure they will form in aqueous suspension. In this model, lipids with a large head group area and a small acyl chain volume, such as lyso PC, are expected to form micelles. Lipids with a more balanced ratio, such as PC, would form bilayers (Figure 1.1.1).

Another model has also been proposed [Gruner *et al.*, 1985; Gruner, 1992]. According to this, a competition between the intrinsic radius of curvature and hydrocarbon packing constraints determines the phase behaviour of different lipids. Such considerations arise naturally from the fact that, within a lipid monolayer, forces arising from interactions in the polar, interfacial, and hydrophobic regions do not usually act through the "centre of mass" of the lipid molecule. Thus, lipid monolayers would be predisposed to curvature as long as the vector sum of the moments of these forces about the centre of mass of the lipid is not zero [Lewis *et al.*, 1997].

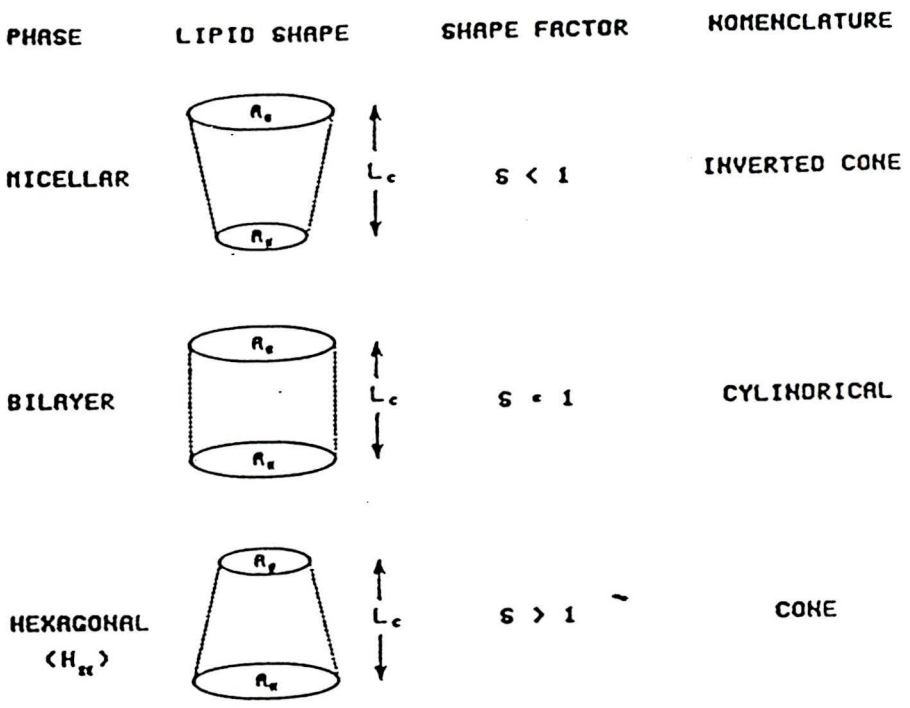


Figure 1.1.1

Shape features exhibited by membrane lipids. See text for further details. Adapted from: Wilshut, J., & Hoekstra, D. *Membrane Fusion*, Marcel Dekker, Inc. (1991) p.49.

1.2 Membrane Bilayer

1.2.1 Fundamental Structure of Bilayers

Eukaryotic cells possess various internal membranes and membrane-bound organelles in addition to the plasma membrane. This thesis is mainly concerned with the cellular plasma membrane. This membrane structure is described by the fluid-mosaic model [Singer *et al.*, 1972; Bloom *et al.*, 1991] (Figure 1.2.1). The extracellular matrix is composed of glycosylated integral and peripheral membrane proteins. Integral proteins are imbeded in the lipid bilayer. The membrane cytoskeleton is located on the cytoplasmic side of the bilayer and plays many functional roles such as determination of cell shape and mechanical support.

Biological membranes contain the lipid bilayer as their fundamental structural element [Epand, 1997]. It is formed as a direct affect of the assembly of amphipathic membrane lipids. This assembly is a thermodynamically favoured process driven by the structure of the lipid molecules as well as the hydrophobic effect [Yeagle, 1993 (b)] . This results in the formation of a stable as well as a selectively permeable structure that is capable of isolating the contents of the cell from that of the exterior and therefore determining the boundary of the cytoplasm [Yeagle, 1993 (b)].

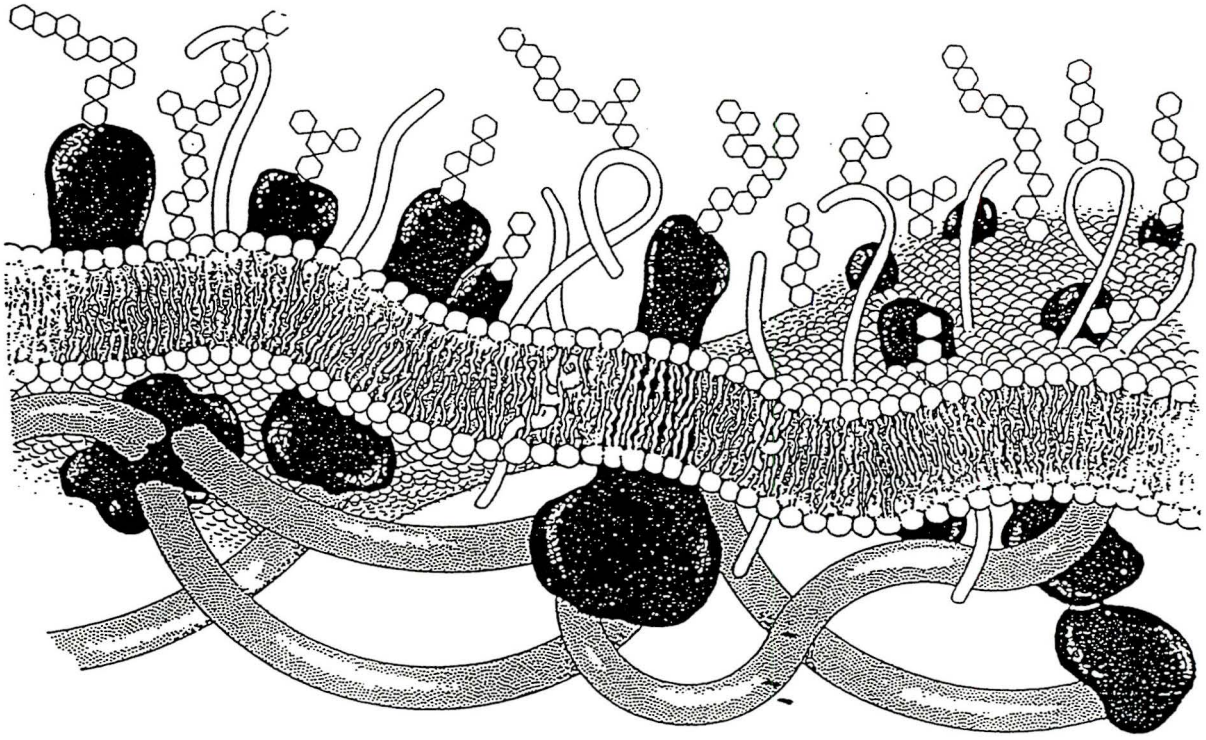


Figure 1.2.1

A schematic representation of eukaryotic plasma membrane [Bloom, *et al.*, 1992]. The top of the diagram represents the extracellular matrix, the middle is the plasma membrane, and the bottom of the picture represents the lumenal side of the membrane with the cytoskeleton. Black objects represent the integral membrane proteins. See text for further details.

1.2.2 Experimental Systems for Studying Membranes

Two general systems are used when studying membranes or membrane associated molecules: Liposomes (artificial systems) or naturally occurring biological membrane systems. Both methods were implemented throughout this work.

1.2.2.1 Artificial Systems (Liposomes)

Liposomes are made of amphiphiles, that is molecules composed of polar and apolar regions. Exposure of the hydrophobic moiety to water is thermodynamically unfavourable. Protection of these portions from aqueous solutions is possible through self-aggregation of the amphiphiles [Israelachcili et al., 1977; Tanford, 1980]. Thus, above a certain critical concentration, which depends on the amphiphiles and the aqueous medium, most amphiphiles self-aggregate. As a rule of thumb, the amphiphiles will form the aggregates in which the hydrophobic portion of the molecule has the minimal contact with water. These can be lamellar or nonlamellar aggregates.

Classifications and nomenclature of liposomes is based on two parameters: number of bimolecular lamellae and vesicle size [Szoka & Papahadjopoulos, 1980]. Unilamellar vesicles, prepared by various methods, vary in size and often divided into two groups, large (50-200 nm) and small (<50 nm) unilamellar vesicles [Wilschut *et al.*, 1991].

Liposomes can be prepared using different methods. After dispersing membrane lipids in an aqueous phase, spontaneous formation of multilamellar vesicles (MLV) takes place. This is then followed by mechanical procedures such as sonication [Huang, 1969], or extrusion through polycarbonate membranes with defined pore size [Oslon *et al.*, 1979] which leads to reduction in size and number of lamellae of the vesicles. A second set of procedures use the addition of suitable detergents. The detergents are added at a concentration far above their critical micellar concentration (c.m.c.) forming mixed micelles with the lipids. The detergents are in equilibrium with free detergent monomers. The slow dialysis of detergent from mixed micelles [Schurtenberger *et al.*, 1984] can produce a homogenous population of vesicles of a predetermined size range [Milsmann *et al.*, 1978; Zumbuehl & Weber, 1981]. This is useful in an important array of applications which require these characteristics [Tauskela *et al.*, 1992]; these include model membrane studies, drug/macromolecule encapsulation, and protein-reconstitution efforts [Schmidt, 1986; Mimms *et al.*, 1981]. The rate of detergent removal has been shown to drastically affect the homogeneity of the glycoproteins distribution and the morphology of the resulting proteoliposomes [Jackson and Litman, 1982]. Fast detergent removal leads to the formation of small unilamellar vesicles while slower removal leads to large unilamellar vesicles [Ames and Dubin, 1960; Levy *et al.*, 1991].

Consistency in the liposome preparation is an important issue when working with artificial membrane systems. Such consistency manifests itself in terms of vesicle size and composition of liposomes. To best mimic biological membranes, liposomes must show some of the physical properties of these membranes. For research involving membrane curvature and fusion, bilayer are expected to posses a curvature strain that is comparable to biological systems. This can achieved by using LUVs above 80 nm in diameter. Above this size, the net curvature strain is minimal in the bilayers.

1.2.2.2 Biological Systems (Biological Membranes)

The relative simplicity of artificial membranes compared to biological membranes allows for easy manipulation of specific intrinsic properties of the system. This would enable one to eliminate the enormous number of variables present in biological membrane studies.

The use of biological membrane systems offers several advantages. First, it allows for the study of membranes in their native state. A central feature in membrane organization is the asymmetrical distribution of phospholipids [Dressler *et al.*, 1983]. Using biological membrane systems would allow for more biologically relevant results. Liposome chemical and physical stability is also a major issue in their application [Lichtenberg, 1988]. Biological membranes, such as the erythrocyte membrane, on the other hand have a

longer shelf life. Finally, it is preferable to have a homogeneous population of vesicles having a narrow distribution in size and number of lamellae [Lichtenberg, 1988]. Such a requirement is easier to control when working with biological systems.

1.3 Membrane Fusion

The bimolecular leaflet arrangement, of lipid bilayers, is quite stable and will resist a gross departure from this organization [Wilschut, 1991]. Any structural change that would severely impede this basic barrier function would be intolerable. On the other hand, in cell biology numerous membrane fusion and fission events occur in order to allow the necessary flow of mass and information between cells and from one intracellular compartment to another.

During a membrane fusion event the two membranes involved have to transiently leave the stable bimolecular leaflet arrangement without compromising their prime function as a relatively impermeable barrier [Wilschut, 1991]. Clearly, the stimulus inducing a fusion event will be restricted to a very short period of time and to a specific localization [Baker *et al.*, 1984; Burgoyne, 1984].

The forces between the membranes are important in determining susceptibility of membranes to fusion. Water between membranes must be removed in order for them to fuse, and the force required is called the hydration

force [Rand and Parsegian, 1989]. In addition, attractive van der Waals, repulsive electric double-layer and entropic forces are also present between interacting membranes and these forces influence fusion [Israelachvili and Wennerstrom, 1992].

Much of our current knowledge regarding the possible molecular mechanisms of membrane fusion has been derived from investigation of model membranes [Nir *et al.*, 1983; Wilschut *et al.*, 1986; Bentz *et al.*, 1988]. Liposomes are relatively simple and relatively simple to make. The membrane composition as well as the aqueous contents can be easily manipulated.

1.3.1 Fusion Intermediates

The versatility of Liposome systems has led to the development of a set of fusion assays based on the incorporation of fluorescent probes into the bilayer of liposomes or sequestering the reporter probe in the aqueous interior [Duzgunes *et al.*, 1988; Silvius *et al.*, 1988]. These assays allow for the investigation of the kinetics of the fusion reaction under well-defined conditions. The kinetics of a particular fusion process can then be correlated with data on the structure of the lipid systems of the same composition at equilibrium so as to provide insight into the possible structural nature of the fusion intermediates.

Membrane fusion requires a transient disruption of the lamellar arrangement of membrane phospholipids, and may involve defects in membrane structure [Hui *et al.*, 1981]. Since the phase preference of a particular lipid influences the properties of the membrane in which it is included [Epand, 1997], the morphological rearrangement of fusing membranes seems to be facilitated by nonbilayer-forming lipids.

The factors that promote the hexagonal phase in lipid dispersions have all been shown capable of inducing fusion in LUVs [Cullis *et al.*, 1978; Ellens *et al.*, 1984]. Evidence regarding the role of nonbilayer structures in membrane fusion comes from the observation of lipidic particles in freeze-fraction studies. Many different types of intermediate structures have been observed during membrane fusion [Frederik *et al.*, 1989]. The most convincing proposed fusogenic intermediate have been the 'stalk' intermediate [Chernomordik *et al.*, 1985; Siegel, 1993]. It proposes the existence of a semitoroidal lipid structure formed by the coalescence of the cis monolayers of the fusing membranes (Figure 1.3.1). The next step in the fusion process involves the increase in the radius of the stalk leading to the formation of trans monolayer contact (TMC). This is in turn followed by a rapid expansion of the TMC giving rise to an area difference between the cis and trans monolayers [Epand, 1997]. The resulting tension would lead to the rupture of the TMC and formation of the fusion pore and therefore membrane fusion.

1.3.2 Curvature and Fusion

The fusion reaction, as previously mentioned, has been associated with instability in the membrane bilayer. Bilayers with high degree of negative curvature stress are less stable and more prone to defect formation [Epand, 1997]. Therefore one would expect that the fusion reaction will increase when curvature stress reaches its maximum. Such maximum will be obtained at the onset of non-bilayer phase formation (Hexagonal or cubic phase) [Bentz *et al.*, 1985].

To better understand the relation between curvature stress and fusion susceptibility, various groups have conducted extensive studies. One example of such studies was conducted by Yang *et al.*, (1997). Polyethyleneglycol (PEG) was used to induce the fusion of vesicles made of PE and PC mixture. The threshold of PEG needed to induce fusion at 25°C was determined with various percentages of PE. The threshold decreased with increasing PE. Different kinds of PE molecules where used. PE molecule promoting more curvature stress, showed the most decrease in the threshold of PEG induced fusion.

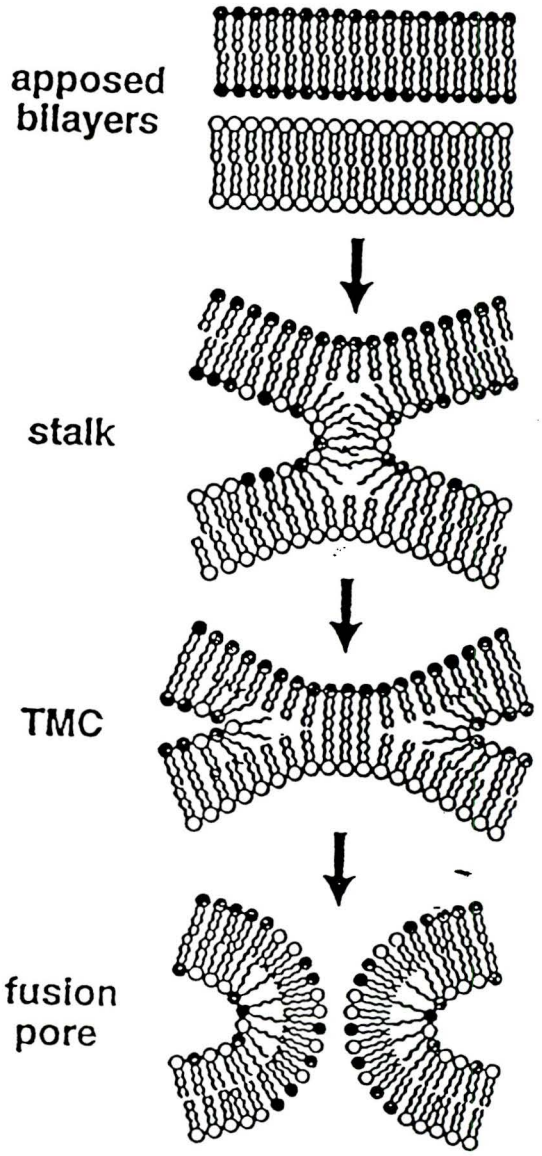


Figure 1.3.1

Proposed mechanism of membrane fusion indicating key intermediate structures (After siegle, 1993). Adapted from: Epand, R. M. *Lipid polymorphism and membrane properties*. Academic Press (1997) p. 367.

1.4 Viral Fusion

1.4.1 Sendai Virus

Sendai virus is a member of the genus *paramyxovirus* [Yeagle,1993 (b)]. It was first isolated in Sendai, Japan and originally named Hemagglutinating Virus of Japan (HVJ) [Okada and Tadokoro, 1962]. The natural host for Sendai virus is the mouse. The major target of the virus are the lungs. The replication of the virus in the lungs causes severe damage but no obvious clinical effects [Kingsburry, 1991]. Sendai virions are usually spherical with an average diameter of 150 to 250 nm, but exhibits some pleomorphism [Hosaka *et al.*, 1966]. Sendai virus has a single helical nucleocapsid with a single 15 kb strand of negative sense RNA [Kingsbury, 1991]. Overlapping genes are expressed from a single mRNA [Giorgi *et al.*, 1983].

Sendai virus possess two membrane glycoproteins, HN and F, which are responsible for binding the virus to cell surface sialic acid and for fusion, respectively [Hsu *et al.*, 1979; Kingsbury, 1991]. The glycoproteins form peplomers which appear as 10 to 15 spikes on the surface of the virions [Hosaka, 1988]. Sendai virus F protein is formed from the protein F_O (~ 62 kDa), then proteolytically processed by the host cell protease to yield two disulphide-linked subunits F_1 and F_2 [Scheid and Choppin, 1974; Kawahara *et al.*, 1992]. This process generates a novel hydrophobic amino terminus on the F_1 subunit and this is required for viral fusion [Scheid and Choppin, 1974]. A non-covalently

linked tetramer of disulphide bounded F_1 and F_2 subunits is believed to be the biologically active form of the fusion protein [Sechoy *et al.*, 1987]. Reconstitution studies have shown the importance of this proteolysis in the fusion process [Hsu *et al.*, 1979].

1.4.2 Mechanism of Viral Fusion

Infection of cells by enveloped animal viruses requires membrane fusion. Viruses such as Sendai, HIV and Herpes simplex virus fuse at neutral pH with the cell membrane [Hernandez *et al.*, 1996; Lawless *et al.*, 1996]. Other viruses, such as influenza, vesicular stomatitis virus fuse with endosomal membranes after endocytosis and subsequent acidification of the endosomal compartment [Marsh and Helenius, 1989] (Figure 1.4.1).

The process leading to the fusion of two lipid bilayers has been suggested to include the following events [Bentz and Ellens, 1988]:

① Aggregation of fusing membranes.

② Close approach of the lipid bilayers of the fusing membranes.

③ Destabilization of the bilayer at the point of fusion.

④ Mixing of the bilayers and ultimate separation from the point of fusion into a new membrane structure.

One proposed mechanism of fusion between Sendai virus and target membrane proceeds as follows [Yeagle, 1993 (b)]. Initial contact between virion and target membrane occurs through the binding of HN protein to a receptor.

The free energy of binding is utilized to promote a rearrangement of the interface between the virion and the target membrane, to allow contact between the F-fusion peptide and the lipid bilayer of the target membrane. A clue of this process can be found in the presence of the temperature-dependent "lag" time preceding the fusion event [Hoekstra *et al.*, 1985]. Contact between the F-fusion peptide and the bilayer of the target membrane leads to destabilization of a small region of the lipid bilayer of the target membrane [Yeagle *et al.*, 1991], utilizing portion of the binding energy. This destabilization allows for the rearrangement of lipids between the two membranes through structures with a small negative radius of curvature. The formation of the fusion pore is then believed to occur as described in Section 1.3.1.

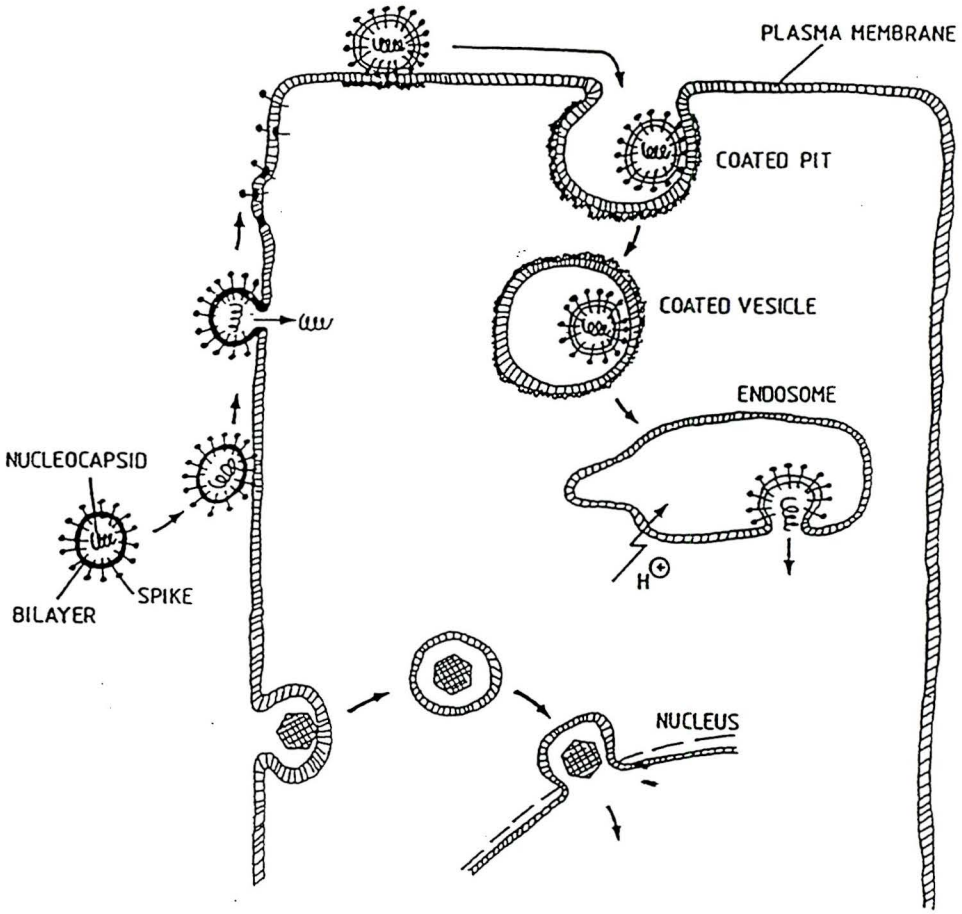


Figure 1.4.1

Pathways of viral infection. Adapted from Hoekstra and Nir, 1991. See text for more details.

1.4.3 Sendai Virus Receptors

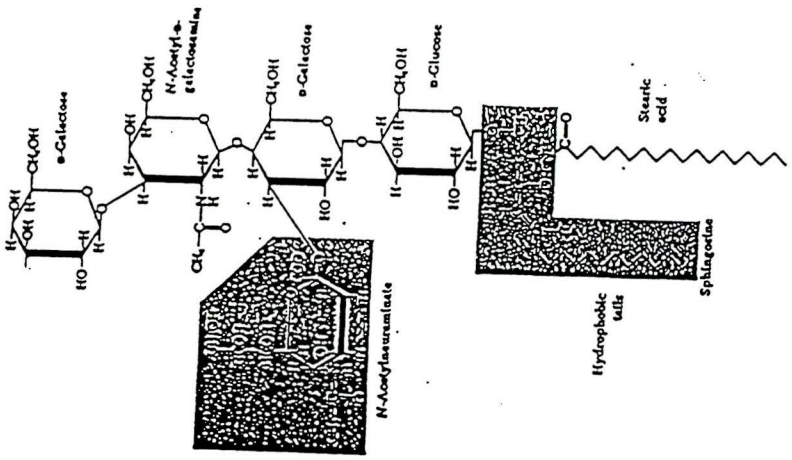
In many cases, members of the same genus of a virus family have been found to select different chemical entities for cell attachment and invasion of host cells [Bitzer *et al.*, 1997; Hernandez *et al.*, 1996]. Moreover, a few viruses have been shown to use more than one distinct receptor population, HIV for example [Wimmer, 1994]. Sendai virus has been shown to recognize sialylated glycoproteins [Wybenga *et al.*, 1996], as well some sialic acid-containing gangliosides, best of which is the ganglioside GD1a [Suzuki *et al.*, 1985]. Sialic acid moieties serve as a receptor for Sendai virus due to the affinity of the HN glycoprotein to this species [Miura *et al.*, 1982]. The importance of sialic acid in the fusion event has been demonstrated using sialidase treatment of certain host cells which prevented viral infection [Marcus, 1959; Haff & Stewart, 1964]. It is important to mention that although sialic acid presence is important for specificity, it seems that other factors must also be involved.

Glycophorin, the major erythrocyte glycoprotein, serves as a excellent receptor for Sendai virus [Oku *et al.*, 1982]. Glycophorin is a 131 amino acid residue protein (31 kDa). 60% of its weight is carbohydrate (18 kDa) containing an average of 30 residues of sialic acid (Figure 1.4.2). It mostly exists as a dimer due to subunit interactions between molecules.

Gangliosides are derivatives of the ceramides with multiple additions of sugar, ending with N-acetylneuraminic acid (sialic acid), a sugar acid carrying a

negative charge [Yeagle, 1993 (b)] (Figure 1.4.2). Gangliosides contain less sialic acid residues than does glycophorin, and have been shown to promote less viral fusion [Wybenga *et al.*, 1996]. Only ganglisides with the proper carbohydrate sequence, such as GD1a, GT1b, and GQ1b, function as natural receptors for Sendai virus in host cells [Markwell *et al.*, 1981].

(A)



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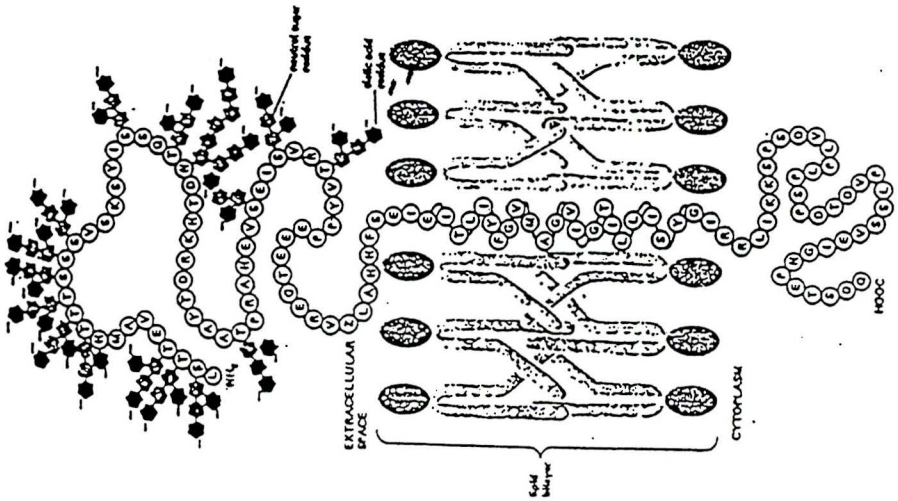


Figure 1.4.2

Structure of viral receptors. (A) Ganglioside, (B) Glycophorin A. Figure adapted from: Lehninger. *Principles of Biochemistry.* Worth Publishers, Inc. (1982) p. 314.

1.5 Transbilayer Movement

1.5.1 Definition of 'flip-flop'

Several cellular processes, such as endocytosis and membrane fusion, involve deformation and/or changes in the curvature of biological membranes. Resistance to such changes is described in terms of bending rigidity. Such rigidity exists on two levels. The intrinsic stiffness of each monolayer to curvature changes, which is termed local bending stiffness (Kc) [Helfrich, 1973]. The second level arises from the different strain each monlayer is subject to when bilayer's curvature is modulated [Raphael and Wagh, 1996]. The two monolayers are not connected and therefore may redistribute independently to try to relieve different strain in the two leaflets. This kind of bending energy is called "global curvature energy" [Evans, 1974].

To relieve global curvature strain biological membranes can implement two mechanisms (Figure 1.5.1). The first includes the propagation of the difference over the vesicle surface by lateral movement of locally expanded leaflet relative to the locally compressed opposing leaflet [Raphael and Wagh, 1996]. The second method involves transmembrane movement of molecules from the compressed leaflet to the expanded leaflet. Such an event is referred to as *"flip-flop"* .

1.5.2 Lipid Asymmetry and Fusion

The concentrations of various lipid species on the cytosolic and plasma or lumenal monolayers of biological membranes are highly regulated [Bailey and Cullis, 1997]. One possible role for such asymmetry may be regulation of membrane fusion through the influence of spontaneous monolayer curvature [Bailey and Cullis, 1997]. Examples of the importance of asymmetry and fusion has been observed in both biological systems as well as model membranes. In eukaryotic plasma membranes, PE and PS (Phosphatidylserine) are found mainly on the cytosolic monolayer and PC and SPM (Sphingomyelin) are present on the outer monolayer [Houslay and Stanley, 1982]. Many experiments have been done to show that the inner monolayers of these membranes are more prone to fusion then the outer monolayers [Hope *et al.*, 1983]. Also, erythrocytes that have lost the asymmetry across their bilayers have shown high tendency of fusion [Tullius *et al.*, 1989].

In model systems it has been shown that by imposing a pH gradient across the membrane, one can induce asymmetry [Hope and Cullis, 1987]. This is true since neutral lipids can partition into the hydrophobic region of the bilayer much more readily than their charged counterparts under such pH conditions. Liposomal systems, exhibiting asymmetry, have since been used to relate such lipid imbalance to membrane fusion [Eastman *et al.*, 1992]. These systems also

indicate a possible role for transbilayer lipid asymmetry in regulating fusion of biological systems.

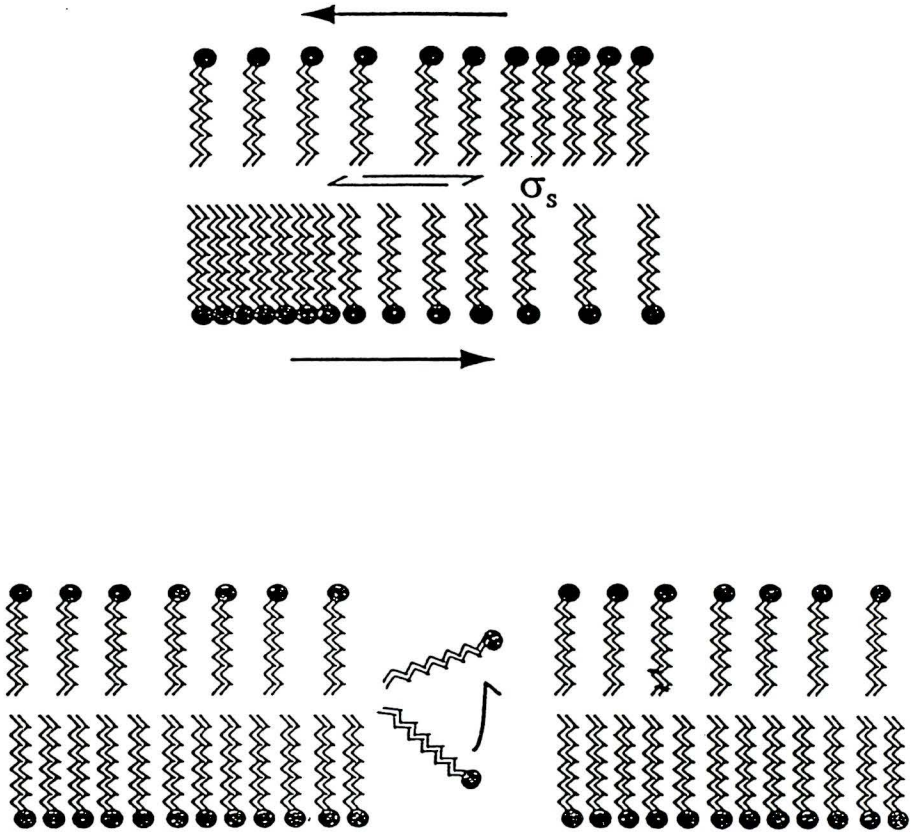


Figure 1.5.1

Two mechanisms for relaxing differential density in membranes [Raphael and Waugh, 1996]. See text for more details.

1.6 Overview and Objectives

Fundamental and diverse cell processes involve rearrangement of biological membranes- membrane fusion. Two membranes merge into one with a joining of the aqueous volumes they initially separated. These complex fusion processes involve proteins that bring the fusing membranes into close proximity and promote fusion through membrane destabilization. Although many fusion proteins have been identified, the exact mechanism of the fusion events remains poorly defined.

Membrane fusion requires transient disruption of the lamellar arrangement of membrane phospholipids. This morphological rearrangement seems to be facilitated by the presence of nonbilayer-forming lipids in the fusing membranes. The ability of biological lipids to exist in various phases has been explained by a lipid shape theory.

Lyso PC has been shown to inhibit diverse biological fusion processes such as exocytosis and virus-mediated syncytia formation. The apparently universal character of the phenomenon suggests that lysolipids inhibit an intermediate step common for all fusion reactions. The focus of the project is to look at the bilayer stabilizer LPC inhibition of Sendai viral fusion. It aims to show that the effectiveness of LPC as a fusion inhibitor is dependent on its shape, and therefore curvature tendencies, as well as its location in membrane bilayers.

The project is divided into two separate yet interconnected parts. The first part is aimed to test if LPC viral fusion inhibition exhibits a time dependency. Both artificial as well as biological membrane systems are used. Proteoliposomes are prepared by reconstituting various Sendai virus receptors into liposomes. Different reconstitution methods, lipid / receptor combinations as well as varying lipid to receptor molar ratios are investigated.

The second part of the work investigates a possible phenomenon that would explain the decrease of fusion inhibition with time. Transbilayer movement, or flip-flop, of LPC is suspect. Flip-flop of LPC in a bilayer would translocate some of the lyso PC to the inner monolayer. Once in the inner monolayer, the LPC is sequestered there and will not contribute to stabilize the curvature strain of the fusion intermediate(s). A method using Bovine Serum Albumin (BSA) and radioactivity labelled LPC, is applied to measure the rates of flip-flop. BSA has been shown to remove LPC from the outer leaflet of bilayers. Purified erythrocyte ghosts are used for these experiments.

In order for the translocation of LPC between monolayers to explain the decrease of inhibition, Lyso PC must only inhibit viral fusion when present in the cis or outermost monolayer. Liposome systems will be used in these experiments. The aim of this is to determine if viral fusion inhibition of LPC is dependent on its position in the membrane.

The work in this project is aimed to support an existing idea as well as add to it. The results will support the stalk intermediate model of membrane fusion. The ability of LPC to inhibit fusion when present only on the cis monolayer, would indicate that fusion inhibition is a consequence of the molecule's shape and therefore its intrinsic curvature properties. Also flip-flop of LPC between the two monolayers may play a role in the effectiveness of LPC as a fusion inhibitor and a bilayer stabilizer.

2.0 MATERIALS AND METHODS

2.1 Materials and Sources

All commercially obtained materials were reagent grade or better. When required, materials were used as specified by the manufacturer.

2.1.1 Lipids

All commercially obtained lipids were of the highest quality and purity available.

Egg Phosphatidylcholine	Avanti Polar Lipids
Egg Phosphatidylethanolamine	Avanti Polar Lipids
Ganglioside GD_{1a}	Bachem
L- 1-[palmitoyl-1-^{14}C]lysopalmitoylphosphatidylcholine	New England Nuclear
L -α-LPC (C-16)	Avanti Polar Lipids
L -α-LPC (C-12)	Avanti Polar Lipids

2.1.2 Chemicals and Solvents

Acetone	Fisher
Ammonium Molybydate	BDH
Ammonyx-Lo *(Lauryldimethlamine Oxide, 30% Solution)*	Calbiochem
Ascorbic Acid	Sigma
BCA Assay Kit	Pierce
Bovine Serum Albumin	Sigma
n - butanol	Fisher
n - butylacetate	Sigma
CHAPS	Sigma
Chloroform	Fisher
Citric Acid	BDH
Cupric Sulphate	BDH

Ethylenediaminetetraacetate (EDTA)	BDH
Glycine	BDH
HEPES	Sigma
HCl	BDH
Lithium Diiodosalicylate	Sigma
Magnesium Nitrate	BDH
MES	Sigma
Phenol	Fisher
PMSF	Sigma
Potassium Phosphate (Monobasic)	Fisher
Resorcinol	Sigma
Scintillation fluid	ACS, Amersham
Sodium Azide	BDH
Sodium Carbonate	Fisher
Sodium Citrate	BDH
Sodium Chloride	BDH
Sodium Dodecyl Sulfate (SDS)	Sigma
Sodium Phosphate (Monobasic)	Fisher
Sodium Phosphate (dibasic)	Fisher
Sucrose	BDH
Sulfuric Acid	BDH
Triton X -100	Bio - Rad
TEMED	Gibco BRL
Tris	Boehringer Mannheim
WGA	Sigma

2.1.3 Fluorescent Probes

Octadecylrhodamine (R_{18})	Molecular Probes

2.1.4 Other Materials

Acrylamide	Gibco BRL
Bio - Gel A1.5	Bio - Rad
Bio - Beads SM - 2	Bio - Rad
Dialysis cassettes *(Cutoff 10 kDa)*	Pierce

Neuraminidase *(Vibrio Cholerae)*	Sigma
Outdated Human Blood	Canadian Red Cross
0.1 μm Polycarbonate Filters	Nucleopore
Sendai Virus	Collaborator
Sephadex G - 75	Pharmacia
Sepharose 2B - 300	Sigma

2.1.5 Commonly Used Buffer Solutions

PBS (Phosphate-buffered Saline)	150 mM NaCl 5 mM sodium phosphate pH 7.4 or 8.0
HEPES/MES Buffer	150 mM NaCl 5 mM HEPES 5 mM MES 5 mM sodium citrate 1 mM EDTA pH 7.4
Sodium phosphate Buffer	5 mM sodium phosphate 1 mM magnesium- sulphate pH 8.

2.2 Methods

2.2.1 Isolation of Glycophorin A from Human Erythrocytes

The first step in the purification is the isolation of the erythrocyte ghosts from concentrated blood cells obtained from the Red Cross Society. Glycoproteins were then removed using lithium diiodosalicylate/phenol extraction [Marchesi *et al.*, 1971; Chen *et al.*, 1956]. Other proteins were removed using differential centrifugation. The separation of Glycophorin A from Glycophorin Band C, was done using a Bio-gel A1.5m gel filtration column using a buffer containing the detergent Ammonyx -Lo (0.1% Ammonyx-Lo, 25mM NaCl, 5mM Sodium phosphate, pH 8, 0.01% Azide). Detergent was then removed by dialysis against double distilled water. Residual lipids were then removed by chloroform : methanol (2:1, v/v) extraction. The Glycophorin A was then stored at -20°C in its lyophilized powder form.

2.2.2 Preparation of LUVs by Detergent Dialysis

2.2.2.1 Large Unilamellar Vesicles Without Curvature Modulators

Large unilamellar vesicles were prepared using modified methods used by Wybenga *et al.*, (1996). Weighed out amounts of Egg phosphatidylcholine (PC) were dissolved in 2:1 (v/v) chloroform/methanol. The phospholipid was then dried out under a gentle stream of nitrogen gas and then placed in a vacuum

evaporator equipped with a liquid nitrogen trap for a minimum of 90 min to ensure complete removal of the organic solvents. In a typical preparation 4 mg of lipid was used.

A stock of glycophorin A solution was prepared by dissolving the lyophilized glycoprotein in HEPES/MES buffer (5 mM HEPES, 5 mM MES, 5 mM sodium citrate, 150 mM NaCl, and 1 mM EDTA, pH 7.4) containing 25 mM CHAPS. GD1a stock was also prepared in a similar fashion. A volume of these stocks was then added to the dried out lipid films. Volumes used were varied to achieve the desired lipid : protein (receptor) ratio in the liposomes. The total volume was then adjusted with the same buffer to achieve desired lipid : detergent ratio.

The lipid-protein mixture was then transferred to a 0.5-3.0 ml dialysis cassette with a 10,000 molecular weight cutoff membrane (Pierce Chemicals Co., Rockford, IL). The sample was then dialyzed against five changes of HEPES/MES buffer (pH 7.4) at 4°C over a period of 40h. Samples prepared in the presence of the Bio-beads SM-2 (16-18 mg / ml) were dialyzed against three volumes of buffer. The first two change occurred after 3-4 hours of the start of dialysis. Dialysis against the third volume occurred over night.

Following dialysis the non-incorporated glycophorin A was removed by running the sample through a Sepharose 2B-300 column (1 x 10 cm; 7 ml bed volume, Sigma). The HEPES/MES (pH 7.4) buffer was used to elute the LUVs. Turbid fractions were pooled together. The samples were stored in silanized test

tubes at 4°C. The lipid concentrations in the samples was then measured using a modified phosphate assay [Chen *et al.*, 1956; Ames *et al.*, 1960].

2.2.2.2 Large Unilamellar Vesicles With Curvature Modulators

The same procedure used in Section 2.2.2.1 was used to prepare LUVs with incorporated curvature modulators with the following modifications. A stock solution (1 mg/ml) of lyso-PC, lysophosphatidylcholine, was prepared in chloroform/methanol 2/1(v/v). Different volumes of the stock were then added to the lipid films. Different volumes were used to achieve different final molar concentrations of LPC in relation to phospholipids in the sample. The mixture was then vortexed for about 1 minute. It was then dried out under a gentle stream of nitrogen gas and placed in a vacuum evaporator equipped with a liquid nitrogen trap for a minimum of 60 min to ensure complete removal of the organic solvents.

2.2.3 LUV Preparation by Extrusion Methods

Lipid films made up of egg PE, egg phosphatidylethanolamine, and the ganglioside GD1a were prepared using the same methods as in Section 2.2.2. The films were then suspended in HEPES/MES buffer (5 mM HEPES, 5 mM MES, 5 mM sodium citrate, 150 mM NaCl, and 1 mM EDTA, pH 7.4) for about 1 hour. The suspension was further processed with five cycles of freezing and thawing,

followed by 10 passes through two stacked 0.1μm polycarbonate filters (Nucleopore) using a microextruder.

2.2.4 Measuring Vesicle Size Using Quasi-Elastic Light Scattering

Vesicle size was determined according to the methods described by Wybenga *et al.*, (1996). Using filtered HEPES/MES buffer (pH 7.4), the LUVs were diluted to a phosphate concentration of 0.132 mg/ml. The instrument used to measure the diameter of the LUV particles, was a Brookhaven Model B1 9000AT digital correlator. The vesicles were assumed to be spherical in shape. Scattering was measured at a 90° angle, and a 514 nm wavelength for an average of 300 s. The data were analyzed by cumulant analysis and non-negatively constrained least squares using software provided by the manufacturer.

2.2.5 Phosphate Assay

Lipid phosphate concentration was measured using an assay based on the reaction of inorganic phosphate with molybdate (Ames *et al.*, 1966). Pyrex tubes (13 x 100 mm) were soaked in 1M HCl solution for a hour and then rinsed extensively with ddH_2O prior to use. 30 μl of 10 % magnesium nitrate $[Mg(NO_3)_2 \bullet 6H_2O]$ in 95 % ethanol was added to samples containing up to 50 nmols of lipid phosphate in a volume of 100 μl. Using a Bunsen Burner, the

samples were then ashed. The tubes were then cooled to room temperature, and 0.3 ml of 0.5 M HCl was added. The tubes were then capped and put in boiling water bath for about 15 minutes to hydrolyse any pyrophosphate that may have formed during the ashing process. Tubes were then cooled and 0.7 ml of a 6/1 solution of 0.42 % (w:v) of ammonium molybdate•$4H_2O$ in 1N H_2SO_4 / 10 % ascorbic acid was added to each tube. The samples were then put in a 50°C water bath for 20 min. Absorbance was then measured at 820 nm against ddH_2O on a Perkin Elmer UV/VIS spectrometer. Standards were prepared using solution of 1 mM potassium phosphate (KH_2PO_4) and treated in same way as the unknowns. Lipid phosphate concentrations were calculated by interpolation using linear regression.

2.2.6 Resorcinol Assay for Sialic Acid

Procedure used was a modified version of the procedure by Svennerholm *et al.*, (1957). A resorcinol stock solution of 1 g in 50 ml ddH_2O and 0.1 M cupric sulphate was prepared (assay reagent). 10 ml of the solution was then added to 80 ml of concentrated 10 M HCl solution containing 0.25 ml of copper sulphate stock solution. The final volume was brought up to 100 ml using ddH_2O. The mixture was placed at room temperature for 4 hours. A standard curve containing up to 20 μg of sialic acid in 1ml ddH_2O was constructed. Samples

were diluted to a volume of 1 ml with ddH_2O. 1ml of the assay reagent was added. The tubes were then capped and put in boiling water bath for at least 20 minutes. Solutions were then cooled to room temperature and 2 ml of N-butyl acetate : 1-butanol (85:15, v:v) was added with vortexing. Samples were then centrifuged in a clinical centrifuge (Maximum setting) for 10 minutes. The upper organic phase was removed and its absorbance measured at 580 nm against ddH_2O on a Perkin Elmer UV/VIS spectrometer.

2.2.7 Determining Lipid to Glycophorin A Ratio

In order to characterize the reconstituted liposomes in terms of lipid to protein (glycophorin A) ratio, resorcinol assay was performed. The assay was done before and after treating the sample with neuraminidase from *Clostridium Perfringens*, which removes glycophorin A's exposed sialic acid residues. Treated fractions were incubated for 1 hour in a 37°C water bath. The proteoliposomes were then passed through a Sephadex G-75 column equilibrated with HEPES/MES buffer (pH 7.4) to remove the neuraminidase and the cleaved sialic acid. The dilution factor was obtained by comparing the phosphate concentrations of the neuraminidase treated and the untreated samples. This allows for the calculation of the percentage of glycophorin A oriented towards either side of the liposome membrane as well as calculation

of the final molar ratio of phospholipids to protein in reconstituted proteoliposomes.

2.2.8 Virus Fusion

2.2.8.1 R_{18} Labelling of Sendai Virus

The virus was labelled with R_{18} (Octadecylrhodamine), according to the procedure of Hoekstra *et al.*, (1984). Octadecylrhodamine was dissolved in methanol to give a final concentration of 1 mg/ml. 10 μl were then added to 1ml solution of Sendai virus that had been warmed up gradually to room temperature. The mixture was then light protected and allowed to incubate for at least 1 hour. Gel filtration chromatography, using Sephadex G - 75 column, was then used to remove the unincorporated R_{18}. The labelled virus was collected in the void volume. The viral protein concentration was then calculated using the BCA protein assay (Pierce chemical Co., Rockford, IL).

2.2.8.2 External Addition of Lysolipids to Liposomes and Erythrocyte Ghosts

Lysolipids were prepared in a stock solution of 1 mg/ml. HEPES/MES (pH 7.4) was used in experiments with liposomes of different lipid compositions, and 5 mM sodium phosphate buffer (pH 8.0) was used in the erythrocyte ghost experiments. Varying volumes of these stocks were added to achieve the desired molar ratio of phospholipids to lysolipids in the final 2 ml volume.

2.2.8.3 Fusion of Sendai Virus with Liposomes

Fusion assays were carried out as described in Wybenga *et al.*, (1996). Silanized cuvettes were used for the assay. The samples were maintained at 37°C under continuous, uniform stirring. Five micrograms of the R_{18} labelled virus was added per sample (liposomes or erythrocyte ghosts) to initiate fusion. Fluorescence was recorded using an SLM AMINCO Bowman Series 2 luminescence spectrometer interfaced with a 386/20 IBM compatible computer. A xenon arc light source was used, with an excitation and emission monochromator wavelength of 565 nm and 600 nm, respectively. Filters were used to minimize any contribution of light scattering to the fluorescence signal. The fluorescence intensity measured after addition of the virus to the reaction mixture is designated as F_0. F_{100} is the intensity measured after the addition of 40μL of 10% Triton X-100. The percent of R_{18} dequenched was calculated at time t from:

$$\% R_{18} \text{ dequenched} = 100[(F_t - F_0)/(F_{100} - F_0)]$$

Each assay consisted of measuring the fluorescence intensity for 600 to 800 seconds from the addition of the labelled virus to determine the extent of lipid mixing. The cuvettes are then light protected and incubated at 37°C for 7h. Finally, the fluorescence is measured at this time as the final extent of fusion.

2.2.8.3 Fusion of Sendai Virus with Erythrocyte Ghosts

Fusion assays of Sendai virus and erythrocyte ghosts were conducted according to Wybenga *et al.*, (1996) with the following exception. The buffer used during the labelling process of the virus was a 5 mM sodium phosphate buffer (pH 8.0). This buffer was used since it is the essential buffer used in the sealed erythrocyte purification process [Steck *et al.*, 1970]. This buffer was used throughout these fusion experiments.

2.2.9 BCA Protein Assay

Protein concentration of Sendai virus and erythrocyte ghosts were measured using a modified Lowry assay [Lowry *et al.*, 1951; Hess *et al.*, 1978]. Two reagents were used. Reagent A (BCA protein assay reagent, Pierce), and reagent B (4 % $CuSO_4 \bullet 5H_2O$). Reagent A and B were mixed at a ratio of 50:1 respectively (Solution C). Samples containing up to 50 μg of protein were made up to 100μl volume using ddH_2O and then 50 μl of 0.5 % SDS solution was added to all samples. Finally 1 ml of solution C was added to all samples which were then put into a 50 °C water bath for 20 minutes for the colour to develop. Absorbances of the samples were measured at 562 nm against ddH_2O using a Perkin Elmer UV/VIS spectrometer. A standard curve containing up to 50 μg of protein was constructed using BSA as a standard. Protein concentration was calculated by interpolation using linear regression.

2.2.10 Purification of Erythrocytes Ghosts

Blood was withdrawn from healthy volunteers, heparinized, and centrifuged (1,300 g_{max}, 10 min, 4°C). Sealed Ghosts were essentially prepared as described by Steck *et al.*, (1970). Blood cells were washed three times by sedimenting in a swinging-bucket rotor (2,300 g_{max}, 10 min 4°C) and resuspending the pellet in five volumes of PBS (phosphate-buffered saline). The buffy coat, containing the white blood cells, was carefully aspirated each time from the surface of the pellet.

Hemolysis was initiated by rapidly and thoroughly mixing 1 ml of packed cells with approximately 40 ml of 5P8-1Mg (sodium phosphate, 5mM, pH 8.0 containing 1mM $MgSO_4$). The membrane ghosts were then pelleted by centrifugation at 22,000 g_{max} for 10 min. The tube is then tipped allowing the ghosts to slide away from the small hard button, rich in proteases that may then be aspirated. To separate sealed from unsealed erythrocyte ghosts, 4 ml of sample was carefully layered on a 30 ml sucrose cushion (43% sucrose solution) as described in Schwoch *et al.*, (1973). These were then centrifuged for 90 min. at 34 g_{max}. The supernatant was found to contain the sealed ghosts. The ghosts were then stored at 4°C till further use.

2.2.11 Measurement of the Transbilayer Reorientation of Lysophosphatidylcholine

The method used was similar to that described by Boscoboinik *et al.*, (1989) and Bergmann *et al.*, (1984) with minor modifications. Erythrocyte ghosts were stored in a 5 mM sodium phosphate buffer (pH 8.0).To obtain the desired final molar ratio of lysolipid to phospholipids in the erythrocyte membranes, [^{14}C]-lysophosphatidylcholine (0.025 μCi), dissolved in methanol, was added to dried out film of unlabelled LPC. The mixture was dried out under a gentle stream of nitrogen gas and then placed in a vacuum evaporator equipped with a liquid nitrogen trap for a minimum of 60 min to ensure complete removal of the organic solvents. The cell suspension was added to the dried lipids and incubated at 37 °C in phosphate buffered saline (pH 8.0) (PBS) in a total volume of 100 μl for varying lengths of time. The reaction was stopped by putting the tubes on ice and adding 800 μl of cold PBS. Two 450 μl samples of this suspension were centrifuged and the supernatant was discarded. One sample (control sample) was mixed with 200 μl of ddH_2O and pipetted into the scintillation fluid to determine the total count. The other sample was incubated with fatty acid free albumin (1.5 % BSA, 4 °C) for 4min to extract labelled lipids from the outer monolayer of the ghosts. The extraction step was repeated 2-3 times and then the suspension was washed with 1 ml of cold PBS buffer to remove any residual BSA remaining. The cell suspension was transferred to a

vial containing aqueous scintillation fluid (ACS, Amersham). The ratio of the counts in the albumin-treated ghosts, normalized to the total counts in the control sample, served as a measure of the transbilayer movement of the labelled lysolipid.

3.0 RESULTS

3.1 Purification of Glycophorin A from Human Erythrocyte Membranes

Figure 3.1.1 shows a 7.5 % SDS-PAGE gel of the purified glycophorin A. To verify the purity of the preparation, commercially obtained glycophorin A was also run on the SDS-PAGE gel as well as glycoprotein obtained from a collaborator (Dr. Sharom). After silver staining, all lanes showed the presence of two major bands at molecular weights of 47,500 and 83,000. These bands correspond to the monomeric and the dimeric forms of glycophorin A, respectively. A very faint lower band corresponding to glycophorin B was observed at a molecular weight of about 25,000. The molecular weight of glycophorin A, according to its amino acid sequence and carbohydrate composition, is 31,000. The migration pattern of glycophorin A varies considerably with the conditions used in the electrophoresis. The presence of bulky oligosaccharide side chains, including large negatively charged sialic acid moieties, slows down the migration of the glycoprotein in both monomeric and dimeric forms.

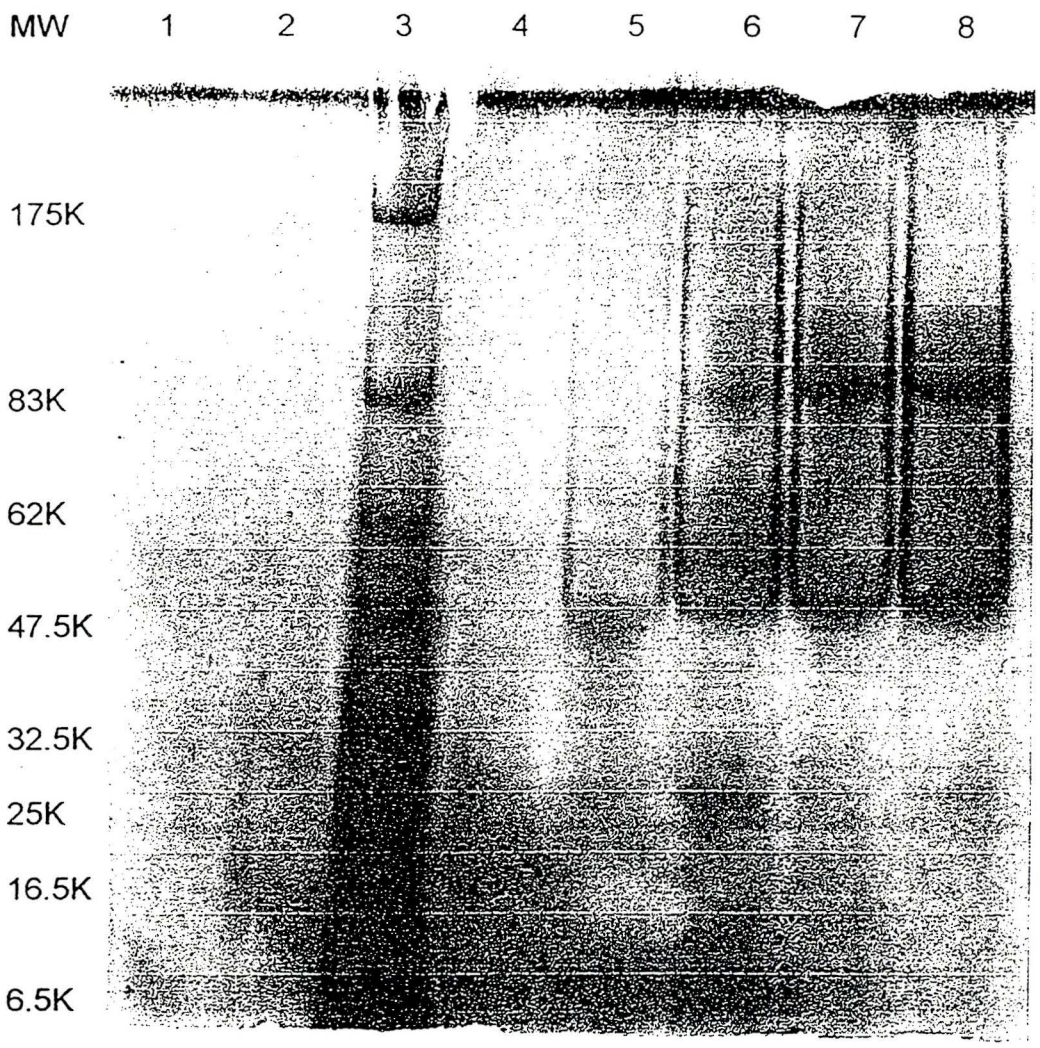


Figure 3.1.1

Purification of glycophorin A. 7.5 % silver-stained polyacrylamide SDS-PAGE gel. Lane (1-2, 4) empty, (3) prestained molecular markers, (5) Gly A from Sigma (6) Gly A from collaborator, (7)isolated Gly A, (8) isolated Gly A prior to gel filtration step.

3.2 Reconstitution of Proteoliposomes

Maintaining consistency in liposome preparation is very crucial when using artificial membrane systems. For research dealing with curvature in bilayers, maintaining a consistent vesicle size is very central [Wilschut *et al.*,1990] Different methods of reconstitution were attempted to achieve a consistent LUV diameter range of 80-200 nm.

3.2.1 Dialysis Method

The success of reconstitution using the dialysis method depends on many variables. One such variable is the rate of detergent removal [Lichtenberg *et al.*, 1988; Schwarz *et al.*, 1988]. Two methods were implemented to achieve consistent proteoliposome size by controlling detergent removal rate.

3.2.1.1 Using Bio-Beads SM-2

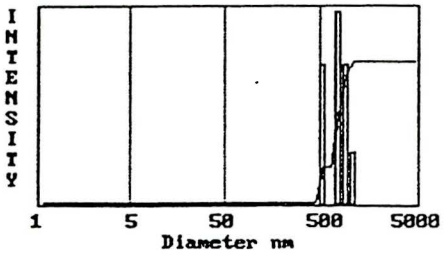
With the addition of detergent-adsorbing beads outside the dialysis cassette, the number of changes of buffer generally required during dialysis can be reduced [Philippot *et al.*, 1983]. Three concentrations of the beads were first used: 10, 20 and 30 mg /ml. The 10 mg /ml was shown to be of little use since a lipid precipitate formed, and the average vesicle sizes observed were > 500 nm (Figure 3.2.1). The 30 mg/ml samples gave rise to very small fragments indicating that the detergent removal was occurring too rapidly (Figure 3.2.1).

The 20 mg/ml sample showed higher turbidity than the 30 mg/ml sample indicating that the vesicles formed were larger as confirmed by the light scattering data (data not shown). All three Bio- Bead concentrations gave rise to samples with phosphate concentrations at almost 75-80 % the theoretical value. Different concentrations of beads were further investigated and it was determined that a concentration of 16-18 mg/ml of beads was sufficient to give consistent preparations (Figure 3.2.1).

A)

Percent	Lower	Upper
By Inten.	100	0
By Weight	100	0
By Number	100	0

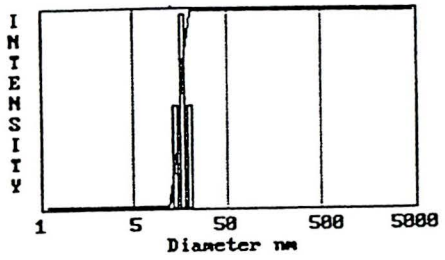
Mean = 883nm
Var. = 0.044
Skew = 0.642
RMS = 5.43E-05



B)

Percent	Lower	Upper
By Inten.	100	0
By Weight	100	0
By Number	100	0

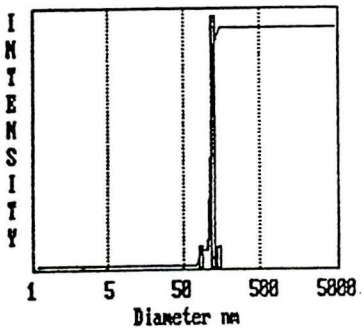
Mean = 22nm
Var. = 0.019
Skew = 0.196
RMS = 7.16E-01



C)

Percent	Lower	Upper
By Inten.	100	0
By Weight	100	0
By Number	100	0

Mean = 147nm
Var. = 0.013
Skew = 0.938
RMS = 3.43E-04



D)

Percent	Lower	Upper
By Inten.	33	67
By Weight	34	66
By Number	99	1

Mean = 197nm
Var. = 0.048
Skew = 1.759
RMS = 3.23E-02

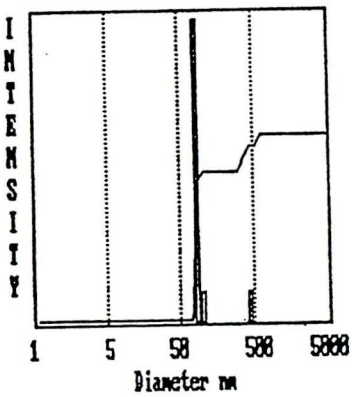


Figure 3.2.1

Optimum concentration of Bio-Beads SM2 for preparing EPC LUVs. Histograms of the size distribution of LUV preparation obtained using QELS. Samples prepared using (A) 10 mg/ml, (B) 30 mg/ml, (C & D) 18 mg/ml and 16mg/ml Bio-Beads SM2 respectively. Sample lipid concentration = 0.132 mM in HEPES/MES buffer pH 7.4.

3.2.1.2 Varying the Dialysis Time

Another approach was to control detergent removal rate by controlling the time between buffer changes. According to Wacker *et al.*, (1998) the first 4 hours are crucial. Different times were attempted between buffer changes. To achieve desired consistency, the first three changes had to be 2.5 to 3.5 hours followed by an overnight incubation (Figure 3.2.2).

3.2.2 Extrusion Method

Extrusion methods were used due to the efficiency of the procedure in producing LUVs with a narrow size distribution (Figure 3.2.3). Liposomes consisting of egg phosphatidylethanolamine and ganglioside GD1a were prepared via this method. The light scattering results of these samples showed a narrower vesicle size range to that achieved with the egg PC :glycophorin A liposomes (Figure 3.2.2).

A)

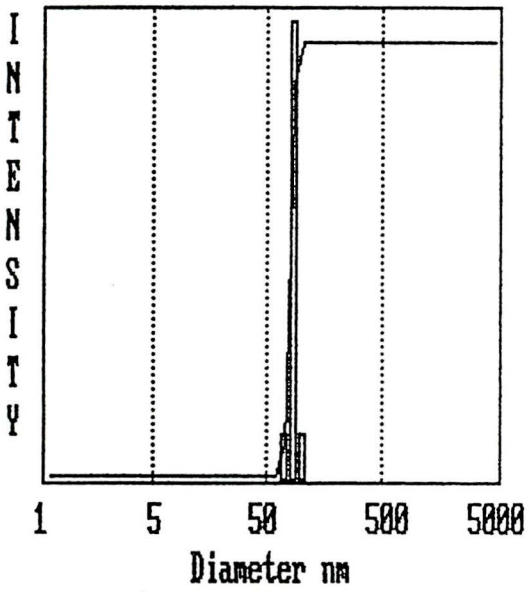
Percent	Lower	Upper
By Inten.	100	0
By Weight	100	0
By Number	100	0

Mean = 110nm
Var. = 0.004
Skew = 0.416
RMS = 1.48E-04

B)

Percent	Lower	Upper
By Inten.	8	92
By Weight	62	38
By Number	98	2

Mean = 189nm
Var. = 0.144
Skew = -.434
RMS = 3.68E-01



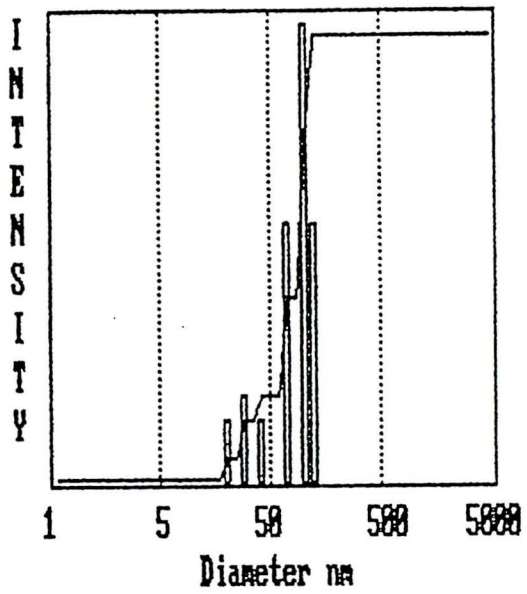


Figure 3.2.2

Optimum dialysis time for preparing EPC LUVs. Histograms of the size distribution of LUV preparation obtained using QELS. (A) PC:Gly A (400:1), (B) PC:GD1a (400:1). The first dialysis buffer change for both samples was carried out after 3.5 hours. Sample lipid concentration = 0.132 mM in HEPES/MES buffer pH 7.4.

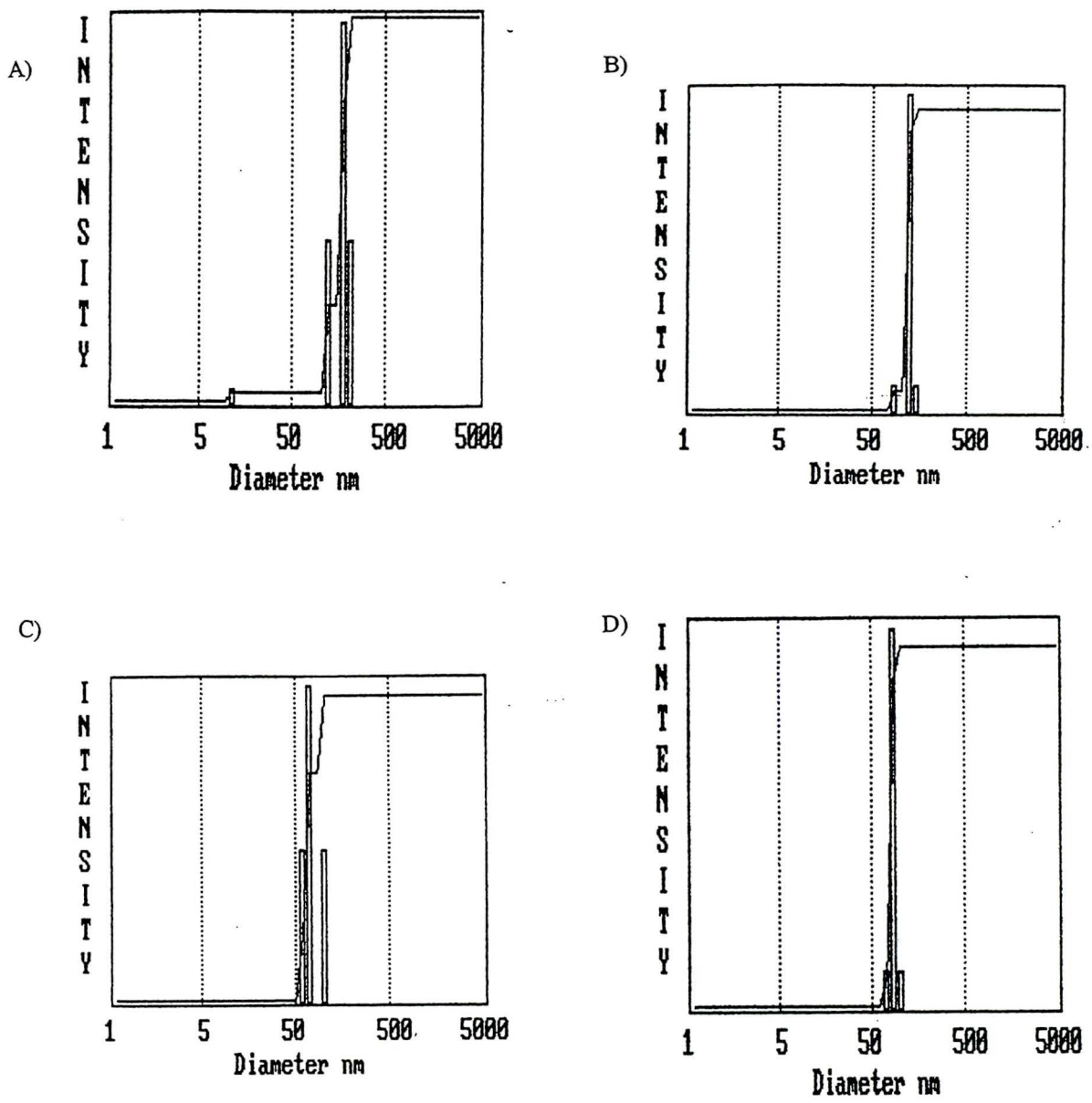


Figure 3.2.3

Using extrusion method to prepare LUVs. Histograms of the size distribution of LUV preparation obtained using QELS. 4 mg EPE, 2% GD1a and (A) No LPC, (B) 1.2 %, (C) 5.0%, (D) 6.3% LPC. Sample lipid concentration = 0.132 mM in HEPES/MES buffer pH 7.4.

3.3 Effect of Time on LPC Inhibition of Sendai Viral Fusion

Lysophosphatidylcholine has been shown to inhibit diverse fusion events in the past [Yeagle *et al.*, 1994; Martin *et al.*, 1995]. One such event involves the fusion of Sendai virus with lipid bilayers. This part of the project was geared towards studying time dependency of this event. The relation of time and viral fusion inhibition of LPC was studied in both artificial as well as biological membrane systems.

3.3.1 Artificial Systems

Various unilamellar liposome systems were used. Large unilamellar vesicles were prepared using different lipid compositions as well as different Sendai fusion receptors. The curvature modulator was added externally and allowed time to incorporate into the different LUVs. Samples were allowed to incubate for intervals of 5, 10, and 15 minutes, after which dequenching assays were performed.

3.3.1.1 External Addition of LPC to Liposomes Prepared Using Dialysis

Liposomes containing the Sendai virus receptor glycophorin A were prepared using the dialysis reconstitution methods (refer to section 2.2.2). Two molar concentrations of Gly A were added originally to the egg phosphatidylcholine lipid films: 400:1 and 50:1. These concentrations represent

the mushroom and brush conformations of glycophorin A respectively. Orientation of the incorporated glycophorin in bilayer was at 48 ± 3 % on the outer monolayer. LPC (C-16) was added to samples at molar ratios of 1, 5 and 9 %. Lipid mixing assay results showed a decrease in fusion inhibition with increased LPC incubation times (Figure 3.3.1). This was observed for all three LPC concentrations used. Samples containing larger molar concentrations of glycophorin A gave rise to similar results (data not shown). LPC (C-12), molar concentrations of 1% and 9%, was also used with 50:1 samples. The extent inhibition of Sendai fusion was similar at an incubation time of 20 minutes for both LPC concentrations (Figure 3.3.2). Higher concentrations of LPC disrupted the membrane structure and led to vesicle aggregation followed by the breakdown of LUVs, as observed by the decrease of the turbidity of the samples.

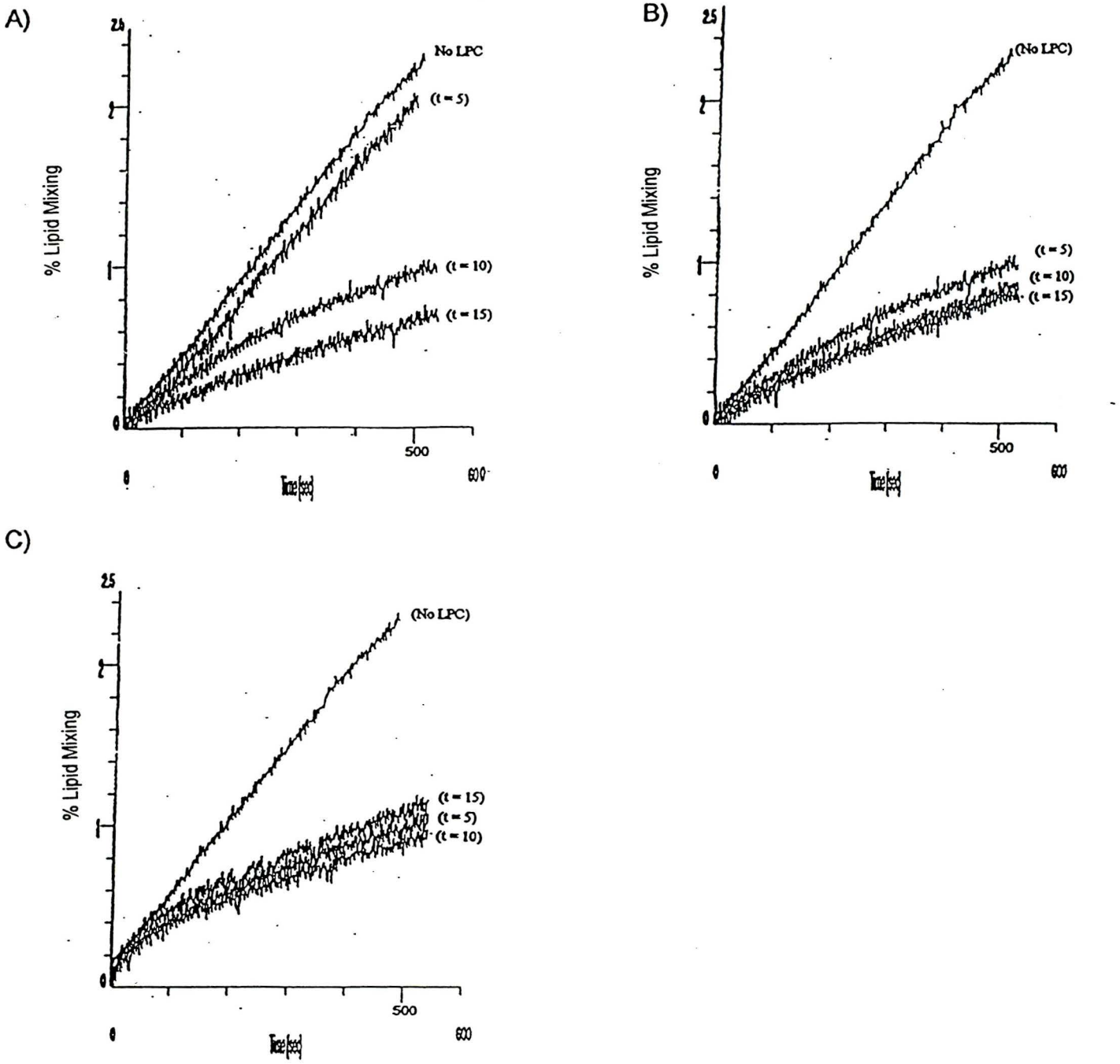


Figure 3.3.1

Effect of LPC incubation time on fusion of Sendai virus. Egg PC LUVs containing a lipid to glycophorin A mole ratio of 400:1 were used.(A) 1% LPC, (B) 5% LPC, and (C) 9% LPC. LPC(C-16) was added and allowed the indicated incubation times (min). Assays were performed at 37°C in HEPES/MES buffer, pH 7.4, using 5μg of Sendai viral protein and 50 μM LUVs.

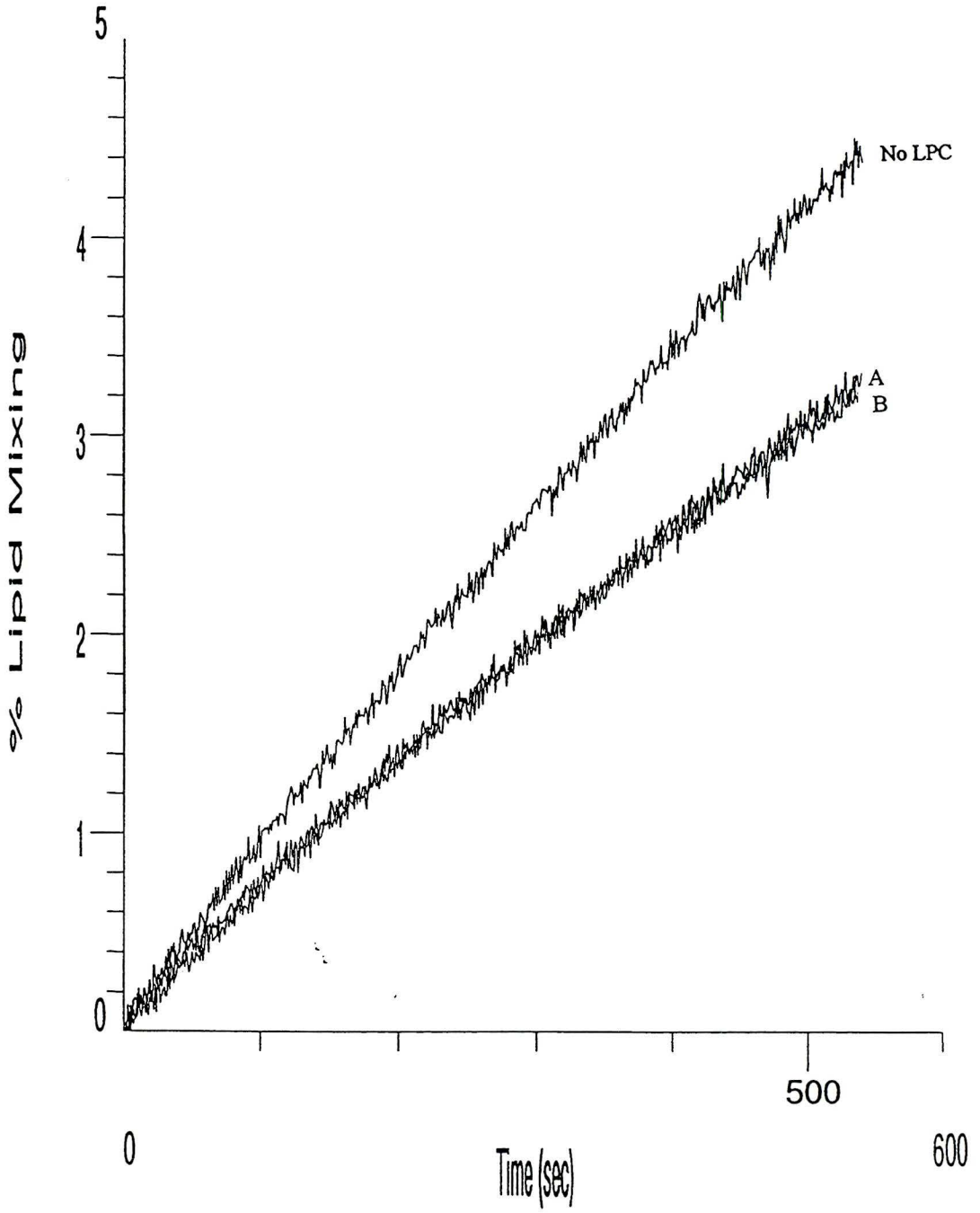


Figure 3.3.2

Effect of LPC incubation time on fusion of Sendai virus. Egg PC LUVs containing a lipid to glycophorin A mole ratio of 50:1 were used. (A) 1% LPC, (B) 9% LPC (C-12) was added and incubated for 20 min. Assays were performed at 37°C in HEPES/MES buffer, pH 7.4, using 5μg of Sendai viral protein and 50 μM LUVs.

3.3.1.2 External Addition of LPC to Liposomes Prepared Using Extrusion

Liposomes containing the Sendai virus receptor GD1a were prepared using the extrusion reconstitution methods (refer to section 2.2.3). Orientation of the incorporated ganglioside in bilayer was measured. Resorcinol assays were performed on samples before and after neuraminidase treatment. The dilution factor was obtained by comparing the phosphate concentrations of the neuraminidase treated and untreated samples. 55 ± 2 % of GD1a was found to be oriented towards the outside of outer monolayer. Fusion assay results showed similar patterns observed in glycophorin A containing samples. The inhibition of viral fusion decreased with increased incubation times of the lysolipid LPC (Figure 3.3.3).

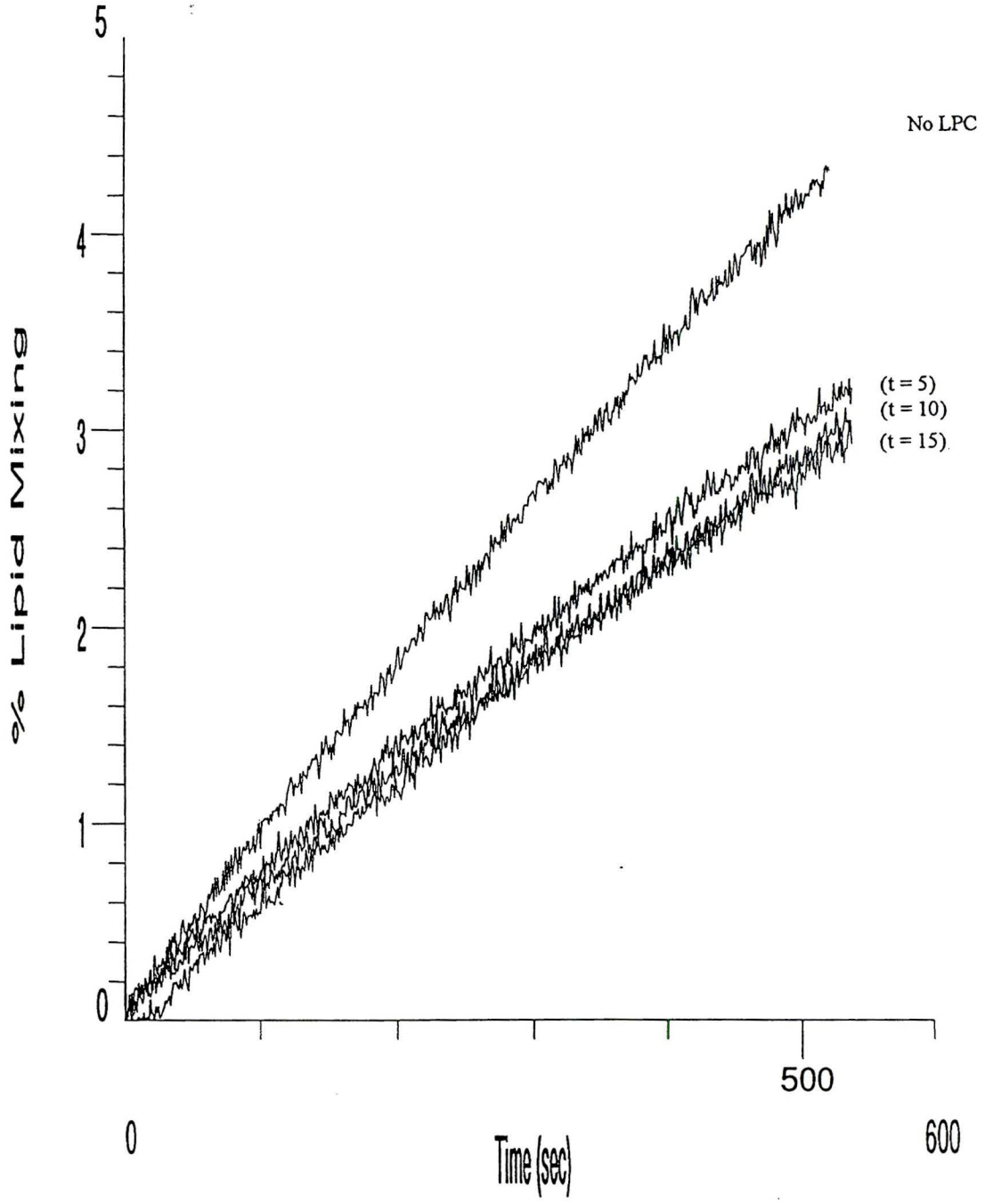


Figure 3.3.3

Fusion of Sendai virus with egg PE LUVs containing 2% ganglioside (GD1a). LPC (C-16) was added and allowed the indicated incubation time (min). Assays were performed at 37°C in HEPES/MES buffer, pH 7.4, using 5 μg of Sendai viral protein and 50 μM LUVs.

3.3.2 Biological Membranes

Erythrocyte membranes are the major source of the glycoprotein glycophorin A [Oku *et al.*, 1982; Gershoni *et al.*, 1985]. This makes them an excellent target for Sendai viral fusion. The first step was to show that the purified ghost exhibited fusion capabilities with the virus. The ability of the ghosts to fuse will help assess the purification procedure. For that different amount of ghosts, identified by the amount of protein they contain, were tested using the lipid mixing R_{18} dequenching assay (Figure 3.3.4). The results showed an increase in the extent of fusion with increasing concentrations of ghosts. This suggested that the resealing process took place as expected and the glycophorin on the surface was accessable to the Sendai virus receptor binding HN proteins.

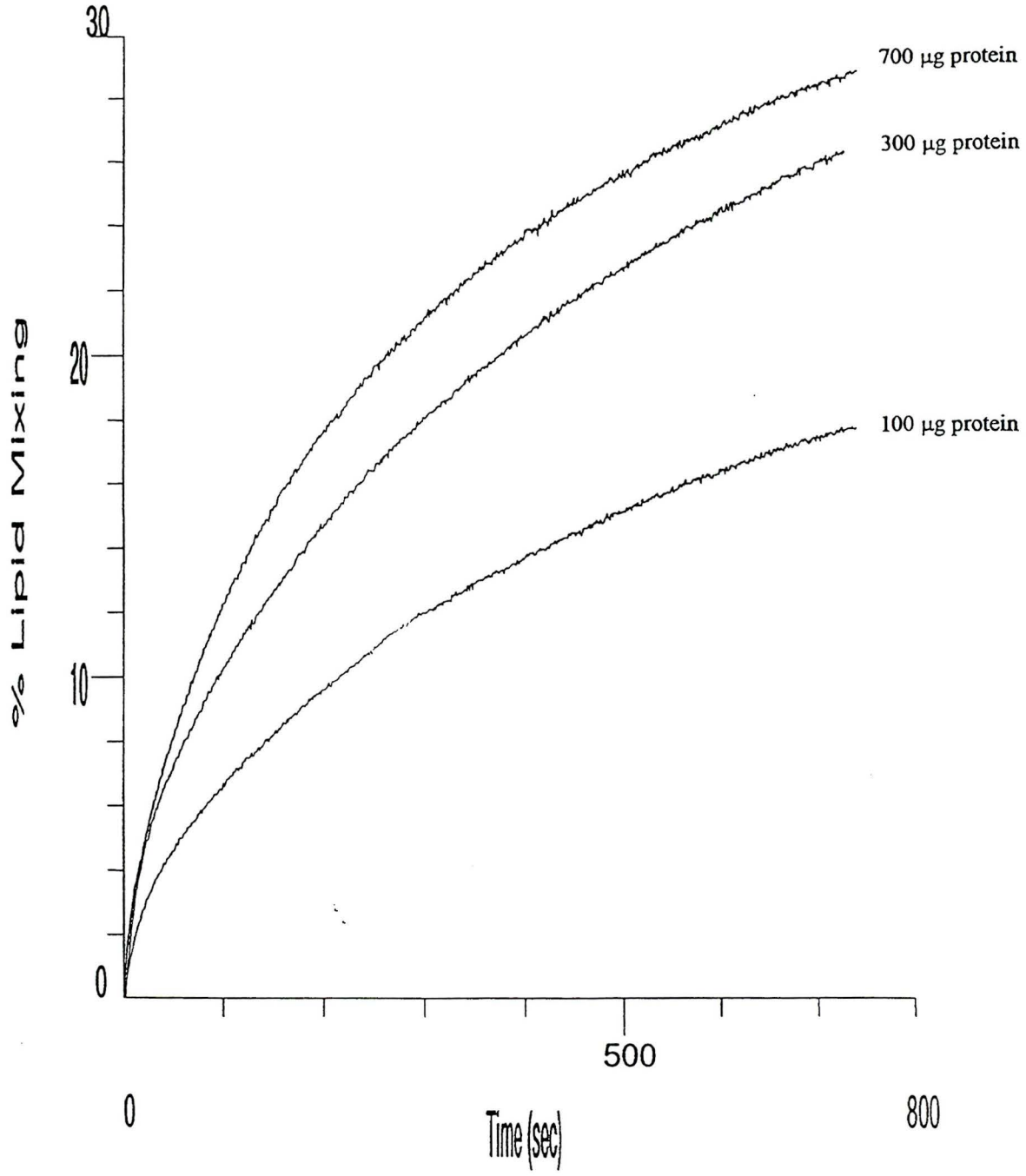
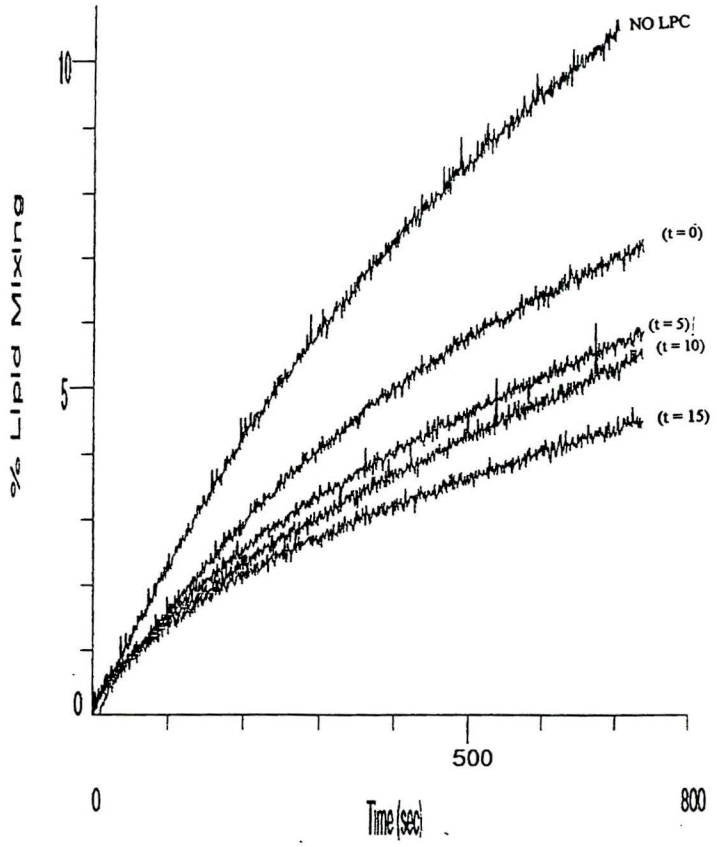


Figure 3.3.4

Fusion of Sendai virus with erythrocyte membranes. Assays were performed at 37°C in sodium phosphate buffer containing 1 mM $MgSO_4$, pH 8.0, using 5µg of Sendai viral protein and different amount of erythrocyte membranes.

Varying concentrations of erythrocyte ghosts and LPC were used to test the time dependency of LPC fusion inhibition. Figure 3.3.5 illustrate the results obtained with 800 μg of ghosts with LPC (C-16) added at a molar ratio of 10%. These results show a decrease in the effectiveness of LPC inhibition with time. Erythrocyte samples showed much higher fusion than that observed with liposome systems. Other concentrations of ghosts were also tested. Samples containing 200μg of ghosts, with LPC present at 2% molar ratio, showed similar inhibition patterns of LPC with increased incubation time (Figure 3.3.5). The inhibition of fusion by LPC (C-12) was also examined. LPC (C-12) was added at molar concentrations ratio of 8 % and 10 % to erythrocyte ghosts. Pattern observed was in agreement with results obtained with LPC (C-16) (Figure 3.3.6).

A)



B)

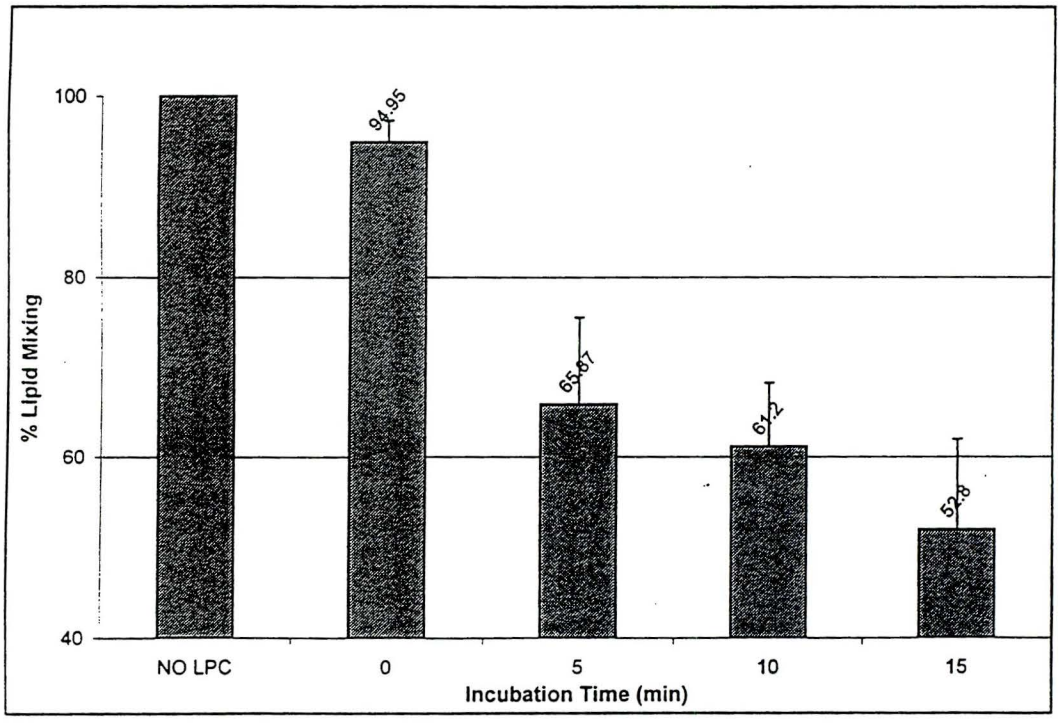


Figure 3.3.5 (panel A)

Fusion of Sendai virus with 800 μg of erythrocyte membranes and 10% LPC(C-16). Assays were performed at 37°C in sodium phosphate buffer containing 1 mM $MgSO_4$, pH 8.0, using 5μg of Sendai viral protein. (A) R18 lipid mixing fluorescence spectra.(B)100% lipid mixing refers to samples with no LPC added. Shown are means ± SE, n= 3.

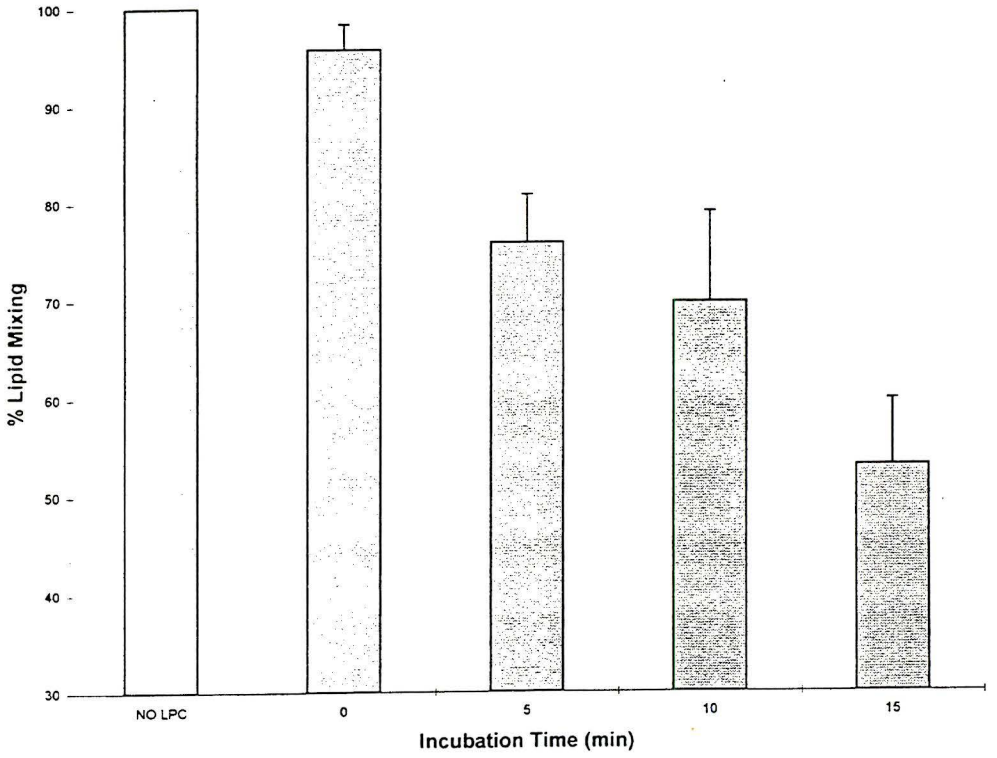


Figure 3.3.5 (B)

Fusion of Sendai virus with 200 μg of erythrocyte membranes and 2% LPC(C-16). Assays were performed at 37°C in sodium phosphate buffer containing 1 mM $MgSO_4$, pH 8.0, using 5μg of Sendai viral protein. 100% lipid mixing refers to samples with no LPC added. Shown are means ± SE, n= 3.

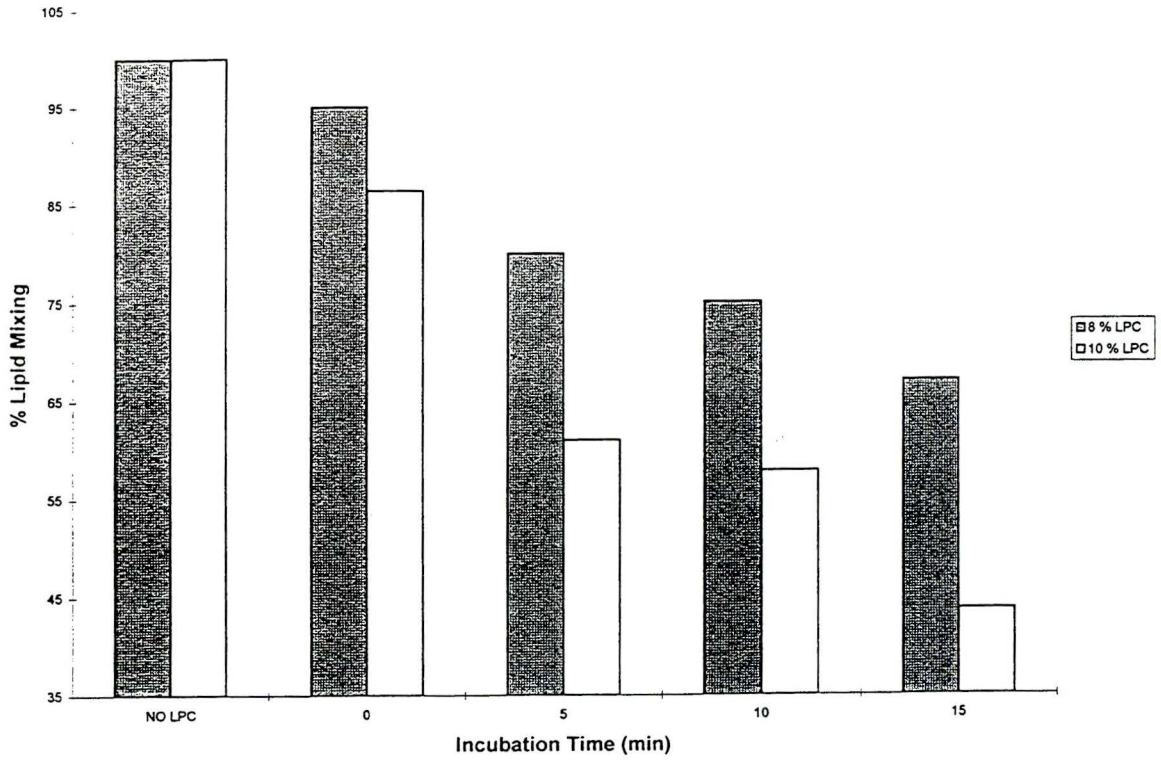


Figure 3.3.6

Fusion of Sendai virus with 600 μg of erythrocyte membranes and (A)8% LPC(C-12), (B) 10% LPC (C-12). Assays were performed at 37°C in sodium phosphate buffer containing 1 mM $MgSO_4$, pH 8.0, using 5μg of Sendai viral protein. 100% lipid mixing refers to samples with no LPC added. Samples were done in duplicates.

3.4 Effect of Bilayer Sidedness on LPC Inhibition of Sendai Viral Fusion

To explore the relation between LPC sidedness in a bilayer and its fusion inhibition properties, the following approach was attempted. Egg PE liposomes were prepared via the extrusion method. The liposomes also consisted of 2% mole percent ganglioside (GD1a). LPC was either added directly to dried lipid prior to extrusion, or it was added externally and allowed an incubation time of 1 minute. Addition of LPC to the lipid film insures an equal distribution of LPC on both sides of the liposome bilayer, as oppose to external addition where only incorporation into the outer (cis) monolayer is possible. Control samples were first prepared. They showed a linear relation between the amount of LPC added and inhibition (data not shown). They also showed the extent of Sendai viral fusion to be a consequence of the mole percent of ganglioside present (data not shown). Samples were then prepared with 1.2% and 0.6 % LPC added to the lipid film as well as externally added. Results from these experiments showed the same level of inhibition between samples of 1.2% LPC added to the lipid film and the sample with 0.6% LPC added externally (Figure 3.4.1). The results also showed that samples containing externally added LPC, exhibited higher inhibition of Sendai viral fusion than samples containing the same amount of LPC in their original lipid film (Figure 3.4.1). Figure 3.4.2 displays a summary of all these results. It required twice as much LPC incorporated directly in the film to achieve the same level of inhibition attained with LPC added externally.

Experiments were also attempted with other LPC concentrations, and similar behaviour was observed (data not shown).

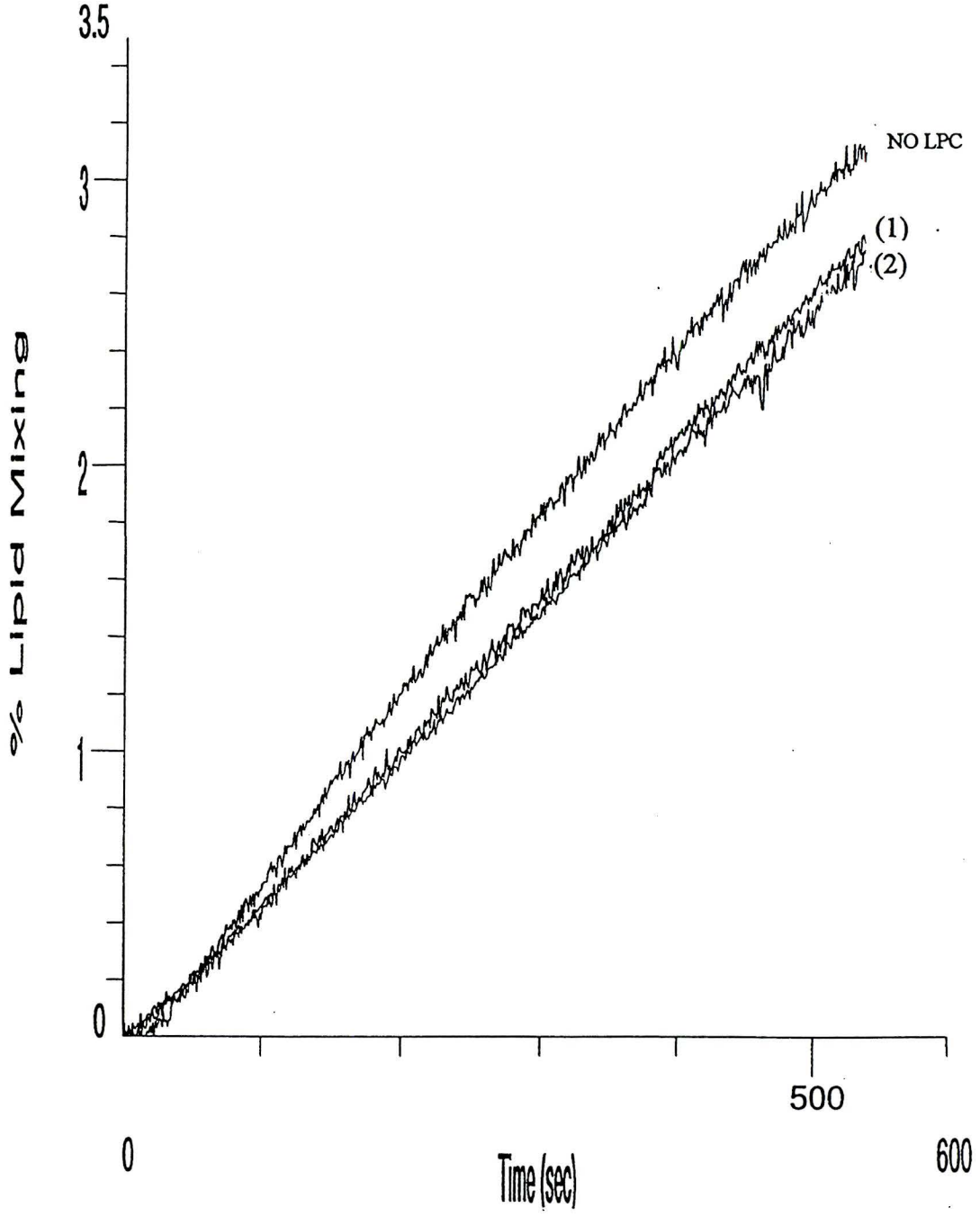


Figure 3.4.1

R18 fluorescence dequenching assay of Sendai virus with egg PE LUVs containing 2% ganglioside (GD1a). (A) 0.6% LPC(C-16) was added to samples using the indicated methods. (B) ① 0.6% LPC(C-16) was added externally and incubated for (t = 1 min),② 1.2 % LPC(C-16) was added to original lipid film. Assays were performed at 37°C in HEPES/MES buffer, pH 7.4, using 5μg of Sendai viral protein and 50 μM LUVs.

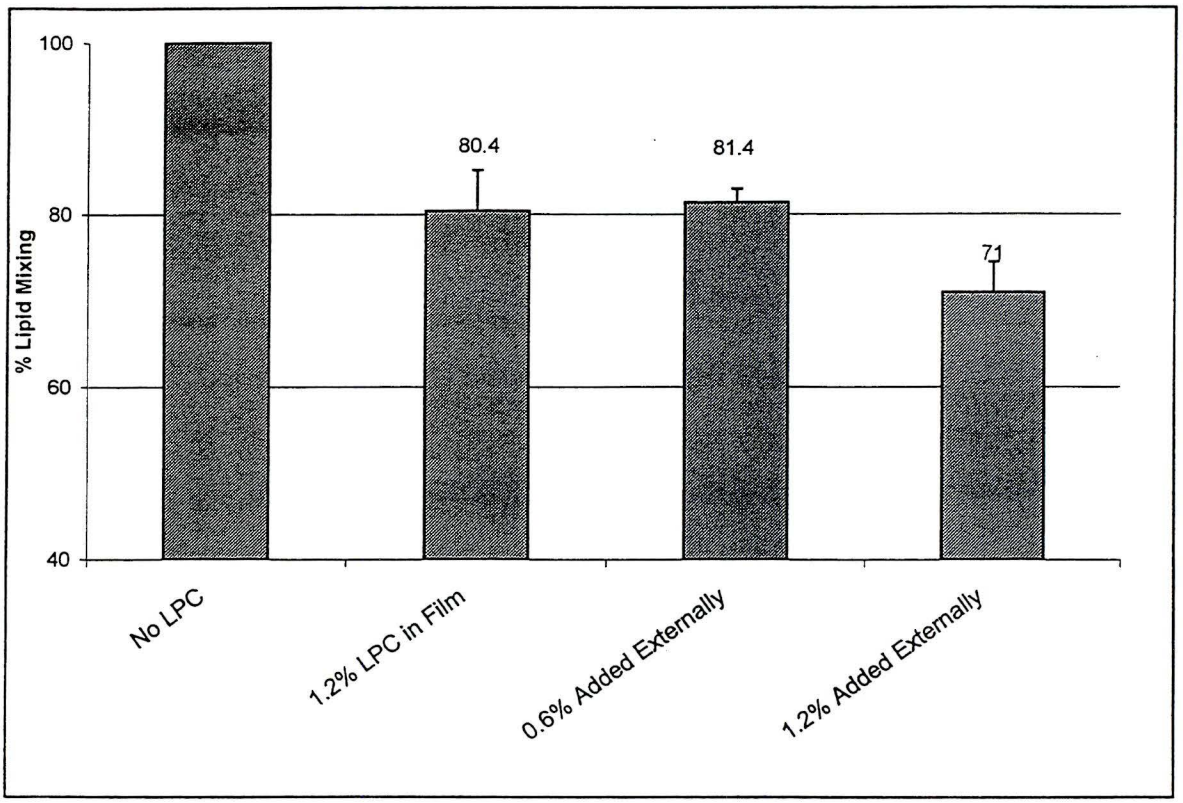


Figure 3.4.2

Relating fusion inhibition of LPC to bilayer sidedness. Percent fusion of Sendai virus with LUVs of egg PE, containing 2% ganglioside (GD1a) and LPC(C-16). Assays were performed at 37°C in HEPES/MES buffer , pH 7.4, using 5μg of Sendai viral protein and 50μg of liposomes. 100% inhibition represent samples with NO LPC added. Shown are means ± SE, n = 3.

3.5 Flip-Flop and Viral Fusion

The results obtained showed a pattern of increased reorientation of LPC with time. Figure 3.5.1 shows more than 8 % flip-flop of LPC in first 20 minutes of incubation. One the other hand control samples showed negligible flip-flop. The control samples were prepared by rupturing the ghost with ddH_2O prior to BSA extraction. The flip-flop rate was calculated to be 0.32 ± 0.08 % LPC / min. Figure 3.5.2 shows a comparison of the approximated flip-flop at different incubation periods, and the fusion inhibition pattern of LPC. The slopes of the two graphs are roughly equal. Incubation times of one and two hours were also attempted (data not shown). These showed very small increase in reorientation beyond that observed for the 20 minute incubation. Control samples with incubation times of 12h and 24h were also done (data not shown). These represented the equilibrium stage. That is, the point at which LPC is equally present on both sides of the bilayer. The results also showed that the erythrocyte ghosts were properly sealed during their isolation.

The effects of Sendai virus on the flip-flop were also studied. Figure 3.5.3 shows results of samples prepared with Sendai virus as well as those that were not. These results showed that the difference in the flip-flop pattern was minimal.

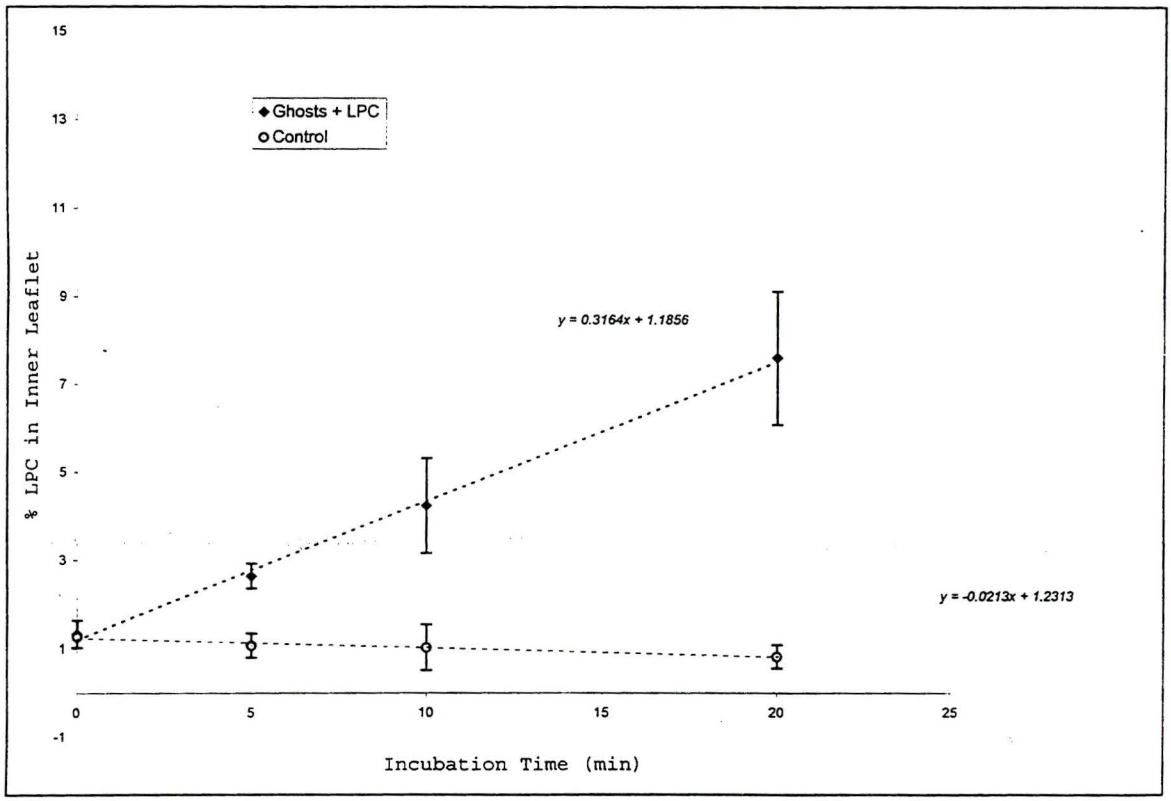


Figure 3.5.1

Reorientation of [^{14}C]-lysophosphatidylcholine in erythrocyte membranes. Samples were prepared using 200 μg of ghosts. Samples also contained 2% molar ratio of LPC (C-16). Shown are means ± SE, n= 6.

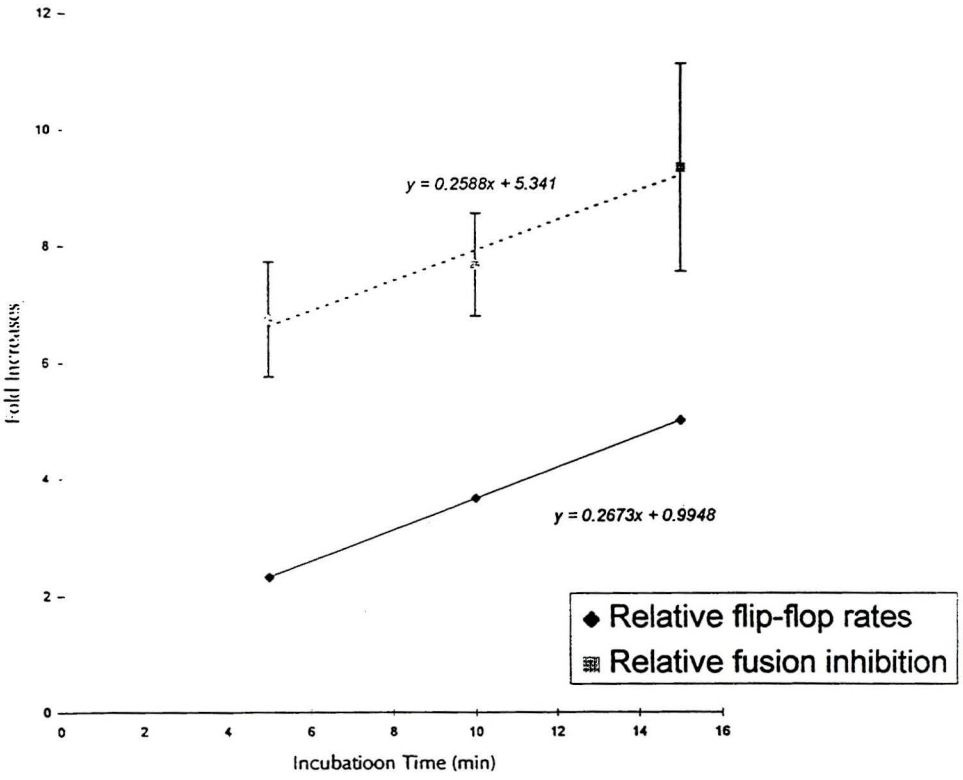


Figure 3.5.2

Comparing reorientation of [^{14}C]-lysophosphatidylcholine, in erythrocyte membranes, to viral fusion inhibition pattern with time. Flip-flop data were prepared by using the linear equation (*y=0.3164x + 1.1856*). Samples were prepared using 800 μg for the lipid mixing assays. Samples also contained 2% molar ratio of LPC (C-16). Shown are means ± SE, n= 3.

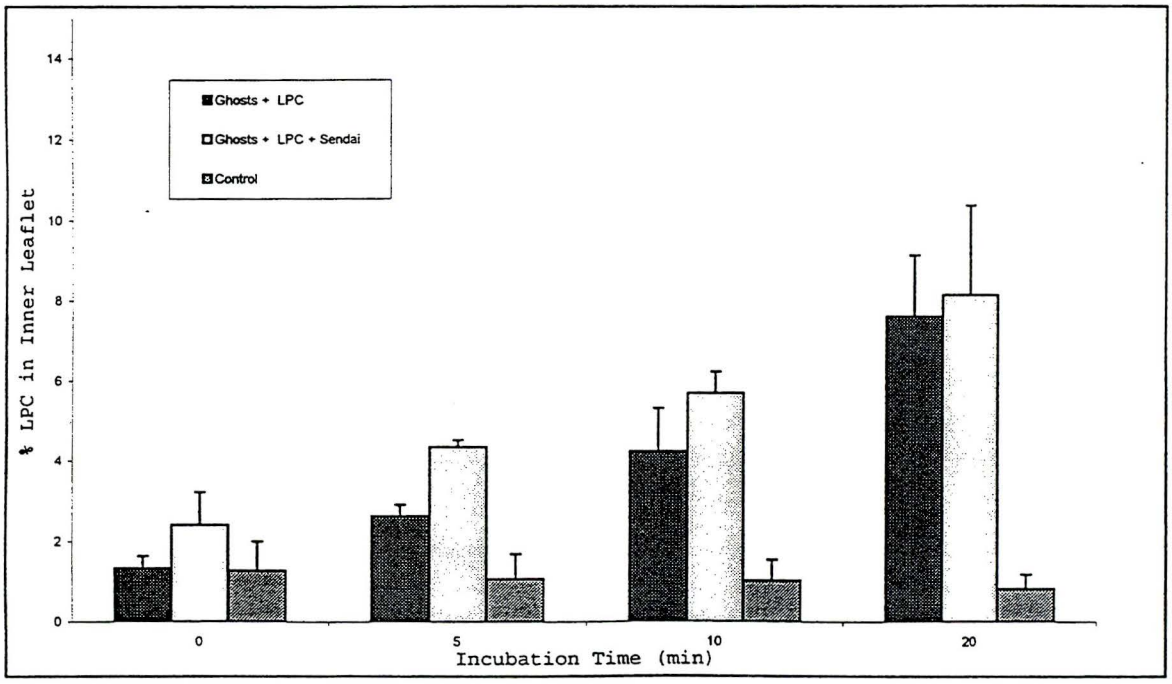


Figure 3.5.3

Reorientation of [^{14}C]-lysophosphatidylcholine in erythrocyte membranes. Samples were prepared using 200 μg of ghosts. Samples also contained 2% molar ratio of LPC (C-16). 5μg of Sendai virus stock were added to the indicated samples. Shown are means ± SE, n= 6.

4.0 DISCUSSION

4.1 Purification of Glycophorin A from Erythrocyte Membranes

The isolation of glycophorin A from erythrocyte membranes was carried out using the methods of Marchesi *et al.*, (1971). This method was implemented since it has been shown, in the literature, to produce a high yield of glycoprotein. Also, the product has been shown to have an acceptable level purity when compared to the commercially obtained glycophorin A. Figure 3.1.1 shows an SDS-PAGE gel of the isolated glycophorin A. The two main bands represent the monomeric as well as the dimeric forms of the glycoprotein. It is believed that the dimeric interactions between molecules are promoted by van der Waals forces between the well defined hydrophobic transmembrane helices [MacKenzie *et al.*, 1997]. The structure of glycophorin A transmembrane interactions had been determined using nuclear magnetic resonance spectroscopy [MacKenzie *et al.*, 1997]. The interaction between the transmembrane domains in the dimers, has been shown to be strong enough to resist the SDS reducing conditions [Brosig & Langosch, 1998].

The apparent molecular weight, as inferred from the gel, of glycophorin A (~47,500) was much higher than the calculated molecular weight (~31,000). The mobility of glycophorin A was hindered due to carbohydrate attached to it. Also,

interactions between the detergent SDS and the transmembrane hydrophobic regions of the glycoprotein can lead to change in the mobility pattern of the protein monomers.

4.2 Reconstitution of Sendai Viral Receptors into Liposomes

Studies of membrane proteins in their native environment can be difficult to interpret due to the restrictions arising from the complexity of the native membranes and interferences with other membrane constituents or other reactions [Rigaud *et al.*, 1995]. Proteoliposomes are therefore a powerful tool for elucidating both structural and functional aspects of their membrane-associated proteins.

Reconstitution of the viral receptors, glycophorin A and GD1a, in liposomes represented the first step in this work. Dialysis reconstitution methods were used with egg PC due to the high stability of these liposomes [Lichtenberg & Barenholz, 1988]. On the other hand, egg PE liposomes were prepared using the extrusion method.

The first decision to be made when planning a reconstitution protocol is the means of solubilizing the glycoproteins [Walter *et al.*, 1991]. Criteria for a good detergent are the ability to solubilize (form co-micelles) and to protect the protein from denaturation while being sufficiently water soluble to be removed efficiently [Walter *et al.*, 1991]. The removal of the detergent is based on its

critical micellar concentration [Rigaud *et al.*, 1995]. CHAPS was used due to its high cmc allowing for the solubilization of the receptors. Furthermore, CHAPS with its high cmc will form small micelles and can be removed easily via dilution techniques [Rigaud *et al.*, 1995]. It also posses high membrane permeability which makes it easy to remove form inner monolayers.

After the detergent has formed co-micelles with the lipid and receptor, the detergent is removed to form the proteoliposomes. Figures 3.2.1 and 3.2.2 illustrate the use of different methodologies to control the rate of detergent removal. In Figure 3.2.1, hydrophobic Bio-Beads SM-2 were used to speed up the detergent dialysis. The adsorption of detergent onto hydrophobic Bio-Beads SM-2 was originally described by Holloway (1973). Bio-Beads allow almost complete removal of detergents whatever the initial experimental conditions [Rigaud *et al.*, 1995]. A concentration of 18-20 mg/ml of Bio-Beads allowed for detergent removal during the micellar-to-lamellar transition, thus giving homogeneous and relatively large liposomes (80-200 nm). In Figure 3.2.2, controlling the time for the crucial first buffer change [Wacker and Schubert, 1998] gave rise to a similar vesicle size range. Changing the buffer after 2.5 - 3.5 hours was found to give this optimum vesicle size range.

4.3 Investigating LPC Viral Fusion Inhibition

Lysophosphatidylcholine has been shown to inhibit diverse fusion events in the past [Yeagle *et al.*, 1994; Martin *et al.*, 1995]. The diversity of these fusion events, suggests that LPC is inhibiting a common step during the fusion processes. This is further supported by evidence that pure lipid bilayer fusion shows remarkable homology to what is known about the sequence of protein-mediated cell membrane fusion events [Lee and Lentz, 1997], suggesting a commonality between all fusion mechanisms. The aim of this work was to try better understand how LPC inhibits viral fusion events with different bilayer systems.

4.3.1 Effect of Time on LPC Inhibition of Viral Fusion

The results of this investigation showed a time dependency of the Sendai viral fusion inhibition properties of lyso PC. Using fluorescence dequenching assays, time dependency was shown to be independent of the nature of the lipid composition of the liposomes. Both liposomes containing egg PC as well as those containing egg PE exhibited a decrease in the effectiveness of LPC as a fusion inhibitor with time (Figures 3.3.1, and 3.3.3). Moreover, the nature of the viral receptor had no effect on this time dependency as samples containing glycophorin A or GD1a gave rise to the same conclusions. Further investigation was also conducted to examine this phenomenon in biological systems.

Erythrocyte ghost membranes showed a similar pattern of decreased inhibition with increase in incubation time (Figure 3.3.5). Different LPC to phospholipid molar ratios, as well as LPCs varying in the lengths of the hydrocarbon chain all had similar time dependency effects on viral fusion (Figure 3.3.5 and 3.3.6).

This data indicates that a common event is taking place making LPC a less effective inhibitor with time. This may imply that with time LPC is becoming unavailable or incapable of exerting its inhibitory effects on viral fusion. Figure 3.3.1 also showed that the fusion inhibition of LPC is affected by the concentration of the lysolipid in solution. Since the difference between the lyso PC concentration in the bathing solution and in the membrane sets the initial gradient for diffusion [Needham and Zhelev, 1995], it seems plausible that the observed phenomenon is related to the presence as well as the location of LPC in the bilayer.

4.3.2 Effect of LPC Bilayer Sidedness on Viral Fusion Inhibition

This part of the work was aimed to investigate how the location of LPC in the bilayer influences the viral fusion inhibition properties. The results indicate that LPC inhibits fusion only when present on the outside monolayer (Figure 3.4.2). The LPC on the inner monolayer does not play a role in inhibiting lipid mixing, and therefore the formation of a stalk fusion intermediate (hemifusion).

This data supports the findings of Chernomordik *et al.*, (1995) that showed that bilayer stabilizers would inhibit stalk formation but promote pore opening.

It also supports the idea that LPC inhibition effects are due to its structure and shape and not attributed to any specific chemical moiety of lysolipids [Chernomordik *et al.*, 1993]. Lyso PC posses a large head group area and a small acyl chain volume and is expected to form micelles [Cullis *et al.*, 1979; Israelachvili *et al.*, 1980]. The tendency of LPC to promote positive curvature inhibits the formation of the stalk intermediate which posses a net negative curvature determined by the competition between negative meridional curvature and positive parallel curvature [Kozlov and Markin, 1983].Therefore, the destabilization of nonlamellar structures, proposed to be the intermediates of bilayer fusion, with a negative radius of curvature may be a mechanism for the inhibition of Sendai viral fusion by lysophosphatidylcholine. Furthermore, these results offer a conceivable explanation for the time dependency of LPC inhibition observed earlier. LPC may be moving from the outer monolayer to the inner monolayer, within the incubation period, and therefore not be able to fully inhibit the formation of the highly curved fusion intermediates.

4.3.3 Reorientation of LPC in Biological Membranes

The aim of these experiments was to help explain the effect of time on the inhibition properties of exogenously incorporated lysophosphatidylcholines.

Flip-flop movement of LPC across the membrane bilayer may explain the time dependency. Reorientation of LPC will occur to relax differential density in bilayer due to incorporation of LPC on the outer monolayer [Raphael and Waugh, 1996]. Reorientation would lead to the sequestering of LPC on the inner monolayer, where it can not inhibit the formation of the stalk intermediate and hence lipid mixing between fusing membranes [Chernomordik *et al.*, 1995]. With time the percentage of LPC incorporated in the inner monolayer increases, hence decreasing the effects of LPC on viral fusion. The results obtained showed a pattern of increased reorientation of LPC with time (Figure 3.5.1). It also suggested that the process proceeds at a high rate initially (0.32 ± 0.08 %LPC / min.) but starts to plateau after that until it reaches equilibrium, the point at which LPC is equally present on both sides of the bilayer. Figure 3.5.2 showed that the flip-flop of LPC across the bilayer and the inhibition pattern proceed at comparable rates. These findings support the hypothesised relation between reorientation of LPC and its effects on the decrease of viral fusion inhibition. Since the reorientation of LPC is independent of the viral fusion event (Figure 3.5.3), the comparable rates might indicate a possible causal effect of 'flip-flop' on the viral fusion inhibition decrease observed in lipid mixing assays.

The effects of Sendai virus on the flip-flop were also examined. Figure 3.5.3 shows results of samples prepared with Sendai virus as well as those that were not. These results show that the difference in the flip-flop pattern was

minimal. From these results it was concluded that flip-flop was occurring, within the incubation period in question, which can explain the observed relation between incubation time of LPC and Sendai viral fusion inhibition.

4.4 Summary and Future Studies

The aim of the project was to investigate the viral fusion inhibition by lysophosphatidylcholine. It looked at the importance of bilayer positioning of the LPC. It also examined the importance of transmembrane movement on the inhibition properties of lyso PC. With the use of different artificial and biological membrane systems it was possible to show that external addition of LPC to membrane systems led to a dependency between inhibition of fusion and the incubation time of the LPC with bilayers. The position of LPC in the bilayer proved to play a very important role in determining the effectiveness of LPC inhibition. Only LPC present on the outer monolayer was shown to participate in the inhibition of lipid mixing and hence the formation of the stalk intermediate during any fusion process. Significant flip-flop was shown to occur within the same incubation times attempted in lipid mixing assays. The ability of LPC to reorient during this time can account for the decrease in fusion inhibition. The sequestering of the LPC in the inner monolayer renders these molecule unavailable to inhibit formation of the hemifusion intermediates with net negative curvature. These result display the importance of structure and shape

of LPC in determining its viral fusion inhibition ability. It also supports the 'stalk' model of membrane fusion. It emphasises the importance of curvature properties of the lipids forming the bilayers in determining the fusion tendency of these bilayers. For these reason as well as the tight regulation of lysolipids concentrations in biological systems [Morash *et al.*, 1989], it is possible that LPC may be used in intracellular vesicle trafficking by controlling its availability as well as location in the bilayer. For this reason further investigating and understanding the properties of lysolipids may prove interesting as well as beneficial.

Further studies may be conducted to further elucidate different parts of this thesis. First, LPC extraction with BSA studies can be conducted in artificial membrane systems. This will help to corroborate the studies done with erythrocyte ghosts. This would also allow us to see if the nature of the lipids or the molar ratio of various viral receptors affect the flip-flop rate of LPC within the incubation time period in question. Second, with the use of flat bilayers one can control the content of both sides of a bilayer. This would enable us to study the effects of LPC on the formation of different fusion intermediates by adding it the different sides of bilayers [Chernomordik *et al.*, 1995]. This in turn will support the hypothesised relation between the shape and fusion inhibition of LPC. Third, NMR may be used to study the flip-flop of LPC in different artificial membrane systems [De Kruijff and Baken, 1978]. Liposomes can be prepared with LPC

equally present on both sides of the membrane. BSA extraction of LPC on the outer monolayer may then be performed at 4°C so no LPC reorientation takes place. Temperature can then be raised and the flip-flop monitored. This will lend support to the proposed flip-flop occurring during the incubation periods studied. Finally, similar experiments should be conducted with a molecule that promotes negative curvature. Such molecule, like oleic acid, should give opposite effect regarding the time dependency. It should also show opposite effects in terms of the relation between sidedness and viral fusion inhibition.

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