VIRAL MEMBRANES: MODEL SYSTEMS FOR STUDYING BIOLOGICAL MEMBRANES

Authors: **Eric J. Patzer**
Department of Biochemistry
Stanford University
Stanford, California

Robert R. Wagner
Department of Microbiology
University of Virginia
Charlottesville, Virginia

Edward J. Dubovi
Department of Microbiology
University of North Carolina
Chapel Hill, North Carolina

Referee: Richard W. Compans
Department of Microbiology
University of Alabama
Birmingham, Alabama

I. INTRODUCTION

Viruses are the simplest biological entities. They are not free-living organisms, but are obligate parasites which depend almost entirely on the host cells which they infect. Some viruses can perform certain metabolic functions in cell-free systems, but almost all replicative processes of viruses use machinery and energy donated by the cell. In addition to their intrinsic interest as pathogens, viruses can serve as incisive probes for studying many of the intricate structures and functions of cells. Many extracellular viruses (virions) can be prepared in large quantities and readily purified to homogeneity for biochemical and biophysical studies.

In addition to essential and unique nucleic acids and proteins, virions of many classes of viruses contain lipids, often as much as 20% of the total virion mass. These virion lipids are invariably derived from the host cell and generally form a lipid bilayer which often assumes the characteristics of a biological unit membrane.[1,2] The term envelope has been applied to the virion membrane, and the presence or absence of this structure represents an important taxonomic principle for classifying all viruses as enveloped or nonenveloped.[3] By the same token, viruses are often classified according to their susceptibility to inactivation of their infectivity by ethyl ether, detergents, or other lipid solvents. Clearly, the lipid bilayer and its associated proteins play critical roles in adherence to and penetration of the host cell surface membrane, which leads to viral infection.

The subject of enveloped viruses has attracted a considerable amount of attention of late, and several excellent reviews have been written.[4-7] Many of these reviews have been concerned primarily with the important subject of comparative biology and chemistry of enveloped viruses. They provide detailed descriptions and are highly recommended for a comprehensive analysis of enveloped viruses and their respective membranes. In the present review, we attempt to provide a unified concept of the viral membrane as a structural and, to some extent, functional entity without presenting another virus-by-virus description of enveloped virions. In this way, we hope to point out how the elegant biochemical and biophysical techniques that have been developed for probing the organization of synthetic lipid and lipoprotein bilayers can be applied to a simple, homogeneous, and definable biological system, the viral membrane.

First, we must define our system by focusing our attention on a few simple and well-characterized viruses and only alluding to all the complicated or ill-defined enveloped viruses. It is well to keep in mind that although all viruses are relatively simple, at least when compared with even the simplest unicellular organism, certain types of viruses are far more complicated than others. At one end of the spectrum, for example, are the poxviruses which are almost as large as small organisms ($\sim$270 × 218 nm for the vaccinia prototype which contains at least 30 structural proteins, including many enzymes). Poxvirus genomes are composed of double-stranded DNA of mol wt $\simeq$ 180 × 10^6 daltons, sufficient genetic information to code for 300 proteins averaging $\sim$30,000 daltons.[8] At the other end are lipid-free and protein-free plant viroids[9] which consist of only a single strand of circular RNA containing only 359 ribonucleotides.[10] The lipid-containing viruses all contain proteins in highly ordered arrays, some of which are complexed with nucleic acids, but they vary greatly in their complexity. It is worth summarizing the classification of lipid-containing viruses in order to provide some basis for deciding which viruses provide the best model systems for understanding their membrane structure and function. Greater details of individual enveloped viruses are provided elsewhere.[4-7]

Table 1 compares certain of the salient features of all classes of enveloped viruses studied to date. All enveloped viruses can be divided into two groups: (1) those that acquire their membranes by budding from cytoplasmic membrane, either surface membrane or endoplasmic reticulum, and (2) those viruses that acquire at least part of their lipids from the inner layer of the nuclear membrane (the herpesviruses)[36-38] or appear to assemble their membranes *de novo* from preformed cytoplasmic lipids and do not bud from cellular membranes. The cytoplasmic budding viruses all contain single-stranded RNA as their genome, whereas all those which assemble their membranes *de novo* or bud from the inner nuclear membranes have genomes composed of double-stranded DNA or, in one case, double-stranded RNA.[40] The RNA budding viruses can in turn be subdivided into two classes: (1) those with negative-strand RNA and helical nucleocapsids which contain an RNA-dependent RNA polymerase (rhabdoviruses,[11,12] paramyxoviruses,[13,14] myxoviruses,[15,16] arenaviruses,[17-18] and bunyaviruses[21-23]) and (2) those RNA viruses with a plus-strand genome, which can serve as messenger (togaviruses,[19,20] oncornoviruses,[27-29] and coronoviruses.[24-26]). All the enveloped RNA viruses contain one to three exterior glycoproteins. The helical negative-strand RNA viruses generally appear to contain a single nonglycosylated matrix (M) protein lining the inner surface of the virion membrane.

The DNA viruses that do not bud from cytoplasmic membranes are an extremely heterogeneous group. Among them are the most complicated virions which can contain as many as 30 proteins. Of considerable interest is the vaccinia (poxvirus) membrane, which selects its own phospholipids and contains a single glycoprotein, the carbohydrate of which is polyglucosamine.[30-32] The floppy membrane of herpesvirus is also of interest because it is derived from nuclear membrane (at least in part) and contains a

TABLE 1

Overview of Lipid-Containing Viruses

Taxonomic group	Prototypes	Nucleic acid[a] (segments)	Capsid	Membrane Proteins[b] Glycosylated	Membrane Proteins[b] Nonglycosylated	Ref.
Bud from cytoplasmic membrane						
Rhabdoviruses	Vesicular stomatitis	ssRNA$^-$(1)	Helical	1(G)	1(M)	11,12
Paramyxoviruses	Sendai, NDV, SV5	ssRNA$^-$(1)	Helical	2(HN,F)	1(M)	13, 14
Myxoviruses	Influenza	ssRNA$^-$(8)	Helical	2(HA,NA)	1(M)	15, 16
Arenaviruses	Pichinde, LCM[c]	ssRNA$^-$(3 + 2[d])	Helical	2(G_1,G_2)	0	17, 18
Togaviruses	Sindbis, SFV[e]	ssRNA$^+$(1)	Isometric	2—3(E_1, E_2, E_3)	C	19, 20
Bunyaviruses	Uukuniemi, LaCrosse	ssRNA$^-$(3)	Uncertain	2(G_1, G_2)	0	21—23
Coronaviruses	Bronchitis[f],DC43[g]	ssRNA$^+$(1)	Uncertain	2(E_1, E_2)	0	24—26
Oncoviruses	Rous, MuLV[h]	ssRNA$^+$(2)	Isometric	1—2(gp85, gp70)	1? (p15E)	27—29
Do not bud from cytoplasmic membrane						
Poxviruses	Vaccinia	dsDNA (1)	Complex	1 (38K)	±5	30—32
Iridoviruses	Iridescent, FV3[i]	ds DNA (1)	Icosahedral	0	yes	33—35
Herpesviruses	Herpes simplex	dsDNA (1)	Icosahedral	4—13	?	36—38
Mycoplasmavirus 2	MVL2	dsDNA (?)	Uncertain	?	?	39
Lipid-containing bacteriophages	PM2	dsDNA (1)[j]	Isometric	0	1 (PII)	40
	ϕ6	dsRNA (3)	Isometric	0	5($C^{3,5,9,10,11}$)	40

[a] Denotes single-stranded (ss), double-stranded (ds), DNA or RNA$^-$ or RNA$^+$ (negative or positive messenger sense, respectively).
[b] Designations: G = glycoprotein, HN = hemagglutinin-neuraminidase, HA = hemagglutinin, NA = neuraminidase, E = envelope, M = matrix-membrane, C = capsid.
[c] Lymphocytic chroiomeningitis.
[d] 18S and 28S RNA species of ribosomal origin
[e] Semliki Forest virus.
[f] Avian.
[g] Human.
[h] Avian and murine.
[i] Frog virus 3.
[j] Circular.

considerable number of rather heterogeneous glycoproteins.[36-38] Little is known about the membranes of the cytoplasmic deoxyviruses (iridoviruses)[33-35] or the very heterogeneous mycoplasma virus group.[39] A great deal is known about the lipid-containing *Pseudomonas* bacteriophages,[40] which have been studied extensively, particularly PM2 and ϕ6. The membrane of the lipid phages, rather than being a surface structure, is surrounded by a protein shell. The well-studied membranes of poxviruses and lipid phages bear little resemblance to cytoplasmic membranes. For this and other reasons, we have decided not to discuss the membranes of DNA viruses in this review. The excellent review by Mindich[40] provides a comprehensive analysis of the lipid bacteriophages.

This review concentrates most of its attention on the membranes of the four simplest, most manageable, and most extensively studied viral membranes, those of vesicular stomatitis virus (rhabdovirus), the Sendai-Newcastle disease — simian virus 5 group of viruses (paramyxoviruses), influenza A (myxovirus), and Sindbis-Semliki Forest viruses (togaviruses). Our discussions are concentrated on the lipid and protein composition of these viruses as well as a critical analysis of their organization, dynamics, and, where appropriate, their potential functions.

II. VIRAL MEMBRANE LIPIDS

Electron microscopic investigations have suggested that most of the lipid-containing viruses are surrounded by what appears to be a typical unit membrane structure similar to cell plasma membranes. The recent intensive interest in membranes and the structural and physiological role of lipids has led to a more detailed investigation of all membranes, including viral membranes. Initially, viral membranes were studied purely on a comparative basis with cellular membranes. Some of the questions of foremost interest to virologists concerned the biogenesis of viral lipids, e.g., where in a cell do viral lipids originate; are preformed cellular lipids incorporated into virions; does the virus have any control over the lipids incorporated into its membrane? As evidence accumulated that viral membranes are typical bilayer structures[41,42] and many viruses obtained their membranes by budding through the plasma membrane and incorporating cellular lipids into their membrane, the research emphasis turned to the study of viral membranes as altered segments of eucaryotic plasma membranes.

The erythrocyte membrane has long been the prototype eucaryotic membrane chosen to study lipid composition due to the ease of obtaining large quantitities of pure cytoplasmic surface membrane. The usefulness of viral membranes stems from similar considerations. In most cases, virions can be purified entirely free of contaminating cellular membranes, simply on the basis of density and/or velocity gradient centrifugation. Since there is only one membrane surrounding virions, the problems of membrane purification are greatly simplified. In addition, many viruses can be grown in large quantities in defined tissue culture media, which allows the manipulation of viral membrane lipids within certain limits.

Since viral proteins are inserted into the virion lipid bilayer to the exclusion of cellular protein, viral membranes cannot legitimately be considered simply as patches of unaltered plasma membrane. However, the viral lipids may reflect the same organization found in the plasma membrane. Certainly more comparative studies on the orientation of viral and cellular lipids and on the dynamic characteristics of the two membrane systems are required before the exact relationship between viral and cellular lipids is known. In any case, viral membranes as model membrane systems provide many of the same membrane-dependent functions as do cell membranes, e.g., membrane-bound enzymes (neuraminidase), receptors (hemagglutinin, cell attachment sites) and cell-fusing capabilities (including hemolysis), which provide an adequate

framework to study the organization of lipids in the membrane and the dependence of membrane functions on lipids.

A. Origin of Viral Lipids

Electron microscopic investigations have indicated that the majority of viruses replicating in animal cells obtain their membrane by budding through the plasma membrane, or in the case of herpesviruses, through the inner nuclear membrane.[4] A more quantitative approach to locate the site of maturation of the viral membrane is to compare the lipid composition of the viral membrane to the lipid composition of purified cellular membranes of the host. This comparison should indicate the lipids of which cellular membrane the virion lipids most closely resemble, i.e., where they were derived, and whether any novel viral lipids are produced, possibly due to viral enzymes. In addition, a comparison of one virus grown in a variety of unrelated cell lines or several viruses grown in the same cell, might suggest whether maturing virions can select for specific host cell lipids.

Table 2 compares the lipid composition of numerous viruses and their host cell plasma membranes. Metabolic labeling experiments have revealed that, except for poxviruses, predominantly preformed cellular lipids are incorporated into viral membranes.[4,53] The lipid composition data for viral membranes support the electron microscopic evidence for the site of virion maturation. Generally, the lipids of most viral membranes closely resemble the lipids of the cellular membrane at which they mature, which in most cases is the plasma membrane.[4,53] Consequently, it is evident that the host cell plasma membrane for the most part determines the lipid composition of the viral membrane.

The lipid composition of the plasma membrane, however, is not identical to that of the viral membrane in all cases. Many of the viruses shown in Table 2 have a higher cholesterol and sphingomyelin content than does the host cell plasma membrane. In addition, the aminophospholipid content is greater for several viruses, e.g., simian virus 5 (SV5), vesicular stomatitis, Rous sarcoma, Semliki Forest, and Sindbis viruses. Some investigators have noted a direct correlation between lipid composition of the host cell plasma membrane and virus yields. Cell lines with plasma membranes enriched in phosphatidylethanolamine were found to produce higher yields of SV5,[46] and a higher content of phosphatidylethanolamine plus cholesterol correlated with greater yields of mumps virus.[54] It was proposed that these lipids were required for production of the viral membrane and were a determining factor in the amount of virus produced.

Other observed differences between the lipids of viral membranes and their host cell plasma membrane involve the fatty acid constituents of both phospholipids and neutral lipids. In particular, the phospholipids of many viral membranes contain more saturated fatty acyl chains than do the phospholipids of the host cell plasma membrane, which was attributed to the higher sphingomyelin content of viral membranes.[53] Tiffany and Blough[55,56] suggested that viral proteins can select for particular fatty acids, thereby altering the relative fatty acid composition of the viral membrane compared to that of the host plasma membrane.

Small differences between the lipid composition of viral membranes and the host cell plasma membrane do not necessarily imply any selective pressure by the virions. Viral membranes and plasma membranes share a notable characteristic, i.e., a high cholesterol, sphingomyelin, and occasionally phosphatidylethanolamine content compared to lipids extracted from all the membranes of the whole cell.[6] Any contamination of the so-called "purified" plasma (surface) membrane preparation with intracellular membranes might provide misleading data of an apparent rather than a real difference in lipid composition of cytoplasmic membrane compared to that of the readily purified viral membrane. Therefore, the notorious impurity of plasma membrane preparations

TABLE 2

Lipid Composition of Viral and Cellular Membranes[a]

Membrane Source	Site of maturation	Host cell	% Phospholipid (PL) content PE	PS	PC	SPM	Amino-PL / Total PL	Chol / PL	Ref.
Coronaviruses									
TGEV	Cytoplasmic vesicles	PK/I	8.9	1.6	28.5	31.7[b]	0.15	0.02	43
Whole cell	—	PK/I	9.3	1.8	21.2	35.7[b]	0.16	0.02	43
TGEV	Cytoplasmic vesicles	APT/2	4.1	—	52.1	16.1[b]	0.06	0.03	43
Whole cell	—	APT/2	7.8	—	54.6	14.8[b]	0.10	0.03	43
Herpesviruses									
Pseudorabies	Inner nuclear membrane	RK	21.0	3.5	51.2[c]	21.0	0.25	—	44
Inner nuclear membrane	—	RK	21.5	3.7	50.5[c]	20.8	0.26	—	44
Paramyxoviruses									
NDV	PM	CEF	33 ± 3	10 ± 2	26 ± 3	26 ± 2	0.43	—	27
Sendai	PM	CEF	31	14	27	24	0.45	—	27
Plasma membrane	—	CEF	31 ± 3	13 ± 2	35 ± 3	20 ± 2	0.44	—	27
SV5	PM	MK	40.3	17.9	25.2	12.2	0.59	0.89	45
Plasma membrane	—	MK	38.8	17.2	32.1	11.8	0.56	0.81	45
SV5	PM	BHK-21F	15.6	5.2	38.5	30.0	0.21	0.64	45
Plasma membrane	—	BHK-21F	11.2	5.1	49.5	24.2	0.16	0.68	45
SV5	PM	MDBK	40.9	2.0	23.8	27.3	0.43	0.84	46
Plasma membrane	—	MDBK	27.2	2.2	44.5	22.8	0.29	0.75	46
SV5	PM	HaK	17.1	5.0	43.8	25.8	0.22	0.60	46
Plasma membrane	—	HaK	13.0	5.0	46.8	24.4	0.18	0.51	46
Poxviruses									
Vaccinia	*de novo*	HeLa	12.4 ± 2.4	4.6 ± 1.0	48.9 ± 3.2	4.7 ± 1.3	0.17	—	31
Whole cell	—	HeLa	19.6 ± 1.6	4.8 ± 0.8	52.7 ± 1.9	5.6 ± 0.6	0.24	—	31
Rhabdoviruses									
VS_{Ind}	PM	L	33.1	17.3	16.4	20.9	0.50	0.65	12
VS_{NJ}	PM	L	31.3	15.4	15.9	22.0	0.47	0.96	12
Plasma membrane[a]	—	L	22.6	7.0	51.0	16.2	0.30	0.58	12
Plasma membrane[b]	—	L	30.1	12.3	32.0	17.1	0.42	0.82	12
VS_{Ind}	PM	CEF	37.0	17.0	18.4	23.9	0.54	0.60	12
VS_{NJ}	PM	CEF	36.0	16.9	21.9	23.6	0.53	0.63	12
Plasma membrane[a]	—	CEF	30.5	10.1	37.8	11.9	0.41	1.01	12

TABLE 2 (continued)

Lipid Composition of Viral and Cellular Membranes[a]

Membrane Source	Site of maturation	Host cell	% Phospholipid (PL) content PE	PS	PC	SPM	Amino-PL / Total PL	Chol / PL	Ref.
Plasma membrane[b]	—	CEF	31.9	10.8	46.6	7.7	0.43	0.54	12
VS_{Ind}	PM	BHK-21	31 ± 2.5	18 ± 1.5	24 ± 2	24 ± 2	0.49	0.72	47
VS_{NJ}	PM	BHK-21	—	—	—	—	0.44	0.81	47
Plasma membrane	—	BHK-21	—	—	—	—	0.31	0.54	47
Rabies	PM	BHK-21	35.1 ± 1.2	7.8 ± 0.2	23.4 ± 2.0	31.3 ± 0.4	0.43	0.48	48
Plasma membrane	—	BHK-21	35.3 ± 2.5	8.2 ± 0.1	34.2 ± 3.0	14.3 ± 0.3	0.44	—	48
Rabies	PM	BHK-21	20.2	19.8[d]	21.7	19.8	0.40	0.92	49
Rabies	IC	BHK-21	20.8	15.3[d]	24.2	24.3	0.36	0.87	49
Whole cell	—	BHK-21	30.5	9.6[d]	20.3	16.0	0.40	0.20	49
RNA Tumor Viruses									
Rous sarcoma	PM	CEF	33 ± 3	12 ± 2[d]	20 ± 2	29 ± 2	0.45	0.88	50
Plasma membrane	—	CEF	26 ± 2	14 ± 2[d]	37 ± 3	20 ± 1	0.40	0.51	50
Rous sarcoma	PM	Quail	26 ± 3	17 ± 3[d]	30 ± 1	22 ± 1	0.43	—	50
Plasma membrane	—	Quail	21 ±1	15 ± 3[d]	47 ± 3	14 ± 2	0.36	—	50
Togarivurses									
SFV	PM	BHK21-Wi2	23	13	33	20	0.36	0.99	51
Plasma membrane	—	BHK21-Wi2	18	9	38	18	0.27	0.70	51
SFV	PM	BHK21-C13	26	12	34	21	0.38	0.97	51
Plasma membrane	—	BHK21-C13	21	7	40	17	0.28	0.56	51
Sindbis	PM	CEF	28.5 ± 0.3	17.9 ± 0.5	29.9 ± 0.5	27.3 ± 0.2	0.46	0.80	52
Plasma membrane	—	CEF	28.4 ± 0.4	9.2 ± 0.2	35.8 ± 0.1	18.7 ± 0.4	0.38	0.60	52

[a] Abbreviations used: TGEV = transmissible gastroenteritis virus; NDV = Newcastle disease virus; SV5 = simian virus5; VS_{Ind} = vesicular stomatitis virus (Indiana serotype); VS_{NJ} = vesicular stomatitis virus (New Jersey serotype); SFV = Semliki Forest virus; PM = plasma membrane; IC = intracellular membranes; PK/1 = primary pig kidney; APT/2 = secondary adult pig thyroid; RK = rabbit kidney; CEF = chick embryo fibroblast; MK = monkey kidney; BHK = baby hamster kidney; MDBK = Madin-Darby bovine kidney; HaK = hamster kidney; HeLa = human cervical carcinoma; L = mouse fibroblast; PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; SPM = sphingomyelin; PL = phospholipid; chol = cholesterol.

[b] SPM + lysolecithin.

[c] PC + phosphatidylinositol.

[d] PS + phosphatidylinositol.

makes comparision with viral membranes very difficult. On the other hand, viruses grown in tissue culture are typically quite free of contaminating membranes. However, the purity of some preparations, such as egg-grown viruses produced during multiple growth cycles from unknown membrane sites, are also suspect. Due to these shortcomings in purifying cell membranes, it is impossible to assess accurately whether viral proteins inserted into infected cell membranes can select for specific lipids during the course of membrane lipid turnover. More sensitive techniques must be employed for directly measuring the interaction of viral proteins and specific lipids.

Other lipid constituents of viral membranes include both neutral glycolipids and negatively charged glycolipids or gangliosides. Since glycolipids are antigenic, some of the first evidence for the existence of unaltered host cell glycolipids in virions was revealed by serological studies. Purified virions contain several glycolipid antigens which either cross-react with or are identical to host cell glycolipids.[57] Using SV5 grown in four unrelated cell lines, Klenk and Choppin[58] demonstrated that the virion neutral glycolipids mimicked the host cell neutral glycolipids. Host cell gangliosides, however, were completely absent in the virions, presumably due to the removal of ganglioside neuraminic acid by the virion associated neuraminidase. In the case of vesicular stomatitis virus, which lacks neuraminidase, unaltered host cell gangliosides are incorporated into the viral membrane in the same relative amounts as in the host cell plasma membrane.[53,59,60] Moreover, there is no indication that any virally coded glycosyl transferases are involved in the production of viral glycolipids.[57]

A notable exception to the viruses discussed so far, all of which bud from cellular membranes, is the vaccinia poxvirus, which acquires its membrane by *de novo* synthesis in cytoplasmic factories.[31] The vaccinia membrane is comprised of typical cellular lipids, although their relative proportion does not resemble the composition of any particular cellular membrane. An interesting speculation by Stern and Dales[31] suggested that during maturation the phospholipids of the vaccinia membrane might exchange with phospholipids of the endoplasmic reticulum catalyzed by an endogenous phospholipid exchange protein. This process could provide a mechanism for virions to select particular phosopholipids, especially if an asymmetric orientation is required for the phospholipids in the viral membrane.

B. Orientation of Viral Membrane Lipids

The first lipids to be analyzed for their distribution between the external and the internal halves of the bilayer were the phospholipids of the erythrocyte membrane.[61,62] The results, which have been corroborated innumerable times,[63,64] showed that the majority of the cholinephospholipids, phosphatidylcholine (PC) and sphingomyelin (SPM), were facing externally, whereas the majority of the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), were facing internally. If this finding were a generalized phenomenon, the implication would be that the asymmetry must somehow be generated and maintained in the cell membrane for some biological function. The membranes subsequently studied, including viral membranes, have revealed that asymmetry in distribution of phospholipids exist in other membranes and for other lipids as well, although the asymmetry is not always in the same phospholipid nor does it always assume the same orientation as the erythrocyte membrane.

1. Phospholipid Distribution

There are a variety of reagents which have been used to determine the orientation of phospholipids in membranes. Many reagents react chemically with primary amino groups of aminophospholipids, either to form a product which absorbs light spectrophotometrically at a specific wave length (e.g., trinitrobenzene sulfonic acid [TNBS]), or which is radioactively labeled (e.g., [^{35}S] formylmethionyl sulfonylmethyl phosphate

[FMMP]), or isethionyl acetimidate (IAI). If the chemical reagent is shown not to penetrate to the internal side of the membrane, then the "labeled" aminophospholipids are assumed to be external. Alternatively, enzymes such as phospholipases have been employed to alter the externally accessible phospholipids by hydrolyzing various portions of the molecule, thereby differentiating between the accessible (external) and inaccessible (internal) phopholipids in the membrane. Also, phospholipid exchange proteins can be used to exchange radiolabeled phospholipids into lipid vesicles or out of the viral membrane to determine the pool size of the accessible (external) phospholipids. No single technique has proved universally applicable to all membrane systems. Some membranes are more sensitive and are perturbed more easily by a given technique than other membranes. In all "sidedness" studies, two factors are key to the interpretation of the results: (1) the membrane must be shown to remain impermeable to the reagent during the entire reaction, and (2) it must be shown that failure of phospholipids to react is due to their location on the internal side of the membrane rather than to shielding of membrane phospholipids by steric hindrance of proteins.

One of the first determinations of phospholipid distribution in virions was undertaken by Tsai and Lenard,[65] who used influenza virus and two phospholipases C, one from *Bacillus cereus* and the other from *Clostridium welchii.* These enzymes, when incubated with intact or protease-treated (spikeless) virions, hydrolyzed up to 55% of the total phospholipids. The remaining phospholipids were unavailable to hydrolysis by the enzymes. This differential susceptibility to phospholipase C suggested the presence of two physically independent pools of phospholipid. Since protease-treated virions gave results similar to intact virions, it seemed unlikely that the unreactivity of phospholipids toward phospholipase C was due to steric hindrance by membrane proteins. Therefore, the authors concluded that the headgroups of the hydrolyzed phospholipids faced externally, whereas the unhydrolyzed phospholipids faced internally. In agreement with data obtained from studying the erythrocyte, the hydrolyzed phospholipids in influenza (~80% PC, ~60% SPM, ~45% PE, ~40% PS) were predominantly cholinephospholipids, whereas the remaining unhydrolyzed phospholipids were mainly aminophospholipids. However, the phosopholipid asymmetry of the influenza membrane was less dramatic than that of the erythrocyte membrane.[63,64] One omission in this study was that the impermeability of the virion membrane was never assayed after enzyme treatment. It was presumed that the enzymes hydrolyzed only the externally facing phospholipids, but without adequate controls, an alternative explanation could be that the enzymes penetrated the virion membrane and hydrolyzed a certain percentage of the internal phospholipids along with some external phospholipids.

In a second more detailed study,[66] the phospholipid distribution of influenza virus was determined by four techniques: availability to the same two phospholipases C and to two phospholipid exchange proteins. The exchange proteins removed intact radiolabeled phospholipids from the virion membrane and replaced them with unlabeled phospholipids from interacting lipid vesicles. Intactness of the virion membrane was controlled, possibly adequately, by assaying the degradation of internal virion RNA by externally added ribonuclease. These investigations indicated that the distribution of total phospholipids in the influenza virus membrane was asymmetric with 30% external and 70% internal. To compensate for this asymmetry, it was suggested that a high glycolipid content (~15%) located entirely in the outer monolayer served to balance the lipid ratios in the two layers of the virion bilayer. The authors assigned the distribution of the individual phospholipids on the basis of the total phospholipid distribution, e.g., 30% of the phosphatidylethanolamine was external and 70% internal, similar to the total phospholipids. Therefore, phosphatidylethanolamine was symmetrically distributed. It seems plausible to us to assign the symmetry of the individual phospholipids based on total lipid content of the bilayer. If glycolipids comprise a

major portion of the lipid in the outer monolayer, the proportion of individual phospholipids in this monolayer should be calculated on the basis of the phospholipid plus glycolipid content, since there is no evidence of lateral phase separation of the lipid molecules. Thus, the phosphatidylethanolamine molecules occupy a greater portion of the inner monolayer than the outer monolayer. Using these calculations, both aminophospholipids and sphingomyelin were oriented asymmetrically (see Table 3).

The location of phospholipids within the vesicular stomatitis (VS) virus membrane has also been studied, using several of the techniques already discussed. Fong et al.[67] employed TNBS to label the aminophospholipids of intact or spikeless VS virions, which resulted in ∼36% labeling of the phosphatidylethanolamine, presumably located externally. The remaining phosphatidylethanolamine could be labeled after disruption of the virions, whereas phosphatidylserine labeling was not detected. To locate the remaining phospholipids within the VS viral membrane and determine the distribution of total phospholipids, an additional technique was necessary. Patzer et al.[47] using both phospholipase C (*C. welchii*) and TNBS exposure of intact and spikeless VS virions, found that 38 to 47% phosphatidylethanolamine, 94% phosphatidylcholine, and 80% sphingomyelin were externally available, whereas the remainder of these phospholipids were unavailable in otherwise intact virions. This susceptibility of VS virions to phospholipase C and TNBS labeling appeared to reflect the external and internal location of the phospholipids, since the virion membrane remained impermeable during the reactions, and the unreactive phospholipids were rendered susceptible to labeling or hydrolysis by mechanical or detergent disruption of the membrane. Further evidence for the location of phosphatidylcholine in the VS viral membrane was provided by studies with a phosphatidylcholine exchange protein.[68] It was found that ∼70% of the virion phosphatidylcholine was present in a rapidly exchangeable pool, presumably due to its external orientation in the membrane. The discrepancy between the accessibility of phosphatidylcholine to phospholipase C and exchange protein could be due to a pool which is available to phospholipase C, but partially shielded from the exchange protein by other membrane components.

The percent of each phospholipid in the inner half of the influenza and VS virus membranes is illustrated in Table 3. This emphasizes the relatively consistent finding that the aminophospholipids are enriched in the inner half of virion membranes in

TABLE 3

Distribution of Lipids in the Inner Half of Viral Membrane Bilayers and Half-Times ($t_{1/2}$) for Transmembrane Migration of Lipids

	Inner half of membrane bilayer					
Virus	PC	SPM	PE	PS	Chol	Ref.
Influenza						
%	16—20	29—49	44—58	63	—	65
%	52—66	77—85	70—72	75—86	—	66
$T_{1/2}$	10 days	30 days	—	—	—	66
%	—	—	—	—	45	81
$t_{1/2}$	—	—	—	—	13 days	81
VS virus						
%	—	—	64	—	—	67
%	6	20	57	85	—	47
%	30	—	—	—	—	68
$t_{1/2}$	7—11 hr	—	—	—	—	68
%	—	—	—	—	∼70	83
$t_{1/2}$	—	—	—	—	4—6 hr	83

agreement with phospholipid asymmetry of the erythrocyte membrane. Similar internal location of aminophospholipids was also apparent for the membrane of Semliki Forest virus by means of labeling with ^{35}S-FMMP.[69] Labeling of aminophospholipids increased seven- to eightfold upon detergent disruption of virions. However, no data were presented to confirm an internal location of the aminophospholipids as opposed to protein shielding of the unlabeled aminophospholipids. The data presented in Table 3 also suggest that the cholinephospholipids exhibit no preferential location within viral membranes.

The phospholipid distributions determined for other membrane systems have generally been supportive of the results obtained in studies with virion membranes. Phosphatidylethanolamine was located preferentially on the cytoplasmic side of the LM cell plasma membrane, and phosphatidylcholine was symmetrically distributed.[70] Similarly, the surface membrane of blood platelets was found to be enriched in aminophospholipids in the internal side, sphingomyelin in the external side, and phosphatidylcholine was distributed nearly symmetrically.[71] On the other hand, intracellular membranes have yielded some conflicting results, possibly due to the difficulty in isolating a pure membrane preparation of specific origin. The endoplasmic reticulum of liver, which has an inverted phospholipid orientation to the plasma membrane,[72] was found in one study to support the erythrocyte phospholipid distribution,[73] whereas a second group claimed a diametrically opposite orientation.[74] In an equivalent membrane preparation from muscle cells, the sarcoplasmic reticulum, two groups found the majority of the phosphatidylethanolamine located externally, a finding quite consistent with the inverse orientation of the erythrocyte membrane; however, most of the phosphatidylserine was internal.[75,76]

Although headgroup orientation has been studied fairly extensively, the distribution of a second chemical constituent of phospholipids, the fatty acyl chains, has received little attention to date. Their distribution can certainly have a profound effect on the fluidity of the entire membrane, or either membrane layer, which could in turn cause alterations in biological functions of the membrane. Basically, the one study of the fatty acid distribution in the erythrocyte membrane concentrated on the fatty acids of phosphatidylcholine and found that they were symmetrically distributed between the inner and outer halves of the membrane.[77] Sandra and Pagano[70] confirmed this observation for the individual phospholipids of LM cell plasma membranes, although the fatty acids of the total phospholipids were more unsaturated on the cytoplasmic side due to the greater unsaturation of the inner phospholipids.

Similarly, Patzer et al.[47] observed that most of the fatty acids in the VS virus membrane phospholipids were distributed about equally between both halves of the bilayer. Palmitic acid was enriched in the external half, primarily due to its high content in the externally facing sphingomyelin. In contrast, the polyunsaturated fatty acids were almost exclusively present in the inner monolayer, even though they were a constituent of phosphatidylethanolamine, which was present in both halves of the membrane bilayer. Consequently, it was concluded that within a given phospholipid, phosphatidylethanolamine in this case, the fatty acids are distributed unequally between the inner and outer monolayers of the VS virus membrane. Figure 1 summarizes data on the distribution of phospholipids and fatty acids between the two layers of the membrane of vesicular stomatitis virus.

The function of phospholipid asymmetry, either headgroups or fatty acyl chains, is unknown at the present time. An asymmetric distribution might be necessary for different functional requirements of each membrane monolayer. Membrane proteins associated with either side of the bilayer may require different lipid constituents for "activity", possibly due to different fluidity requirements. Similarly, the interaction of the membrane lipids with soluble (extrinsic) proteins or other macromolecules may be

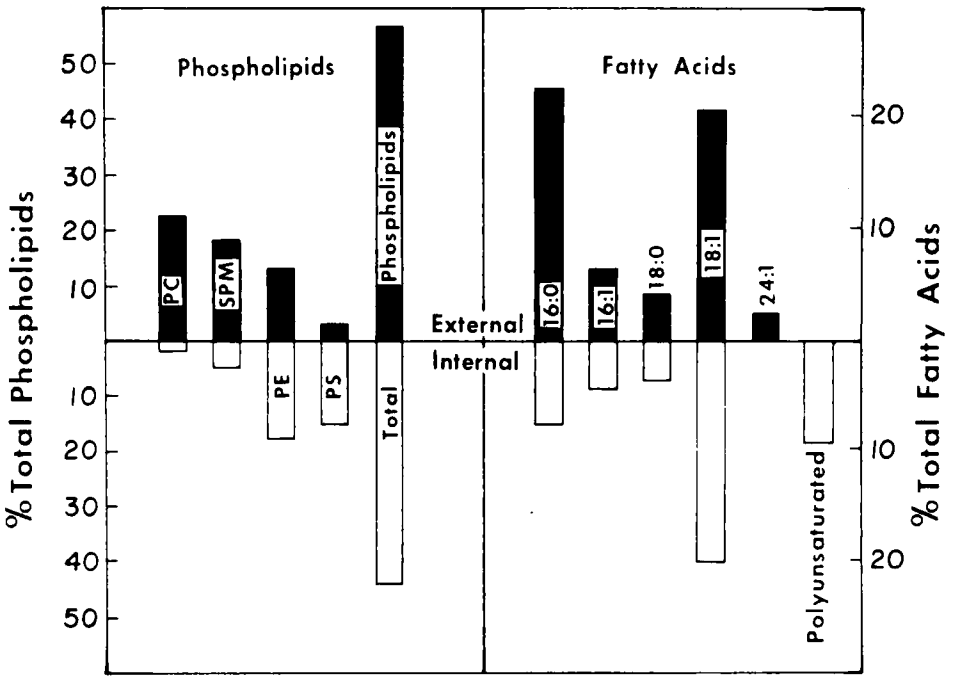


FIGURE 1. Putative location of phospholipids and fatty acids in the two halves of the membrane bilayer of vesicular stomatitis virus. The phospholipids were assigned to external or internal orientation based on their availability in intact virions to reaction with phospholipase C and to labeling with trinitrobenzene sulfonic acid in the case of phosphatidylethanolamine (PE) and phosphatidylserine (PS). The fatty acids of the hydrolyzed and unhydrolyzed phospholipids were analyzed by gas-liquid chromatography and expressed as a percentage of the control fatty acids. (Reprinted from Patzer, E. J., Moore, N. F., Barenholz, Y., Shaw, J. M., and Wagner, R. R., *J. Biol. Chem.*, 253, 4544, 1978. With permission.)

dependent on a specific asymmetry. In particular, it appears that cholesterol can be oxidized by cholesterol oxidase only when cholesterol is in association with aminophospholipids in the erythrocyte membrane[78-80] and the VS virion membrane.[79,80]

2. Cholesterol Distribution

The second major lipid component of eucaryotic plasma membranes and virions derived from plasma membranes is the neutral lipid, cholesterol. Determination of the cholesterol distribution requires the same precautions as the determination of phospholipid distribution. The membrane must remain impermeable to the reagent used during its incubation with virions, and the possibility of protein shielding of cholesterol should be eliminated. One method to locate cholesterol was employed by Lenard and Rothman[81] with influenza virus. They observed that cholesterol would spontaneously exchange between virions and sonicated lipid vesicles without the aid of a catalytic protein. This provided a means of determining the availability of cholesterol from the external side of the membrane. The cholesterol of influenza virus was metabolically labeled during growth and exchanged (one for one) with unlabeled cholesterol from lipid vesicles or depleted from the viral membrane into lipid vesicles lacking cholesterol. The kinetics of the cholesterol exchange in both cases revealed two pools: a readily exchangeable pool containing ~55% of the total cholesterol and a slowly exchangeable pool containing ~45% of the cholesterol. The rapidly and slowly exchangeable pools were assigned to the external half and internal half of the bilayer, respectively.

Moore et al.[82] used a different approach in attempting to locate cholesterol in the

membrane of VS virus. Cholesterol oxidase, an enzyme which oxidizes the 3β-OH group of cholesterol, was incubated with intact or spikeless VS virions previously exposed to phospholipase C to remove phospholipid headgroups. Although this prior treatment of virions with phospholipase C was essential for cholesterol oxidase activity, the virion membrane remained impermeable to surface labeling reagents during all stages of the reaction. At least 90% of the VS viral membrane cholesterol could be oxidized by externally added cholesterol oxidase. This observation led to the hypotheses that either the cholesterol was located entirely in the outer monolayer or there was rapid (<2.5 min)[358] transmembrane movement of cholesterol from the inner layer of the virion membrane. Later studies[79,80] revealed that vesicle cholesterol associated with intact aminophospholipids can be oxidized by cholesterol oxidase, confirming the observations of Gottlieb[78] that cholesterol in the cytoplasmic layer of the erythrocyte membrane is accessible to cholesterol oxidase. Moreover, it was difficult to visualize how cholesterol, which represents ∼40% of VS virion lipids, could be packed into the outer layer of the VS virion membrane along with 55% of the phospholipids.[47]

Since the cholesterol oxidase method provided only suggestive results, Patzer et al.[83] employed a second technique for studying the distribution and transmembrane movement of cholesterol in the VS virion membrane. Viral cholesterol was depleted or exchanged by interaction with lipid vesicles under conditions similar to those used by Lenard and Rothman[81] for influenza virus. In our experiments, radioactive cholesterol was exchanged into as well as out of the VS virion membrane. Figure 2 depicts kinetic studies which reveal a rapidly exchangeable pool, representing ∼30% of the viral membrane cholesterol and a slowly exchangeable pool, accounting for the remaining 70%.[83] The half-time for equilibration of the two pools was 4 to 6 hr, a rate which was considerably slower than that predicted by the cholesterol oxidase reaction[82] (<2.5 min). It would appear that the perturbing nature of the cholesterol oxidase reaction does not provide an accurate picture of cholesterol distribution and its transmembrane movement, as confirmed by studies of the erythrocyte membrane.[78] Based on the two kinetic pools of exchangeable cholesterol, the VS virus cholesterol appears to be asymmetrically distributed within the membrane with more, perhaps 70%, residing in the inner half of the bilayer. In contrast, the influenza virus membrane cholesterol appears to be more symmetrically distributed.[81] Some comparative data are presented in Table 3.

Similar vesicle exchange techniques used to study cholesterol distribution in the erythrocyte membrane have led to conflicting data,[84,85] presumably due to firmly bound cholesterol.[86,356] Evidence for symmetrical distribution of cholesterol between the two freeze-fractured layers of the erythrocyte membrane appears to be somewhat inconsistent.[87] Better results were otained by the use of unesterified cholesterol in high-density lipoproteins for exchange of cholesterol from the membrane of *Mycoplasma gallisepticum*, which revealed that ∼50% of cholesterol was present in each half of the bilayer.[88]

3. Membrane Glycolipid Distribution

Glycolipids have long been assumed to reside in the externally facing half of plasma membranes and viral membranes, but only indirect evidence supports this belief. The carbohydrates of all known glycoproteins face the external milieu and, by analogy, glycolipids are assumed to do the same. Certainly, plasma membranes are enriched in glycosphingolipids,[58] and it is tempting to infer that they are located exclusively on the external surface of plasma membranes and budding virions.[58] Although recent evidence is scanty, especially for viral membranes, our initial assumptions appear to be correct. Two studies have shown that the glycolipids in the erythrocyte membrane are exposed to external labeling techniques.[89,90] Similarly, the neuraminic acid residues associated with hematoside, the only glycolipid in VS virus grown on BHK cells,[59] can

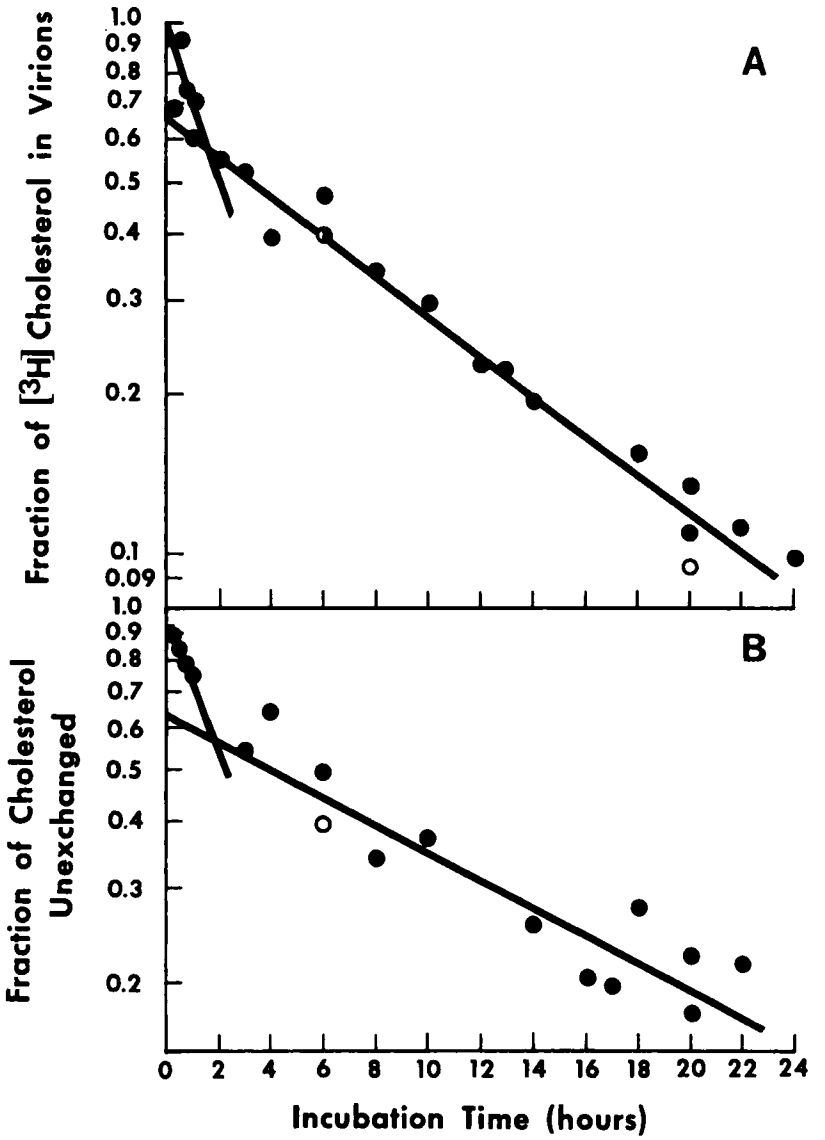


FIGURE 2. The kinetics of [^{3}H]xholesterol exchange from trypsinized VS virus into PC/PS/cholesterol vesicles (panel A) and the kinetics of exchange of [^{14}C]cholesterol from PC/PS/cholesterol vesicles into unlabeled trypsinized VS virus. Semilogarithmic plots were drawn, using linear regression analysis of the data points. The open circles represent twice the concentration of vesicles; all values were corrected for vesicles adhering to virions. (Reprinted with permission from Patzer, E. J., Shaw, J. M., Moore, N. F., Thompson, T. E., and Wagner, R. R., *Biochemistry*, 17, 4192, 1978. Copyright by the American Chemical Society.)

be removed by externally added neuraminidase.[91] In addition, intact VS virions containing glycolipid antigens from the host cell will react with host antibody and fix externally added complement.[60] Thus, virion membrane glycolipids appear to be predominantly exposed on the external surface, although they may be partially shielded by glycoproteins.[92]

C. Translocation of Lipids Across the Membrane

The asymmetric distribution of phospholipids in viral membranes, as well as other

membranes, raises an important question: does any transmembrane movement (inside to outside or vice versa) of phospholipids occur within the membrane, and if so, what maintains the asymmetry? It is clear from studies of single-bilayer lipid vesicles that in the absence of proteins, any transbilayer movement occurs at exceedingly slow rates with half-times greater than 5-10 days.[93-96] The transmembrane movement in biological membranes containing protein, however, is less clearly resolved. Two studies have recently shown that membranes active in lipid biosynthesis, the membrane of *Bacillus megaterium*[97] and rat liver microsomes,[98] have very rapid half-times (*B. megaterium* — 3 min; microsomes — <1 hr) for transmembrane movement. On the other hand, membranes not active in lipid biosynthesis have relatively slower or nonobservable rates. Conflicting studies with the erythrocyte membrane have reported half-times of 2.3 to 5.3 hr[99,100] or undetected rates[101] for transmembrane movement.

Viral membranes, which are also inactive in lipid biosynthesis, in some cases reflect the slower rates observed in the erythrocyte membrane. The phosphatidylcholine and sphingomyelin in influenza virions showed half-times for transmembrane movement of >10 days and >30 days, respectively,[66] whereas the phosphatidylcholine in VS virions had a half-time of 7 to 11 hr for transmembrane migration[68] (see Table 3). Whether significant transmembrane movement of phospholipids occurs in viral membranes on a biologically relevant time scale will require further study. A recent study, however, reported that glycophorin, a transmembrane glycoprotein in the erythrocyte, can catalyze the movement of lysophosphatidylcholine across a lipid vesicle bilayer.[102]

Transmembrane movement of cholesterol within the viral membrane bilayer could be dependent on other membrane constituents, including the presence of proteins or specific phospholipids on either side of the bilayer. The two viruses investigated with the use of similar techniques have yielded different results, which are shown in Table 3. The transmembrane movement of cholesterol in the VS viral membrane was found to be rapid with a half-time for equilibration between inner and outer monolayers of 4 to 6 hr.[83] In contrast, the transmembrane movement of cholesterol in influenza virus was assigned a half-time of >13 days.[81] Since similar techniques and incubation conditions were used for the exchange reactions in influenza and VS virus, the differing results might be due to inherent characteristics of each membrane. Unfortunately, other membrane systems suffer from similar inconsistencies. Lipid vesicles containing cholesterol were found to exhibit no transbilayer movement by one group,[103,104] whereas a second group observed an extremely rapid movement.[100] Similar conflicting results have been reported for erythrocytes,[86,105] with no clear indication of which is correct. Hopefully, future studies of other viral membranes will lead to a better understanding of the dynamic characteristics of phospholipids and cholesterol within the membrane bilayer.

D. Dynamic Characteristics of Viral Membranes

1. Mobility of Lipids in Membranes-Techniques for Measuring

The preceding discussion of lipid distribution in membranes tends to give the misleading impression of a very static condition of the organization of lipids within the bilayer. It is true that transmembrane movements of lipids appear to be relatively sluggish events. In reality, membrane lipids are quite mobile, being capable of rapid lateral diffusion (10^{-8} cm^2/sec) in the plane of the membrane.[106] To better understand some of the dynamic characteristics of lipid bilayers, three techniques have been employed in recent years to measure the mobility of lipids in membranes: electron spin resonance (ESR) spectroscopy, fluorescence depolarization, and nuclear magnetic resonance (NMR) spectroscopy. Each technique uses a "probe" molecule either endogenously or exogenously incorporated within the lipid bilayer. The mobility of the probe can be

correlated with the average degree of fluidity in its environment within the membrane, allowing comparisons to be made between two or more membranes. It is well to remember that no techniques are available to measure fluidity in selected regions of a single membrane, which may exhibit microheterogeneity.

Each of the techniques employed in these fluidity studies has certain advantages and disadvantages. The ESR studies for the most part have employed spin-labeled fatty acids or phospholipids which have been exogenously incorporated into membranes. The nitroxide "reporter" group can be positioned virtually anywhere along the fatty acyl chains of the probe molecule, which allows insertion at any depth in the bilayer. In this way, the fluidity of the central hydrocarbon core region of the bilayer, as well as the interfacial region close to the phosopholipid headgroups, can be measured. This is particularly useful when attempting to correlate the depth of penetration of the lipophilic tails of glycoproteins with their effect on lipid mobility. Nuclei which can be observed with NMR can also be positioned at various locations along the length of phospholipid molecules, but in addition, they can be endogenously incorporated into viral lipids during metabolic assembly of membranes. This circumvents the problem of perturbation of the membrane by exogenously adding lipids to already assembled membranes.

Fluorescence depolarization measurements rely on probe molecules which are inserted exogenously and incorporated in the membrane. However, each fluorophore positions itself at only one characteristic location in the lipid bilayer. For example, 1,6-diphenyl-1,3,5-hexatriene (DPH) is incorporated into the most hydrophobic regions of the bilayer where it undergoes anisotropic motion.[107] Other fluorophores must be employed to selectively probe other regions closer to the phospholipid headgroups. One advantage of the fluorescence depolarization technique is the relatively small amount of membrane material required for fluidity measurement, in contrast to both ESR and NMR spectroscopy which usually require much larger quantities in concentrated suspensions.

In general, studies using these three physical techniques for studying virus systems have followed two approaches: (1) the fluidity of the viral membrane has been compared to the composite cytoplasmic membrane system of the host cell or to just the isolated host cell surface plasma membrane; (2) specific viral membrane components have been altered or removed to correlate a fluidity change with a given membrane constituent. The first of these approaches in theory is straightforward, but in practice suffers from the same drawback as does comparing the lipid composition of the virus with that of the host cell plasma membrane. The purity of plasma membrane isolation techniques is difficult to judge. Nevertheless, some interesting observations have resulted from comparisons of the fluidity of viral membranes and the membrane(s) of host cells.

2. Membrane Fluidity of Virions and their Host Cells

The fluidity of several viral membranes has been compared to that of their host cell plasma membrane, using both ESR spectroscopy and fluorescence depolarization. The conclusions generally have been consistent: the membranes of all viruses are almost invariably less fluid than the membranes of whole host cells or the host cell plasma membranes. One of the first fluidity measurements of a viral membrane used the spin-label androstane incorporated in influenza virus grown on MDBK cells and compared its fluidity to that of the erythrocyte membrane.[42] Although the study concluded that the virus membrane was less fluid, it could not be determined whether this was due to the heterologous nature of the erythrocyte membranes or was an inherent characteristic of viral membranes. In subsequent studies, however, the fluidity of viral membranes has been compared directly to the fluidity of the whole host cell or its plasma mem-

brane. Landsberger and Compans,[108] using spin-labeled fatty acids, observed that the ESR spectrum of the VS virus membrane indicated a less fluid environment than that of the host cells, presumably labeled in the plasma membrane. Similarly, the ESR spectra of the membrane of Sindbis virus suggested a less fluid environment than the membrane of the host chick embryo fibroblast (CEF) cells.[109] In addition, the extracted lipids of Sindbis virus were less fluid than the plasma membrane lipids of CEF cells, which suggested that lipid differences were at least partially responsible for the dissimilar fluidities.[109]

Fluorescence depolarization measurements with the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) have confirmed the ESR results with these and several other virus-host cell systems. VS viruses grown in either L cells[110] or BHK-21 cells[47] were less fluid than the host cell plasma membranes. Again, the extracted lipids exhibited the same relationship, illustrating that the lipids were primarily responsible for the less fluid environment in the most hydrophobic region of the VS viral membrane. This differential membrane fluidity also obtained when comparisons were made with a fish rhabdovirus, infectious hematopoietic necrosis (IHN) virus, and its host CHSE-214 cells,[111] and with two togaviruses, Sindbis and Semliki Forest (SF) viruses and their host BHK-21 cells,[112] confirming the ESR results of Sefton and Gaffney.[109] Thus, in all cases studied to date, the viral membranes were found to be less fluid than total membranes of their host cell or host cell plasma membrane alone. An interesting analogy has been drawn between viruses and vesicles exfoliated from a cell surface:[113] the membranes were found to be less fluid than the cell plasma membrane, suggesting that exfoliation (including exfoliation of budding virions) selects for less fluid areas of the plasma membrane. Possibly, less fluid regions of the plasma membrane are simply more active in the exfoliation process.

A comparison of two rhabdoviruses, VS viruses and IHN fish virus, grown in different cell lines at different characteristic growth temperatures, has led to an interesting observation.[110,111] At a given temperature, both IHN virus and its host CHSE-214 cells were more fluid than VS virus and its host L cells, respectively. If the fluidity of the two systems were compared at their respective growth temperatures, i.e., $\sim$18°C for IHN virus and CHSE-214 cells and $\sim$37°C for VS virus and L cells, then the fluidity of the viruses was similar and the fluidity of the cells was similar.[110,111] These findings suggest that the cells may be capable of altering their lipids to maintain a constant fluidity at their growth temperature and thereby cause an alteration in the fluidity of the viral membrane derived therefrom. A similar relationship was reported for a single virus grown in two cell lines at different temperatures.[112] Sindbis virus grown in *Aedes albopictus* mosquito cells (growth temperature $\sim$22°C) had a fluidity at 20°C which was similar to that of the fluidity of Sindbis virus at 37°C when grown in BHK-21 cells at 37°C. Although a similar correlation was not observed in comparing mosquito cell fluidity with that of the whole BHK cells, the plasma membranes could conceivably exhibit this relationship. Alterations in fluidity have been shown to effect plasma membranes differently than fluidity of whole cell membranes when using the fluorescent probe, DPH.[114]

3. Factors Responsible for Viral Membrane Rigidity

The relative fluidity of model membrane systems, such as protein-free liposomes, has been shown clearly to depend on specific lipid constituents. Alterations in phosopholipid headgroups[115] or fatty acyl chains[116] or relative amount of cholesterol[116] and sphingomyelin[107] all affect the observed fluidity. In addition to various lipid constituents, viral membranes presumably all have proteins in close association with the lipid bilayer. Two classes of viral proteins could interact with the lipids and alter their mobility: (1) one or more glycoproteins facing externally with a hydrophobic tail inserted

in or through the lipid bilayer, and in some cases, (2) a membrane or matrix (M) protein (or nucleocapsid core protein), which lines the inner side of the membrane.

Considerable interest in the contribution of viral proteins to their membrane fluidity has been generated, due in part to the relative simplicity of these membranes, which contain only a limited number of proteins associated with the lipid bilayer. In addition, it is of key interest in morphogenesis of enveloped viruses to determine to what extent the viral proteins can recognize and interact with the lipid bilayer of the infected cell. The glycoprotein tail embedded in the membrane may interact with specific fatty acyl chains or the M protein on the inner side of the membrane may bind internal phospholipid headgroups. Generally, three approaches have been used to identify specific membrane components in order to determine the degree to which they affect membrane fluidity. First, the lipids of viral membranes have been extracted and reconstituted as protein-free liposomes in aqueous buffer. A difference between the fluidity of the lipids alone and the intact virions can be attributed to membrane protein. Secondly, the external virion glycoproteins can be selectively cleaved with protease to determine whether their removal causes a specific fluidity change. Finally, specific lipid components can be altered to determine if the fluidity change is analogous to that observed in model membrane systems. This latter approach will be discussed in detail in a later section.

Influenza virus has three proteins which can potentially interact with the lipid bilayer: two glycoproteins on the external side of the membrane and an M protein on the internal side. Landsberger et al.,[42] using ESR spectroscopy, observed that the fluidity of intact virions and spikeless virions, whose glycoproteins were proteolytically removed, exhibited identical patterns. This was interpreted as evidence that the glycoproteins had no effect on the viral membrane fluidity. A later study, however, revealed the presence of small glycoprotein-derived peptides associated with virion membrane after proteolytic treatment.[117] These small peptides, if embedded in the membrane, could exert the same influence on fluidity as intact glycoproteins. Therefore, external proteolysis of glycoprotein spikes may not cause any measurable change in the membrane dynamics. The latter study also compared the fluidity of standard influenza virions with incomplete virions containing a substantially increased concentration of glycoprotein[118] with the same conclusion: the glycoproteins have no effect on the fluidity of influenza virions.[117] The assumption made was that any effect of the glycoproteins on fluidity in standard virions would be magnified by the higher concentration of glycoprotein in incomplete virions.

On the inner side of the influenza virus membrane, the M protein was purported to be in close association ($\leq$11 Å) with the lipid bilayer, using a fluorescent molecule incorporated in the membrane.[119] Fluorescence energy transfer was found to occur between 12-(9-anthroyl)-stearic acid and a virion protein. Since the transfer persisted after protease treatment, the internal M protein was postulated to be the energy donor. However, as already indicated, glycoprotein fragments can remain inserted in the virion membrane after proteolysis[117] and could conceivably continue as energy donors.

A comparative study of influenza and SV5 virions, both grown in two different cell lines, has provided the most convincing data on the contribution of proteins and lipids to the fluidity of these two virus membranes.[120] A comparison of SV5 or influenza virus grown in either BHK-21F or MDBK cells indicated that the fluidity of the virions near the phospholipid headgroups was lower in the MDBK-grown virions than in virions grown in BHK-21F cells. Thus, under conditions in which the lipids were derived from different sources but the proteins remained constant, the fluidity changed. In contrast, SV5 and influenza virions grown in the same host cell line, either BHK-21F or MDBK, had the same fluidity. In this case, the lipids were derived from the same source, but the proteins were different in each virion exhibiting the same fluidity.

Therefore, the proteins appear to exert little or no influence on the fluidity of these two viruses. However, a similar approach, comparing VS and influenza viruses grown on the same two cell lines, yielded different results.[108] Both viruses grown on the same cell line exhibited different fluidities, presumably due to membrane-associated proteins in the VS virus membrane.

The most detailed account of the effect of proteins on the mobility of viral lipids has accumulated for the Indiana serotype of VS virus. Several independent studies have revealed that the degree to which VS viral proteins influence lipid mobility is dependent on the location of the probe within the membrane. Figure 3 presents a schematic diagram of the VS virus membrane illustrating the approximate location of the probe molecules employed to study membrane fluidity. The greatest effects of the viral proteins were recorded for the interfacial region of the bilayer in proximity to the aqueous environment. In the more hydrophobic regions near the center of the bilayer, little or no protein influence was detected.

The headgroups of phosphatidylcholine and sphingomyelin in the outermost regions of the VS virus membrane were metabolically labeled with [^{13}C] choline during growth, and their mobility was determined by their spin-lattice relaxation times (T_1), using NMR. Stoffel and Bister[121] and Stoffel et al.[122] reported that no change in mobility was detected in protease-treated virions or in liposomes prepared from extracted lipids

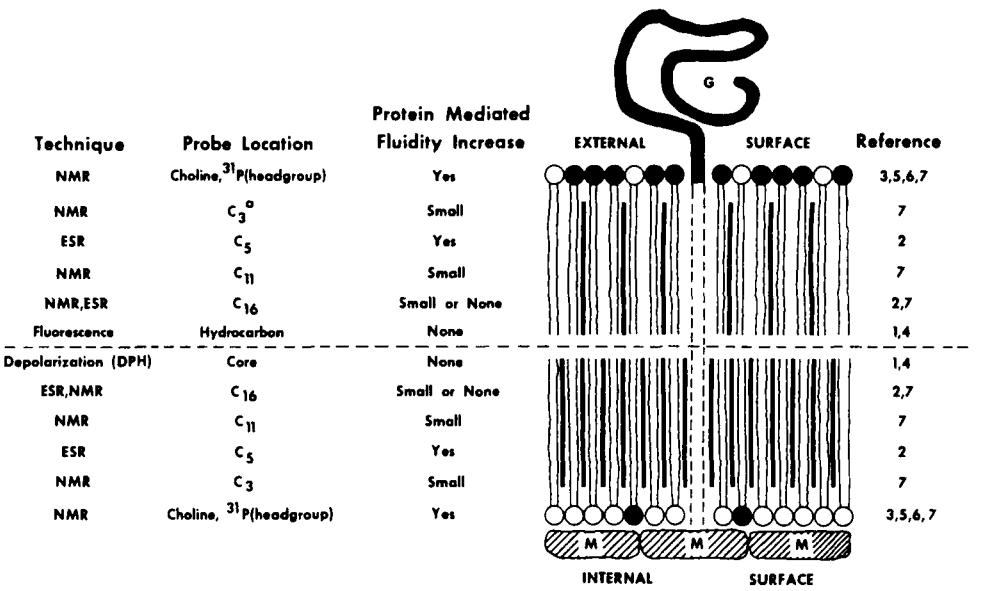


FIGURE 3. Schematic diagram of the membrane of vesicular stomatitis virus depicting the distribution of lipids in the bilayer and the effect of membrane proteins G (external) and M (internal) on the mobility of the lipids determined by nuclear magnetic resonance (NMR), electron spin resonance (ESR), and diphenylhexatriene (DPH) fluorescence depolarization. Also depicted is the location of each probe in headgroup or at different regions along the length of the fatty acyl chains. The solid circles represent choline headgroups, the open circles represent amino headgroup, and the dark lines indicate the theoretical positions of cholesterol molecules intercalated between the fatty acyl chains. * C_3 refers to the carbon atom labeled from the carboxyl end. References: 1. **Barenholz, Y., Moore, N. F., and Wagner, R. R.,** *Biochemistry,* 15, 3563, 1976. 2. **Landsberger, F. R. and Compans, R. W.,** *Biochemistry,* 15, 2356, 1976. 3. **Moore, N. F., Patzer, E. J., Wagner, R. R., Yeagle, P. L., Hutton, W. C., and Martin, R. B.,** *Biochim. Biophys. Acta,* 464, 234, 1977. 4. **Patzer, E. J., Moore, N. F., Barenholz, Y., Shaw, J. M., and Wagner, R. R.,** *J. Biol. Chem.,* 253, 4544, 1978. 5. **Patzer, E. J., Schmidt, C., and Wagner, R. R.,** unpublished experiments 1978. 6. **Stoffel, W. and Bister, K.,** *Biochemistry,* 14, 2841, 1975. 7. **Stoffel, W., Bister, K., Schreiber, C., and Tunggal, B.,** *Hoppe Seyler's Z. Physiol. Chem.,* 357, 905, 1976.

free of viral proteins. In contrast, when these experiments were repeated in this laboratory with ^{13}C-labeled headgroups, a small increase in mobility was observed upon trypsin treatment of VS virions.[123] Moreover, the naturally occurring phosphate (^{31}P) in all phospholipid headgroups showed a dramatic increase in mobility by NMR when the glycoprotein was removed by trypsin or the extracted lipids alone were examined.[124] At positions slightly deeper in the bilayer, C_3 of viral phospholipids[122] and C_5 of exogenously added free fatty acids[108] there are also detectable increases in mobility of the trypsin-treated virions or extracted lipids. Even at position C_{11} of the fatty acyl chain in viral phospholipids, there was a slight increase in mobility in the extracted lipids.[122] However, the most hydrophobic region, near the center of the bilayer, appears to be unaffected by proteins. Neither fluorescence depolarization with DPH[47,110] nor fatty acids spin-labeled at the C_{16} position[108] were able to detect any change in fluidity due to removal of the viral proteins, although Stoffel et al.,[122] using NMR, did report a small increase in fluidity, but only in the extracted lipids labeled at the C_{16} position of phospholipids. Similarly, the proteins of a fish rhabdovirus, IHN, exert no effect on the fluidity of the interior hydrocarbon region of its membrane, measured by fluorescence depolarization.[111] Taken as a whole, these results suggest that the VS virus proteins can influence the mobility of lipids to varying degrees in different locations in the membrane. Deeper penetration into the viral membrane results in progressively less influence of the viral proteins (G and/or M) on the mobility of the lipids. Specifically, the glycoprotein alone appears to immobilize at least part of the phospholipid headgroups since trypsin treatment can alleviate the immobilization. In some cases, however, there was a greater fluidity increase in the extracted lipids than the protease-treated virions. This could be interpreted as the result of residual glycoprotein-derived lipophilic tail fragments in protease-treated virions or, alternatively, it could be due to immobilization by M protein. No conclusive data concerning the lipid binding properties of M protein are available at this time.

Further information concerning the effect of proteins on membrane fluidity was obtained by studying two togaviruses, Sindbis and Semliki Forest viruses. In contrast to the previous viruses, togaviruses contain two or three glycoproteins and no M protein, although a core protein (C) may functionally replace M on the inner side of the virion membrane. Both ESR[109] and fluorescence depolarization[112] studies revealed that removal of proteins by protease or extraction of lipids resulted in an increase in membrane fluidity. Although Sefton and Gaffney[109] detected a progressively smaller protein effect in the more interior areas of the Sindbis virion membrane, unlike the membrane of VS virus, all regions of the Sindbis virion membrane became more fluid upon protein removal.

As data have accumulated, it has become increasingly evident that no generalizations can be made about protein contributions to fluidity in viral membranes. The membrane-bound proteins of some viruses have no effect, whereas the proteins of others decrease the fluidity to varying degrees, depending on their location within the viral membrane. Keith and Snipes[125] have provided an interesting theoretical discussion of the dynamic aspects of biological membranes with particular emphasis on membrane fluidity and protein sequestration in relation to virus budding from cell membranes.

E. Selection of Cellular Lipids by Viral Proteins

1. Role of Protein-Lipid Interaction

Some of the available evidence for protein-lipid interactions in viral membranes was presented in two previous sections. Section II. A presented data on the bulk lipid composition of viral membranes compared with the host cell plasma membrane in order to identify differences, which could be a result of selection by viral proteins. Small

differences could occasionally be detected, but the insensitivity of the technique and the impurity of plasma membrane preparations did not allow an unambiguous interpretation. Section II. D (above), however, indicated that the proteins of certain viruses clearly have an effect on the fluidity characteristics of their membranes, although this provides only limited information on the mechanism of protein-lipid interaction. In general, membrane proteins cause relatively subtle alterations in a lipid bilayer, leaving the majority of the lipids in a relatively unperturbed state.[126] Moreover, variations in lipid composition of viruses grown in different cells (see data on SV5 in Table 2) indicate that viral proteins alone do not determine the lipid composition of virion membranes.

A better understanding of the interactions between viral proteins and the lipid bilayer is necessary before any meaningful descriptions can be undertaken of the events taking place at the host plasma membrane. These events presumably lead to maturation and budding of newly synthesized virus, as well as influencing the initiation of viral infection by attachment to and penetration of the host cell. It is important to understand the mode of interaction of the viral glycoproteins with lipids on the external membrane surface as well as the M proteins or the ribonucleoprotein core protein at the internal membrane surface. Do these proteins interact with a particular phospholipid headgroup or fatty acyl chain, or will just any phospholipids serve as an anchoring matrix? Possibly the critical binding functions occur solely through protein-protein interactions discussed in Section III. One approach to these questions is to purify the individual proteins and reconstitute them in the presence of lipids to determine whether the isolated protein has any affinity for particular lipids, and whether its functions are dependent on these lipids.

2. Lipid Binding Properties of Viral Glycoproteins

The glycoproteins of many viruses have been shown to be amphipathic molecules containing a hydrophilic portion with carbohydrate chains and a lipophilic tail embedded in the lipid bilayer.[127-129] The lipophilic portion which binds detergents and is soluble in organic solvents[128] allows the entire molecule to be soluble only in the presence of detergents. Similar proteins with hydrophobic segments have been isolated from other membranes, e.g., glycophorin from erythrocytes, cytochrome b_5 and cytochrome b_5 reductase from microsomes, intestinal amino peptidase, and HLA histo-compatibility antigens.[126] The common feature of this type of membrane protein is the relative independence of the hydrophilic and lipophilic domains. Generally, the function of the lipophilic tail is to anchor the glycoprotein in a hydrophobic environment, either a detergent micelle or a lipid bilayer. This anchorage allows the hydrophilic portion to express its activity, which may be attachment to or fusion with a cell surface. The independence of these two domains complicates the determination of any lipid binding properties of the proteins. Unlike the ATPases or cytochrome oxidase, which display increased activity upon reconstitution with specific lipids,[126] a requirement for lipids by amphipathic glycoproteins necessitates some sort of binding assay.

Much of the work on the lipid requirements of viral glycoproteins has been done with two paramyxoviruses, Sendai (HVJ) and measles viruses. In a series of studies, Hosaka and co-workers[130-132] found that the Sendai virus glycoproteins solubilized with NP40 exhibited hemagglutinin (HA), hemagglutinin-inhibition (HI) blocking, and neuraminidase activities in the presence of detergent, but required lipids for restoration of the hemolytic and fusion activities expressed in native virions. Furthermore, hemolytic activity could be restored with all phospholipids except phosphatidylserine, although phosphatidylethanolamine appeared to be most efficient.[131] Low concentrations of cholesterol also stimulated the formation of hemolytic activity in the presence of phosophatidylethanolamine, whereas high concentrations were inhibitory. Besides

lipids, restoration of the fusion activity of the glycoproteins required an uncharacterized factor extracted with NP40 from the virions.[132] Similar results have been reported for measles virus in which NP40-solubilized glycoproteins contained only the HA activity[133] (measles virus does not contain neuraminidase). To restore the hemolytic and fusion activities to the F glycoprotein, the original viral lipids or phosphatidylethanolamine were required.[133] The nature of the paramyxovirus HN (hemagglutinin-neuraminidase) and F (fusion factor) proteins will be discussed in a later section.

It is interesting that the functions not requiring lipid in both studies were receptor or enzymatic activities (HN protein), whereas fusion or hemolysis (F protein), both of which involve membrane fusion, require lipid. Possibly the expression of these latter functions merely requires an intact lipid bilayer for fusion with cellular membranes in addition to a receptor activity. In support of this hypothesis, a recent series of papers by Martin and MacDonald[134-136] showed that the hemagglutination, hemolytic, and fusion activities of paramyxoviruses could be mimicked by protein-free lipid vesicles. The initial interaction of vesicles with cell membranes appeared to be electrostatic, whereas the final stages of hemolysis and fusion involved close juxtaposition of the vesicles and cell membrane in the presence of lysolecithin. The fusion data were consistent with a mechanism involving simultaneous fusion of the vesicle and two cell membranes.

Other viral glycoproteins have also shown little lipid specificity during reconstitution. The hemagglutinin (HA) and neuraminidase (NA) glycoproteins of influenza virus could be inserted into lipid vesicles of egg phosphatidylcholine and dicetylphosphate without any further lipid requirements, although the activity of the two glycoproteins was not measured.[137] Similarly, the vaccinia virus hemagglutinin (VHA) appeared to require lipids for both HA and HAI activity, although no specific phospholipids were necessary.[138] This is in contrast to the findings of Smith et al.,[139] who identified a lipid component as the hemagglutinin. However, this study used organic solvents and denaturing SDS to isolate and reconstitute the hemagglutinin. The interpretation of these reconstitution studies should be done with caution. Many of the isolation techniques for membrane proteins require harsh organic solvents or detergents which can denature proteins and are difficult to remove even by extensive dialysis. The use of detergents is especially precarious when assaying hemolysis or cell fusion, since the detergents alone can cause these activities. Furthermore, the conclusion that a particular phospholipid can activate a glycoprotein suspended in detergent at lower concentrations than other phospholipids may not be a glycoprotein characteristic. The detergent may also exert some selectivity for the replacement lipid. The dialyzable detergent, β-D-octylglucoside, offers a more satisfactory method for inserting viral glycoproteins into membranes of denuded virus[140] or phospholipid bilayers.[141]

A potentially better approach to detect interactions of isolated viral glycoproteins with reconstituted lipid systems is by using labeled lipids suitable for ESR or NMR spectroscopy. These techniques have proven successful for detecting lipid binding to other membrane proteins and should be applicable to viral proteins. For example, spin-labeled lipids detected by ESR were immobilized by cytochrome oxidase[142,143] or the lipophilic tail of cytochrome b_5.[144] In addition, NMR studies have revealed a "fluidized" region of phospholipid surrounding glycophorin in which the more unsaturated phospholipids appear to be located.[145] Diphosphoinositide was also shown to be in close association with glycophorin during isolation from the erythrocyte membrane.[146] In the future, these and more sophisticated techniques will hopefully provide more information about the lipid-binding properties of viral glycoproteins. Such procedures are essential because transmembrane location of a viral glycoprotein, such as that of VS virus, cannot be detected by freeze-fracture electron mircoscopy.[147]

3. Lipid-Binding Properties of Viral M and Core Proteins

Viral matrix (M) proteins and core (C) proteins in viruses lacking M appear to occupy a critical position in terms of the structure and assembly of virions. The M proteins all appear to reside in close juxtaposition to the inner side of the viral membrane of rhabdoviruses,[11] paramyxoviruses,[13] and myxoviruses,[15] as does the C protein lie close to the membrane of togaviruses.[41] The M and C proteins appear to provide the key to virus self-assembly. It is critical to determine whether these inner membrane proteins bind to the respective glycoprotein tails which may span the virion membrane or to specific lipid molecules which are also recognized by the glycoproteins. It is intriguing to note that cytochrome C, a peripheral membrane protein in mitochondria, is capable of selecting certain lipids within a bilayer by producing a lateral phase separation of the lipids.[148] Certainly a lateral phase separation of viral glycoproteins to the exclusion of cellular membrane proteins must occur during virus maturation. However, no evidence for lateral phase separation of the viral lipids is available. Considering the location of these M and C proteins, it would be informative to know the lipid-binding characteristics of the M and C proteins. The M proteins of rhabdoviruses and paramyxoviruses require both relatively high salt and detergent for solubilization,[11,13] suggesting that the detergent may be necessary to bind a lipophilic region of the protein. However, the M protein of VS virus is also soluble in the presence of salt alone,[357] although it does spontaneously associate with purified cytoplasmic membranes.[149] Clearly, more work is needed to establish the lipid-binding properties of this key group of proteins. Further information on the membrane location and properties of M proteins are presented in Section III below.

F. Perturbation of Virion Membranes by Lipid Alteration or Substitution

Virion membranes have two obvious functions: (1) to serve as a permeability barrier to protect the enveloped nucleocapsid and (2) to serve as the anchor for viral glycoproteins which are required for recognition of host-cell surface receptors and, for some viral glycoproteins, to mediate virus-cell membrane fusion. It is possible that virion membranes subserve other functions, but to date none have been definitively identified.

Three general approaches can be employed to determine lipid dependent viral functions: (1) the lipids in virions can be altered by enzymatic or other means, e.g., phospholipase hydrolysis of phospholipids, (2) exchange or removal of lipids from the viral membrane, and (3) the host cell lipids incorporated into virions can be varied by using as hosts dissimilar cell lines varying in lipid metabolism or by loading up the host cell with foreign lipids. Thereafter, the "altered" virions can be tested for a measurable change in physical or biological activities, e.g., membrane fluidity, infectivity, HA, cell adsorption, ability to fuse cells, or hemolyzing activity. A change in these activities must be interpreted with some caution, since a number of secondary or alternative perturbations could occur and produce this same end result. For example, a specific lipid could be directly responsible for the activity being examined or it could have a secondary effect on a protein which is required for expressing the same activity. Conceivably, any lipid alteration could also cause complete disruption of the virion membrane, resulting in the loss of functions unrelated to specific lipids but dependent on an intact membrane. This trivial alternative is potentially the most serious criticism of techniques used ostensibly to specifically alter the lipids of mature virions, although such an eventuality could be circumvented if means could be devised to restore the virions to normal activity.

1. Phospholipid Alteration

Much of the early evidence on the effect of lipid alteration was obtained using phospholipases. These hydrolytic enzymes remove either the polar headgroups of phospholipids (phospholipase C), or fatty acyl chains (phospholipase A_2), which produce dig-

lyceride or lysophospholipids, respectively. Treatment of Newcastle disease virus (NDV) with phospholipase A_2 resulted in the loss of the viral fusion factor, but retention of HA, hemolysin, and neuraminidase activities.[150] It was concluded that the fusion factor activity was dependent on the integrity of the viral membrane. In another study, the infectivity of NDV, as well as influenza virus, was reported to be sensitive to phospholipase C, and the sensitivity was considered to be dependent on the sphingomyelin content of the virion membranes.[151] Unfortunately, it was never shown whether sphingomyelin resulted in resistance to enzyme degradation or simply stabilized the membrane.

Friedman and Pastan[152] found that phospholipase C apparently affected the stability of the Semliki Forest virus membrane, but only during prolonged incubation at 37°C. Initial hydrolysis of ~60% of total virion phospholipids had no effect on infectivity. However, further incubation in the presence of active or inactive enzyme at 37°C resulted in marked loss in infectivity. There was a parallel loss of structural proteins from the Semliki Forest virions during prolonged incubation, suggesting that the phospholipids were not directly involved in the infectivity loss, but were necessary for a stable virus structure. Short incubation (<60 min) of the related Sindbis virus with phospholipase C also produced only a slight decrease in infectivity.[151] Contaminating proteases could, of course, explain some of these effects on infectivity.

Studies on incubation of VS virus with phospholipase C yielded conflicting reports of infectivity loss,[151,153] although neither study recorded the degree of phospholipid hydrolysis. In more recent experiments, treatment of VS virus with phospholipase C confirmed that only a minor reduction in infectivity occurred despite hydrolysis of ~55% of the phospholipids.[82] In contrast, phospholipase A_2 hydrolyzed ~50% of the phospholipid, but resulted in a 2 to 3 log loss in infectivity.[82] The differential effect of these two enzymes on infectivity could be due to the loss of virion membrane integrity after phospholipase A_2 treatment.[358] Phospholipase C did not significantly affect virion membrane integrity.[82] Additional experiments revealed that the fluidity of the viral membrane increased dramatically upon treatment with phospholipase C.[82] Similar fluidity increases due to phospholipase C treatment have also been observed for the erythrocyte membrane[154] and mitochondrial membranes.[155] Other changes in the physical state of membranes following phospholipase C treatment include simultaneous appearance of immobilized regions in the mitochondria[155] and decreased hydrophobicity in membrane vesicles of *Mycobacterium phlei*.[156] In all cases, there was only a minor effect on membrane protein functions following phospholipid hydrolysis. There seemed to be no correlation between the fluidity characteristics of the membranes and maintenance of native protein structure. Possibly, many proteins, including those needed for virus infection, can function independently of the bulk phospholipids, requiring only a lipid substratum with a small percentage of closely associated phospholipids.

2. Cholesterol Alteration

The study of cholesterol in membranes has been hindered considerably by the paucity of reagents that react specifically and selectively with cholesterol. Recently, three methods have been attempted with virion membrane cholesterol with some success. In one, the polyene antibiotic, filipin, which has been shown to interact with sterols in phospholipid-sterol vesicles,[157] was incorporated into the VS virus membrane in equimolar ratio to viral cholesterol.[158,159] As a result, the fluidity of the virion membrane was reduced with a simultaneous 500-fold decrease in viral infectivity.[159] It might be a bit premature to venture direct correlation between these two events; general perturbation of the virion membrane by filipin could be severe. Earlier studies in which VS virions were exposed to the sterol glycoside, digitonin, which also complexes with cholesterol, resulted in disruption of virion membrane and release of G and M proteins.[160]

A second technique applied to the VS virus membrane involved the oxidation of cholesterol to cholest-4-en-3-one by cholesterol oxidase.[82] Prior or simultaneous exposure of intact virions to phosopholipase C was necessary for cholesterol oxidase activity, presumably due to steric hindrance by the phospholipid headgroups. This prior treatment with phospholipase C resulted in an increase in virion membrane fluidity with little loss of infectivity, whereas the cholesterol oxidase caused no further fluidity changes, but resulted in a 4 to 5 log loss of infectivity. This infectivity loss was complicated by the production of H_2O_2 as a by-product of the reaction. Attempts to reduce the effects of H_2O_2 by the addition of catalase or β-mercaptoethanol had little effect, whereas H_2O_2 added externally caused similar infectivity losses, but only at tenfold higher concentration than H_2O_2 generated internally. Although these results indicate that H_2O_2 may not be the cause of the infectivity loss, some confirmatory evidence is still required.

The ability to exchange cholesterol between membranes and interacting lipid vesicles has been exploited in various membrane systems in order to study the effects of altered cholesterol levels. The depletion of cholesterol from the VS virus membrane[83] resulted in an increased fluidity and a simultaneous decline in infectivity.[161] The infectivity loss (10^{-3}) was complicated by lipid vesicles adhering to the glycoprotein spikes. However, adhering vesicles which did not deplete virion cholesterol caused only 90% loss of infectivity.[83,161] Similar fluidity increases with decreasing cholesterol content have been observed in lipid vesicles[162] as well as erythrocytes[162] and human platelets.[163]

3. Growth of Viruses in Cells Containing Altered Lipids

It would be helpful to be able to study the properties of viral membranes with lipids altered by biological means rather than by the chemical, enzymatic, and physical techniques described above. Since viruses derive their lipids from host cells, ways must be sought to change the lipid composition of the host cell without altering its capacity to support viral replication. The two basic approaches to altering host cell lipids, neither yet very successful, are either to use cell lines with intrinsically different lipid compositions or to load up a single cell line with different, even unusual, lipids incorporated from supplemented media during growth of the cell before and during viral infection. The difficulty with the first approach is the need to identify cell lines with sufficiently different lipid composition, but retaining the capacity to support virus growth to the same extent. Moreover, interpretation may be complicated by multiple alterations occurring in lipid composition. Luukkonen et al.[20,164] have described a system of growing Semliki Forest virus in BHK cells and mosquito cells, which could provide useful methods for studying lipid composition and virion membrane properties. The phospholipid and cholesterol content of the virions produced by the two cell lines were quite different, which should permit a comparison of their infectivity.

The second approach, selectively altering the lipids of a host cell by supplementing the growth medium, can potentially alter the content of a single lipid species in order to observe the effect on the virion membrane. An early study by Blough et al.[165] reported that addition of vitamin A to embryonated eggs resulted in altered phospholipids incorporated into influenza virions propagated in these cells. These changes were interpreted as a possible mechanism for promoting the formation of filamentous myxoviruses. However, the putative filamentous viruses appear to be pleomorphic and vitamin A has detergent activity. More recently, Steinhart et al.[166] found that the production of herpes simplex virions could be reduced by the addition to the cell growth medium of clofibrate, an inhibitor of host cell lipid metabolism. Apparently, active production of phospholipids is required for production of these virions.

A unique feature of sterol metabolism in L cells was exploited by Bates and Rothblat[167] to study the sterol requirement of VS virus. In the absence of exogenously added

cholesterol, L cells synthesize desmosterol, the immediate precursor of cholesterol containing an extra double bond, whereas in the presence of exogenous cholesterol, >90% of the cell sterol is cholesterol. When VS virus was grown on L cells containing both cholesterol and desmosterol, it incorporated a higher percentage of cholesterol than was present in the whole cells. Virions containing desmosterol were not altered in their growth, stability, or infectivity characteristics.

L cells[168] and LM cells[114] provide an opportunity to alter the phospholipid fatty acyl chain and headgroup composition, respectively, by their incorporation of exogenously added lipid metabolities into cellular lipids. In addition, cell mutants altered in sterol metabolism have recently been isolated, which require exogenously added sterol.[169] If these cells lines will support the growth of viruses, then systematic variations in cellular lipids may well produce novel plasma membrane lipids for selection by budding virions. A study of the stability, infectivity, and fluidity characteristics of these altered virions should provide some insight into the lipid requirements of virion membranes.

III. VIRAL MEMBRANE PROTEINS

The proteins of enveloped viruses have been studied intensively in recent years along with the parallel increase in the study of cell membrane proteins. Those viruses which contain a unit membrane structure have the distinct advantage over cell membrane fragments of yielding very homogeneous preparations without employing herculean efforts. Of particular interest to the virologist, as well as to the biochemist, is the nature of the complement of viral proteins found within the virion envelope.

Table 1 lists the major virus groups which have been used to ascertain the structure and function of virion membrane proteins. Certain of these virus groups have been studied more extensively than others because of the ease of propagation, ease of purification, and relative simplicity of the virion constituents. As a result of these considerations, much of the following discussion will deal mainly with viruses of the myxovirus, paramyxovirus, togavirus, rhabdovirus, and oncornovirus groups.

A. Location of Proteins in the Virion Membrane

A general feature of the proteins of viral envelopes is that those proteins on the exterior are glycosylated and those on the interior are not glycosylated. Table 1 lists the glycosylated and nonglycosylated constituents of several virus groups. A common characteristic of enveloped viruses is that at least one glycoprotein exists on the surface of the virion. This consistent finding could be due to a critical role for virion surface glycoproteins in cell recognition. However, it might also be due to a common cellular mechanism of glycosylation for inserting proteins through the outer layer of membranes in a functional orientation to the external environment. If this possibly were correct, then the carbohydrate portion of these proteins might not be a key element in the function of these proteins. The role of the carbohydrate side chains will be discussed later.

1. Surface Labeling of Glycoproteins

The exterior location of the glycoproteins has been established, using a variety of techniques. A common technique used to study the exterior proteins of a number of enveloped viruses is the in vitro labeling of surface proteins with radioactive compounds. In several studies, lactoperoxidase and ^{125}I were used to label the proteins of VS virus. However, significantly different results were obtained, suggesting that this technique cannot be used without proper precautions. In the initial study, Walter and Mudd[170] concluded that four of the five VS viral proteins occupied sites on the exterior of the virion. These data were not entirely confirmed by Moore et al.,[171] who found

that lactoperoxidase catalyzed and chloramine T iodination labeled predominantly the glycoprotein (G), and to a much lesser extent the membrane protein (M). These data were interpreted as indicating that the G protein was on the surface of the virion, and that either a small amount of M protein or a small segment of M protein might be accessible to external labels. The external location of G was confirmed by Eger et al.,[172] employing pyridoxal phosphate-$NaB(^3H)_4$, but these workers concluded that M protein was not located at an external site, despite a certain degree of labeling. Finally, McSharry[173] demonstrated that the lactoperoxidase technique can be used to specifically label the surface proteins of VS virus. In this study, only the G protein was labeled with ^{125}I, but only if EDTA were excluded from the virus preparation. The inclusion of EDTA resulted in significant labeling of the M protein. These studies clearly indicate that care in the preparation of the virus samples must be exercised or internal proteins may be labeled and thus erroneously identified as being on the exterior of the virus particles. It seems clear that all the tyrosine residues of VS viral G protein are exterior to membrane permeability barriers, whereas the M-protein tyrosines are internal. No tyrosines are present in the membrane-associated fragment of the VS viral glycoprotein.[129]

Another difficulty with enzyme-mediated labeling of surface proteins is the inaccessibility of certain surface proteins due to conformational restrictions. Sefton et al.[174] showed that the two glycoproteins of Sindbis virus were iodinated to different extents, even though both proteins are displayed on the surface of the virion. In this instance, the E_2 protein of Sindbis was labeled four times more heavily than E_1. This inaccessibility of E_1 to enzymatic activity was also demonstrated by the relative resistance of the carbohydrate side chain to cleavage by endoglycosidases.[175] An analogous situation exists in Semliki Forest virus where E_1 protein is not accessible to galactose oxidase following treatment with neuraminidase[176] and in influenza virus where HA_1 glycoprotein is not labeled as extensively as HA_2 by lactoperoxidase iodination,[177] which could be due to differences in tyrosine residues.

Similar results have been obtained in the radiolabeling of oncovirus surface proteins. Lactoperoxidase iodination labeled gp69/71 of the murine leukemia viruses, but no label was detected in the p15(E) proteins.[178] That the p15(E) protein is a surface protein has been demonstrated by the ability of specific antisera to recognize p15(E) by precipitating whole virus.[179] The glycoproteins of mouse mammary tumor virus also exhibit a differential labeling pattern. Lactoperoxidase iodination labels gp64 and gp52, but gp36/38 can be labeled only after detergent disruption.[180] Identical results were obtained using a sialyl transferase to attach sialic acid to carbohydrate side chains.[181] These results indicate that several procedures may be necessary to correctly identify all surface proteins.

2. Susceptibility to Proteases

Another technique used to identify surface proteins is exposure of virions to proteolytic enzymes. The basis for this test is the assumption that proteolytic enzymes cannot penetrate the viral membrane. Treatment of VS virus with trypsin,[128,153] pronase,[182] thermolysin, chymotrypsin, or bromelain[129,140] resulted in the removal of G protein without affecting any of the other VS viral proteins. These data agree with the surface labeling techniques that demonstrate the G protein on the exterior of the virus particle. However, protease studies can also be difficult to interpret due to structural peculiarities. Bussereau et al.[183] demonstrated that within the Indiana serotype of VS virus strikingly different results were obtained following protease treatment. The Brazil and Argentina strains of VS virus were completely resistant to trypsin and chymotrypsin treatment, as measured by infectivity. Protein analysis revealed that protease treatment removed two thirds of the surface glycoprotein. The surface projections which re-

mained had a slightly different amino acid composition. In light of the coding capacity of the VS viral genome, it is unlikely that this resistant fraction of G protein is a different polypeptide from the sensitive fraction. These data suggest that the G protein of the Brazil and Argentina strains of VS virus may exist in two different conformations. As with surface labeling, data from protease studies may give additional data on the surface structure of enveloped viruses.

The surface glycoproteins of influenza virus are also susceptible to proteolytic attack. Bromelain treatment resulted in the removal of HA, HA_1, and NA glycoproteins, but HA_2 was resistant.[184] Treatment with chymotrypsin removed all four glycoproteins, but did not affect the nonglycosylated proteins.[185] Protease-treated virions lack infectivity, neuraminidase, and hemagglutinating activity. Pronase treatment of the paramyxovirus SV5 also resulted in a loss of surface glycoproteins.[186] Protease treatment has also been used to identify surface glycoproteins on togaviruses,[187,188] arenavirus,[18] oncornovirus,[189] coronavirus,[25] and bunyavirus.[190]

3. The Nonglycosylated Membrane Proteins

The location and characteristics of the nonglycosylated proteins purported to be structural elements of viral membranes are much less certain than the glycoproteins. As indicated in Table 1, not all membrane-enclosed viruses contain an identifiable membrane (matrix, M) protein. Certain of the data concerning the M protein has been alluded to in the discussion of the glycoproteins. That the M proteins occupy an internal position was demonstrated by their resistance to proteolytic digestion and their inability to be labeled with surface-labeling techniques.[172,173] These data do not place the M proteins in the membrane. However, studies on the morphology of influenza virus demonstrated the existence of an electron-dense layer on the inner surface of the viral envelope.[191-194] That this electron-dense layer was the M protein was suggested by the following: (1) lipid extraction of particles which had been treated with proteolytic enzymes and then fixed with glutaraldehyde revealed the existence of a layer which still surrounded the ribonucleoprotein,[195] and (2) calculations of the amount of M protein per virion indicated that it is the only protein within the virion which could form such a structure.[195]

The location of the membrane protein on the inner surface of the virion envelope has also been suggested by studies on the differential solubility of viral proteins with nonionic detergents. The G protein of VS virus can be selectively removed from the viral membrane using Triton® X-100,[196] Triton® N-101,[197] or Nonidet® P_{40}.[198] If the virions devoid of G protein are subsequently treated with Triton® X-100 in 0.43 *M* NaCl, the M protein is solubilized, leaving the nucleocapsid intact.[199] These data suggest that M protein lies between the G protein and the nucleocapsid. However, unlike influenza, no electron-dense region has been observed on the inner surface of the VS virion membrane. Detergent treatment of two salmonid rhabdoviruses demonstrated that they, unlike VS virus but like rabies virus, contain two M proteins.[11,200] However, one of these so-called M proteins is likely to resemble the NS protein of the VS virus nucleocapsid.[11]

Similar experiments with paramyxoviruses have also shown differential solubilities of the glycoproteins, membrane (M) proteins, and nucleocapsids.[201-204] Treatment of Sendai virus with Emasol® 1130 or Tween® 20 buffered at pH 10.0 resulted in the preferential solubilization of the envelope glycoproteins.[201,202] Similar results can be obtained by using Triton® X-100 in low salt buffers as was demonstrated with VSV.[202,204] Nucleocapsids isolated from virions treated with alkali-Tween 20 can be separated on CsCl gradients into fractions of different densities, one which contains M protein and one which does not.[202,205] Nucleocapsids containing M protein had a greater width than ones without M protein.[205] Also, nucleocapsids containing M pro-

teins appeared folded or as having a sheath-like covering around the nucleocapsid structure.[202,205] The ability of M protein to form a sheath-like structure is not dependent on the presence of nucleocapsid, as was demonstrated by Hewitt and Nermut,[204] using purified M protein. Again, these studies suggest that the M protein lies between the exterior glycoproteins and the interior nucleocapsid.

4. Minor Protein Components of Virion Membranes

Although enveloped viruses make use of host lipids in the construction of the viral membrane, there is no evidence to suggest that host proteins play any role in the structure of viral membranes. Analysis of purified virus by polyacrylamide gel electrophoresis does not reveal a significant presence of host protein in the virus. However, minor amounts of host protein are most certainly present. One such protein which has been detected is actin. Careful analysis of purified Sendai and measles viruses reveals the presence of a 42,000-dalton protein, which has been identified as actin.[206-208] The significance of the presence of actin has yet to be determined.

The phenomenon of phenotypic mixing, i.e., the assembly of a virus particle with the genome of one virus and the envelope proteins of an often unrelated virus, is well known.[209] The existence of this phenomenon suggests that virus assembly is not as precise as once thought. Therefore, it is not surprising to find evidence for the presence of host cell glycoproteins in enveloped viruses. Hecht and Summers[210,211] demonstrated the existence of H-2 histocompatibility antigens of mouse cells in highly purified preparations of VS virions. These antigens were detected by the inhibition of immune cytolysis and not by standard analytical procedures. The loss of H-2 activity from infected cells was reflected in the appearance of H-2 activity in mature virus particles.[210,211] Various purification procedures could not remove the H-2 antigens, suggesting that the H-2 antigens were an integral part of the viral membrane or were bound extremely tightly. A similar analysis of Friend leukemia virus particles also demonstrated the existence of H-2 antigens.[212] However, unlike VS virus, these seemed to be a selective incorporation of a single H-2 specificity.

Another indication of the presence of host proteins in viral particles is the detection of enzyme activity. A commonly detected enzyme is protein kinase which was first described by Strand and August[213] in Rauscher murine leukemia virus, avian myeloblastosis virus, and VS virus. Initial attempts to separate the enzyme from the virion suggested that it was an integral part of the virion. This association of the enzyme with the virion was confirmed by Imblum and Wagner,[214] who demonstrated that the enzyme was solubilized by detergents under conditions which liberated the G and M proteins. A cellular origin for the protein kinase was suggested by different kinetics for the enzyme in virions grown in different host cells.[214] A similar conclusion concerning the host cell origin of virion bound protein kinase was reached by Moyer and Summers.[215] Mizutani and Temin[216] described the existence of numerous enzyme activities in purified virions of Rous sarcoma virus. Such reports make it very unlikely that these activities are coded by the viral genome. As more sensitive assay procedures are developed, it is very likely that other host-derived proteins are scavanged by viruses and will be found within viral particles or associated with cell-derived virions membranes.

The apparent existence of variable quantities of minor proteins in virions may not reflect the existence of host proteins. Conflicting results over the number of virion proteins has resulted from artifacts in the analysis of the viral polypeptides. A striking example of this problem was reported by Sturman[217] in the analysis of the proteins of the murine coronavirus A59. If the standard procedures for preparing samples for polyacrylamide gel electrophoresis (heat in the presence of reducing agents and detergents) were employed, at least six protein bands were observed. In the absence of heat

and reducing agents, only four bands were detected. Subsequent analysis revealed that the smallest glycoprotein (gp23) forms oligomeric structures under the influence of heat and reducing agents, resulting in the appearance of variable amounts of 38,000- and 60,000-dalton components. Additional evidence suggested that these higher order complexes were generated by the interaction of a fragment of gp23 which lies within the lipid membrane. Sturman[217] hypothesizes that these aggregates result from interaction of hydrophobic domains on the polypeptide. Similar interactions have been detected with the erythrocyte membrane sialoglycoprotein glycophorin A.[218] These data clearly indicate that the analysis of the proteins in enveloped viruses must be approached with caution.

B. Virion Glycoproteins

1. General Principles and Synthesis

A vast literature has been accumulating on the structure and biosynthesis of membrane glycoproteins, including those of viruses. A complete analysis of this literature is beyond the scope of this review, and the reader is referred to the reviews of Lenard,[5] Rott and Klenk,[6] and Capaldi.[219] Analysis has begun on the primary structure of certain readily available virion glycoproteins. A partial amino acid sequence of the amino-terminus of an influenza virus hemagglutinin has been accomplished by Skehel and Waterfield,[220] as has the fusion (F) protein of Sendai virus.[221] The best available evidence would indicate that the carboxyl terminal ends of virion glycoproteins are inserted into and probably through the virion membrane of influenza,[220] Sendai,[221] and vesicular stomatitis virus.[222]

The best studies on synthesis and membrane insertion of viral glycoprotein have probably been done with cells infected with VS virus. Early studies[223,224] revealed that the VS viral G protein is rapidly inserted into a smooth membrane fraction, probably cytoplasmic surface membrane. Kinetic studies revealed association of VS viral G protein with a plasma membrane fraction within minutes of a pulse label.[225] These findings have been confirmed and extended by the same and other investigators, using increasingly refined techniques.[226-231] These studies indicate that the VS viral glycoprotein is synthesized on polyribosomes initially bound to endoplasmic reticulum, probably sequestered in inside-out vesicles, whence the complex migrates to the plasma membrane where it is inserted.[232] Concurrent synthesis of the M protein takes place, but M protein becomes located on the inner surface of the plasma membrane.[232] Confirmation of this mechanism of maturation of the VS viral G protein comes from studies of temperature-sensitive mutants in complementation group V, which are restricted in insertion and transmembrane migration of the glycoprotein at nonpermissive temperatures.[233,234] The role of glycosylation in maturation and membrane insertion was also demonstrated by Morrison et al.[235] who showed that when glycosylation was inhibited by the antibiotic tunicamycin, migration to smooth intracellular membrane was inhibited, but there was no effect on attachment of the carbohydrate-free glycoprotein to intracellular membrane (endoplasmic reticulum).

The biosynthesis of VS viral glycoprotein and its insertion into membranes have been confirmed by the elegant studies of Rothman and associates[222,236] and Toneguzzo and Ghosh.[237,238] Their experiments were based on the "signal" hypothesis of Blobel,[239] by which the amino-terminal end of the nascent polypeptide chain attaches the messenger and polyribosomes to endoplasmic reticulum. Translation of the glycoprotein mRNA in a cell-free system in the presence of pancreatic membrane vesicles results in the insertion of the amino-terminus into the interior of the vesicle; the carboxyl terminal 5% of the polypeptide remains anchored in the membrane, and an oligopeptide of perhaps 30 amino acids is extruded from the cytoplasmic surface of the vesicle. Glycosylation of the G proteins remains confined to the growing amino terminal segment within the lumen of endoplasmic reticulum vesicle.

The final step of insertion into the plasma membrane occurs only in vivo by fusion of the cytoplasmic vesicles at the inner surface of the plasma membrane. Thus, the amino terminus of the glycoprotein is exteriorized. The addition of terminal sialic acid to the carbohydrate chains occurs by the action of sialyltransferase at the plasma membrane. The maturing virion buds from this region of the converted plasma membrane. The result is a virion membrane with an amphipathic glycoprotein, the external 95% or so of which is glycosylated and hydrophilic, and the remaining hydrophobic 5% or so is anchored in and probably traverses the virion membrane and protrudes from the inner surface. Some additional details are recorded elsewhere.[240,241]

2. Carbohydrate Composition of Virion Membrane Glycoproteins

The limited coding capacity of the genome of the less complex viruses makes it obvious that most viruses must depend on many host cell functions to complete their replication cycle. One area where this is especially true is in the synthesis of the carbohydrate moieties of the glycoproteins. Numerous enzyme activities would need to be involved in this biosynthetic pathway. Clearly, viruses such as VSV and Sindbis do not contain the genetic information to code for the enzymes necessary to glycosylate their envelope proteins. If the host enzymes performed these functions for the virus, then one might assume that significant host-dependent differences would exist in viruses grown in various cells. Alternatively, one might expect similarity of glycosylation patterns of different viruses within the same host. It would appear that neither of these hypotheses is wholly tenable.

Sindbis virus contains two envelope glycoproteins designated E_1 and E_2.[242] Both E_1 and E_2, when isolated from virus grown in chick embryo fibroblasts, contain two different oligosaccharide structures.[243] One oligosaccharide contains the monosaccharides common to many glycoproteins, namely, glucosamine, mannose, fucose, galactose, and sialic acid. The other oligosaccharide is less complex, containing only glucosamine and mannose. Initial studies on the effect of the host cell on the oligosaccharide structure of Sindbis virus glycoproteins suggested that host cell differences did exist. Analysis of the glycopeptides of Sindbis virus grown in BHK-21 cells revealed the presence of more sialic acid and less mannose than was found in the virus grown in chick embryo fibroblasts.[244,245] A comparison of protease-generated glycopeptides from virus grown in BHK and CEF cells demonstrated a greater quantity of higher molecular weight glycopeptides in BHK-derived virus. This was shown to be due to an increase in sialic acid content of the oligosaccharides from virus grown in BHK cells, as neuraminidase treatment of the glycopeptides eliminated these differences.[246] Analysis of purified E_1 and E_2 Sindbis glycoproteins showed that the cause of the lower quantity of mannose in virus propagated in BHK cells was due to the absence of the mannose-rich core oligosaccharide in the E_1 protein.[247] The significance of this host modification on the biological properties of the virus is unknown.

Studies on the properties of cells transformed by oncogenic viruses have demonstrated differences in cell surface glycopeptides in contrast to nontransformed cells. A comparison of the glycopeptides of Sindbis virus grown in chick cells transformed by Rous sarcoma virus or in BHK-21 cells transformed by polyoma virus demonstrated little difference in glycopeptide patterns as compared to the respective nontransformed cells.[246] The differences which were noted could be explained by variations in the sialic acid content of the glycopeptides. Similar differences in the extent of sialylation were demonstrated with Sindbis virus grown in the same cell but at different temperatures.[246] These data suggest that the basic oligosaccharide structure of Sindbis virus glycoproteins grown in various cell lines is very similar. Where differences were seen, these were due to variations in the extent of completion of the oligosaccharide chains since sialic acid is normally a terminal residue.

Similar analyses of the oligosaccharides of VS virus do show host dependent differences in glycosylation patterns.[244,248-250] The VS virion membrane contains a single glycoprotein (G protein) of 65 to 69,000 daltons of which carbohydrate comprises approximately 10% by weight. The G protein of VS virus contains two major oligosaccharide side chains of similar composition,[248,251,252] but the possibility of additional glycosylation sites has been suggested.[250-253] Moreover, rabies virus, an unrelated rhabdovirus, appears to contain at least three different oligosaccharide chains.[254] The linkage of the oligosaccharides to the polypeptide of VS viral G protein was shown to be through an asparagine-*N*-acetylglucosamine linkage.[250] The terminal sugar residue of the oligosaccharide is *N*-acetylneuraminic acid.[252] As with Sindbis virus, the degree of sialylation produces heterogeneity in the oligosaccharides from VS virus.[248,249,251,255] Another source of heterogenicity is the content of fucose. The *N*-acetyl glucosamine residue linked to the polypeptide is substituted with fucose,[249] but there is evidence for only one fucose residue per two oligosaccharide side chains.[248,251] Further evidence for heterogeneity within the oligosaccharide structures was obtained using glycolytic enzymes. Moyer and Summers[249] demonstrated that glycopeptides obtained from VS virus grown in BHK-21 cells transformed by polyoma virus contained glycopeptides resistant to endo-β-*N*-acetylglucosaminidase, whereas no such glycopeptides were demonstrated in nontransformed BHK-21 cells. Similar glycosidase-resistant glycopeptides were detected in VS virus grown in nontransformed HeLa cells.[250] The cause of this differential susceptibility to enzymatic degradation has not been elucidated. Although heterogeneity does exist in the oligosaccharides of VS virus grown in different hosts, the evidence to date points to a high degree of similarity within the core structure of the major oligosaccharides.[252]

The evidence just discussed suggests that although the virus may not code for the glycosylating enzymes, it does have some role in the final glycosylation process since the structure of the oligosaccharides are similar in dissimilar host cells. That virus does play a role in this process was clearly demonstrated by Sefton.[255] In this study, Sefton analyzed the oligosaccharides of the membrane glycoproteins of Sindbis, VS, and Rous sarcoma viruses grown in primary chicken embryo cells. The data clearly demonstrate that each virus acquired a different set of oligosaccharide side chains. The differences observed were not due just to heterogeneity or to the degree of sialylation. The glycopeptides acquired by Rous sarcoma virus were larger than those of Sindbis and VS virus. Neither VS nor Rous sarcoma virus contained the same mannose-rich glycopeptides of Sindbis virus. These data demonstrate that either the amino acid sequence of the glycoprotein influences the glycosylation pattern or the glycoproteins of each virus are synthesized at different sites within the cell which contain a different set of glycosylating enzymes.

A similar conclusion was reached from a comparison of the glycopeptides derived from Sindbis virus with the glycopeptides from the uninfected host cell.[256] These data showed that the Sindbis glycopeptides were similar to a subset of host glycopeptides whether the virus was grown in chick embryo fibroblasts or BHK-21 cells. This work also supports the hypothesis that the virus uses host cell enzyme systems for glycosylation, but the amino acid sequence of the viral proteins directs the addition of specific, presumably terminal, oligosaccharides.

Preliminary evidence for the existence of major host-dependent difference has also been obtained with influenza virus.[257] The analysis of the glycopeptides of HA_1, HA_2, and NA of fowl plague virus demonstrated at least three types of glycopeptides. HA_1 contained only Type I, whereas HA_2 and NA contained all three types. One of the oligosaccharides may be similar to the mannose-rich structure in Sindbis virus. Type I oligosaccharides from influenza virus grown in MDBK cells were considerably larger than Type I oligosaccharides from chick embryo cells (3400 vs. 2600 daltons). In this

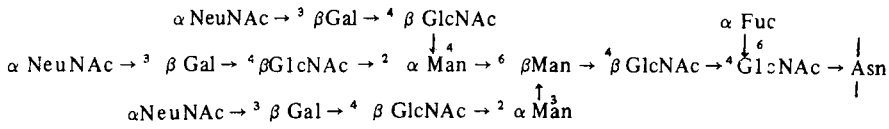
case, the content of sialic acid is not responsible for these differences, since influenza virus contains a neuraminidase which removes all sialic acid residues from viral glycoproteins. However, other terminal additions could be a cause of these differences.

A similar analysis of the glycopeptides of influenza strain WSN revealed significant differences between the glycosylation patterns of fowl plague and WSN influenza viruses grown in the same MDBK host cells.[258] Classes of only two sizes of glycopeptides were detected, although heterogeneity within the classes was evident. Unlike fowl plague, WSN HA_1 contained both size classes, while HA_2 contained only one. Virus grown in different cell lines contained similar sets of glycopeptides, but size differences were noted. A mannose-rich glycopeptide was also detected in WSN virus, similar to that found in Sindbis and fowl plague viruses. These data again suggest that the amino acid sequences of the viral proteins direct the glycosylation sites to some extent.

Other modifications of the glycoprotein of influenza virus have also been noted. Compans and Pinter[259] detected the incorporation of sulfate into the HA glycoprotein. Subsequent analysis indicated that all of the sulfate in the HA protein was associated with the glycopeptides isolated after pronase digestion, and most of the sulfate was found in the larger glycopeptide.[260] The existence of sulfate in the glycoproteins of enveloped virus is rather ubiquitous. It has been demonstrated in SV5, Sendai, Sindbis, Rauscher leukemia virus, VS,[259] and herpesvirus viruses.[261] In the case of VS virus, the sulfation was shown to be host cell dependent. The G protein of VS virus was labeled in MDBK cells, but not in BHK-21F cells.[259] The role of sulfate in enveloped viruses is unknown.

Despite these apparent differences in the glycopeptides of the various virion glycoproteins, there is definitive evidence for a common inner core structure for the proximal oligosaccharide chain bound to glycoproteins by asparagine linkages.[262] Although the side chains vary, the asparagine-linked core oligosaccharides are preassembled in an activated state bound to the isoprenoid lipid dolichol pyrophosphate.[263] Conclusive experiments by Robbins et al.[264] revealed that this common lipid-linked, mannose-rich oligosaccharide is transferred from the lipid carrier to the glycoproteins of chick embryo cells infected with Sindbis virus and vesicular stomatitis virus. Therefore, it seems likely that most, if not all, viral glycoproteins, like other mammalian membrane glycoproteins, contain a common oligosaccharide core linked to asparagine.

The decisive analysis of the carbohydrate structure of vesicular stomatitis virus glycoprotein was carried out by Reading et al.[265] By means of chemical and enzymatic procedures for controlled degradation, the polysaccharide chain of VS virus was sequenced and the sugar and peptide linkages determined. The glycoprotein of VS virus grown in BHK-21 cells has two identical carbohydrate chains with the following structure:



3. Search for Biological Functions of the Carbohydrate Components of Virion Glycoproteins

No biological role has yet been assigned to the completed polysaccharide chains of virion glycoproteins. Two approaches to this problem have been tried. One is to study the effect of removing some of the carbohydrate from the glycoprotein already inserted in the viral envelope. The other approach is to inhibit glycosylation of the nascent polypeptide. This latter approach was used to study the carbohydrate function of influenza viral glycoproteins by testing two glycosylation inhibitors, D-glucosamine and 2-deoxy-D-glucose, to prevent the synthesis of detectable HA protein.[266,267] Instead, a

precursor of HA was formed which lacked the normal carbohydrate constituents. Interestingly, these unfinished proteins were incorporated into viral particles, indicating that some surface proteins can be inserted in the membrane in the absence of glycosylation. These particles demonstrated reduced hemagglutinating activity and infectivity. These results do not indicate whether the carbohydrate plays a primary or secondary role, i.e., whether the hemagglutinin spike requires carbohydrate at the cell-binding site, or whether the HA protein requires the carbohydrate to enable the three HA proteins to associate in the proper configuration.

Tunicamycin, an antibiotic which inhibits the formation of *N*-acetylglucosamine-lipid intermediates,[268,269] has been used to prevent glycosylation of VS virus and Sindbis virus glycoproteins. At 0.5 μg of tunicamycin, viral yields were inhibited by more than 99%.[270] An unglycosylated form of the precursor protein, PE2 of Sindbis virus, was found within infected cells, but processing of this precursor was not detected. Within VS virus-infected cells, an unglycosylated form of the G protein was detected, but no evidence was found for transfer of this protein to the cell surface. These data suggested that glycosylation was essential for processing and/or transport of the viral glycoproteins to the cell surface. However, in a subsequent study, Gibson et al.[271a] did detect a low level of infectious VS virus released from tunicamycin-treated cells. These particles had a specific infectivity similar to that of VS virus grown in the absence of tunicamycin. Moreover, analysis of the proteins in these released virions revealed the presence of the unglycosylated form of the VS viral glycoprotein. These data suggest that glycosylation is not a requirement for transport and insertion of the viral glycoprotein into the cell membrane, although the process may be inefficient in the absence of glycosylation. Similar evidence that glycosylation is not essential for membrane insertion of glycoproteins has been obtained with influenza[271b] and Semliki Forest[271c] viruses. More importantly, these data imply that the carbohydrate portion of the glycoprotein is not necessary for the expression of biological activity of the virus particle.

The role of sialic acid on the glycoproteins of enveloped viruses has been extensively examined. Schloemer and Wagner[272] reported a loss of infectivity of VS virus following removal of sialic acid. Resialylation resulted in recovery of infectivity. Similar data with measles virus as well as VS virus have been reported by Dore-Duffy and Howe.[273] However, Cartwright and Brown[274] found that, whereas removal of sialic acid abolished hemagglutinating activity, neuraminidase did not reduce infectivity. It seems possible that these discrepant infectivities were due to varying degrees of aggregation due to altered surface charge of desialylated VS virus. McSharry et al.[275] demonstrated that VS virus grown in different host cells exhibited different titers of hemagglutinin but similar levels of infectivity. These differences appear to be a result of the carbohydrate composition of the glycoproteins, since a significant variation was demonstrated in sialic acid content. It was also shown that VS virus grown in lectin-resistant cell lines did not exhibit a reduction in specific infectivity even though the glycoprotein lacked sialic acid.[276]

Removal of neuraminic acid from the envelope of Visna virus had no affect on infectivity, cell fusion, or viral adsorption.[277] In like manner, Sindbis virus lacking neuraminic acid possessed similar particle to plaque-forming unit and particle to HA ratios, as did virus-containing neuraminic acid.[278] In addition, the viral glycoproteins lacking neuraminic acid retained the antigenic properties of neuraminic acid-containing virus. Thus, it would appear that neuraminic acid, which is the most highly variable sugar residue on glycoproteins, exerts a minimal effect on the function of envelope glycoproteins. Moreover, studies with influenza virus exposed to pneumococcal endoglycosidase, which removed approximately 50% of the HA carbohydrate, had no effect on hemagglutinating activity or infectivity.[279]

The role of carbohydrate in the antigenic functions of the glycoproteins from Friend

murine leukemia virus was studied, employing glycosidase enzymes to remove the carbohydrate residues.[280,281] Treatment of gp71 with glycosidase enzymes reduced the relative molecular weight by 5 to 10,000 daltons and removed most of the [^{3}H] glucosamine label in the polypeptide. Removal of the carbohydrate did not alter significantly the type, group, or interspecies determinants on gp71.[280] In a follow-up study,[281] it was demonstrated that removal of carbohydrate from gp71 did not abolish its ability to interfere with the infection of mouse cells by Friend leukemia virus or AKR murine leukemia virus. In addition, glycosidase treatment of intact virus was not found to have any affect on viral infectivity. However, in a study on the ability of glycoprotein gp85 from avian myeloblastosis virus to act as an antigen, it was demonstrated that removal of the carbohydrate residues by glycosidases reduced the ability of the treated glycoprotein to compete with untreated pg85 antigen. A similar treatment of gp85 from B_{77} avian sarcoma virus left antigenic activity intact.[282] These results suggest that the carbohydrate residues may have a minor role in determining biological activity once the glycoprotein is synthesized and inserted in the virion membrane. Further studies on other virus systems are clearly needed.

4. Processing of Virion Glycoproteins

The posttranslation processing of polypeptides is a common event in the synthesis of many viral proteins. The role of these processing events is clearly demonstrated in myxoviruses and paramyxovirus groups. The largest glycoprotein of influenza, the HA protein, is synthesized as a primary gene product and then is cleaved into two polypeptides designated HA_1 and HA_2.[283-286] These two polypeptides remain associated in the viral envelope by means of disulfide bonds.[287,288]

Studies on the primary structure of the HA polypeptide showed that the HA_1 polypeptide is the amino terminal end of the HA, while HA_2 comprises the carboxyl terminal end.[220] Treatment of the HA polypeptide with bromelain resulted in an HA_2 polypeptide approximately 30 amino acids shorter than uncleaved HA_2. These results confirmed the role of HA_2 in holding the HA structure in the lipid membrane. The uncleaved HA protein has full hemagglutinating activity, but viral infectivity is enhanced with the production of HA_1 and HA_2.[289,290]

An analogous situation exists with the paramyxoviruses. Both the F protein and the HN protein require proteolytic cleavages to manifest full activity. Homma[291] observed that trypsin treatment of noninfectious Sendai virus enhanced infectivity. Subsequently, it was demonstrated that the proteolytic cleavage of the F protein was necessary for infectivity, cell fusion, and hemolytic activities.[292-294] The active F protein of Sendai virus consists of two polypeptides held together by disulfide bonds.[295] The smaller cleavage fragment (F_2) is derived from the amino terminal of the F_0 protein. This would suggest that the F_1 fragment is associated with the membrane while the carbohydrate-rich F_2 faces the exterior.

The proteolytic processing of the HN protein of NDV was detected, studying the avirulent strains Ulster and Queensland.[296] Trypsin treatment of virions containing the HN_0 protein enhanced neuraminidase and hemagglutinating activity. However, the effects were not large, leaving some doubt as to the significance of the observation. This doubt was removed with the purification of the HN_0 protein and demonstration that the uncleaved protein lacked neuraminidase and hemagglutinating activities.[297] Cleavage of the HN_0 with a variety of proteases resulted in a large increase in these activities. These results show that both of the envelope glycoproteins of the paramyxoviruses must be processed by proteolytic enzymes before their functions are expressed. Processing of envelope glycoproteins has also been detected in togaviruses,[298,299] coronaviruses,[217] and oncornaviruses.[179,300]

5. Envelope Protein Interactions and Spatial Relationships

It is becoming evident that in many instances individual envelope proteins do not exist in a monomeric form. Homo-oligomers and hetero-oligomers have been detected in numerous virus systems. That these types of structures existed has been shown by studies on the spike glycoproteins of influenza virus. The isolated hemagglutinin of influenza was shown by electron microscopy to consist of a structure larger than the 75 to 80,000-dalton polypeptide of the HA protein.[301] Sedimentation analysis of the hemagglutinin resulted in an estimate of 215,000 daltons for the morphological subunit,[302] and electron microscopy showed a triangular shape for this structure.[303] Cross-linking of the proteins of influenza resulted in the production of trimers as the predominant oligomer of the HA protein.[304] These data indicate the existence of a trimeric structure for the hemagglutinin, but do not elucidate the nature of the bonds holding the HA polypeptides together. By similar analysis, the neuraminidase of influenza was shown to consist of four NA polypeptides, two pairs of dimers held together by disulfide bonds.[305-307]

The HN glycoprotein of Sendai, SV5, and NDV is also an oligomeric structure. Sedimentation analysis of isolated HN protein in detergent containing sucrose gradients suggested that this subunit consisted of more than one polypeptide.[201,308,309]

Sendai virus proteins analyzed under nonreducing conditions on polyacrylamide gels showed a reduction in the HN band and the appearance of higher molecular weight species.[310] These high molecular weight species contained hemagglutinating and neuraminidase activities. Mild reduction with glutathione abolished these activities and produced the monomeric HN polypeptide. These data strongly suggest that the active hemagglutinin and neuraminidase is an oligomeric structure held together with disulfide bonds. Similar observations on the electrophoretic mobility of the nonreduced structural proteins of Newcastle disease,[311] Sendai,[312] and measles[313] viruses confirm the role of disulfide bonds in maintaining these oligomeric structures.

Data derived from studies on Sindbis virus and Semliki Forest virus also indicate that the surface glycoproteins exist in an oligomeric form. Solubilization of Semliki Forest virus with Triton® X-100 resulted in the release of the intact nucleocapsid and a 4S complex.[314] The 4S material contained 10^5 daltons of protein, an amount consistent with the complex being composed of a dimer of E_1 and E_2. Garoff,[315] employing a protein cross-linking agent, also found complexes consistent with a dimeric structure. Cross-linking of E_1 and E_2 could also be achieved in preparations solubilized with Triton® X-100. This could occur only if the E_1 and E_2 proteins existed as a complex in the detergent solution. However, data could not determine whether homodimers (E_1 to E_1) or heterodimers (E_1 to E_2) were being generated. This point has not been resolved, but data on the biochemical defects of certain *ts* mutants of Sindbis virus are consistent with the concept that E_1 and E_2 exist as heterodimers in the infected cell membrane and in the mature virus particles.[316]

The location of the envelope proteins of the oncornoviruses has been extensively studied. However, only recently has the spatial relationship of these proteins been described. Leamnson and Halpern[317] analyzed the envelope proteins of avian sarcoma virus B_{77} under nonreducing conditions and found that gp85 and gp37 appear to be linked by disulfide bonds in the mature virus particle and in the infected cell membrane. In addition, disruption with nonionic detergents resulted in the isolation of complexes which, by sedimentation analysis, are consistent with structures composed of two or three disulfide bonded complexes. Montelaro et al.[318] found a similar glycoprotein complex in the analysis of nonreduced proteins of the Prague strain of Rous sarcoma virus. However, these authors also found some p19 in these complexes. Antiserum against gp85 precipitated complexes containing gp85, gp35, and small amounts of p19 and p27. Similar results to those found with the avian sarcoma viruses were

also found with the murine leukemia viruses. Pinter and Fleissner[319] described a gp90 complex in AKR murine leukemia virus disrupted under nonreducing conditions. Reduction of the gp90 complex resulted in the separation of gp71 and a recently described protein p15. The amount of gp90 was variable and it has been suggested that this complex may be an intermediate.[318] Protein p15E is further processed to p12E and in this processing the disulfide bond to gp71 may be broken because no disulfide-bonded complexes of gp71-p12E have been detected. Other reports on Friend murine leukemia virus[318] and Moloney leukemia virus support the idea of a disulfide-bonded complex on the virion surface. In addition, McLellan and August,[178] employing a protein cross-linking agent, demonstrated the possible existence of gp71 oligomers, although no gp71-p15E complexes were detected in this system. It is interesting to note that these disulfide-bonded complexes probably arise through a proteolytic processing event as is seen with the HA protein of influenza virus and the F protein of the paramyxoviruses. The basis for this idea is the evidence that gp85 and gp37[320] and gp 71 and p15E-p12E[179] are synthesized by way of a precursor polypeptide.

A key question in the interaction of envelope proteins which has not been resolved is whether the glycoproteins on the exterior of the envelope interact with matrix membrane (M) proteins or nucleocapsid proteins on the interior of the virion. Many techniques have been used to address this problem, but no resolution has been forthcoming. A major problem appears to be uncertainty about the sensitivity of the techniques and uncertainty about the significance of the measured parameters. The alphaviruses (Sindbis and Semliki Forest) seemed to be ideal viruses for the resolution of the question because virion consists of two (Sindbis) or three (Semliki Forest) glycoproteins and one nucleocapsid protein arrayed in an iscosahedral structure with the ribonucleic acid genome. The envelope of Sindbis virus was shown to have a typical lipid bilayer structure. However, data from x-ray diffraction studies did not detect a direct interaction between the surface glycoproteins and the nucleocapsid.[41] Evidence for the penetration of the glycoproteins into the lipid bilayer was provided by the isolation of the hydrophobic tail fragment of the glycoproteins following proteolytic digestion of the intact virion.[127,187] In discussing the data from these studies, Utermann and Simons[127] suggested that the area of the lipid envelope occupied by the hydrophobic portion of the glycoproteins would be too small to be detected by X-ray diffraction.

The structure of the virion membrane has also been examined using the technique of freeze-etching, a procedure which allows the visualization of the intramembrane surfaces and the two leaflets of the lipid bilayer. Examination of the envelope of Sindbis virus revealed an absence of the intramembranal particles which are an ubiquitious feature of plasma membrane architecture.[321] Similar negative results have been reported on the intramembrane structure of influenza[191] and vesicular stomatitis virus.[322] However, again the question of the limits of resolution comes into play. Deamer,[323] in a discussion on the resolution of the technique of freeze-fracture, suggested that the intramembranal particles are not composed of single polypeptides, but may actually be oligomeric structures. Only one or two helical polypeptide chains spanning the lipid bilayer may present too small an area to be resolved by this technique.

Garoff and Simons[324] and Garoff[325] studied the structure of the envelope of Semliki Forest virus by examining the spatial relationships of the viral proteins. In vitro radiolabeling studies using formyl[^{35}S] methionyl sulfone methylphosphate suggested that the envelope glycoproteins E_1 and E_2 spanned the lipid bilayer.[324] This conclusion was reached by comparing the peptides that were labeled using intact virus and viral membranes solubilized with Triton® X-100. The validity of their conclusions rests on the assumption that the spatial relationships of the envelope glycoproteins are the same in these two preparations. Given the apparent complexity of the surface structure of Sindbis virus,[175,326] this assumption may be invalid. Putative support for the conclu-

sions drawn from the radiolabeling experiments was obtained by employing dimethylsuberimidate, a protein cross-linking agent. High concentrations of this nonreversible cross-linker produced viral particles that could not be disrupted with detergents.[325] These experiments were taken as evidence that the envelope glycoprotein and nucleocapsid protein were cross-linked, and the large complex would not penetrate polyacrylamide gels. Other investigators failed to detect cross-linking between envelope and capsid proteins by employing different cross-linkers with specificities similar to that of dimethylsuberimidate.[327,328] Further experiments are needed to provide additional evidence for possible proximity leading to interaction of the envelope glycoproteins and nucleocapsid of togaviruses.

The spatial relationship of the membrane proteins of other enveloped viruses has also been examined using protein cross-linkers. McLellan and August[178] also demonstrated that cross-linking of Rauscher murine leukemia virus with methyl-4-mercaptobutyrimidate resulted in a virion with altered susceptibility to detergents. However, unlike data with Semliki Forest virus,[325] analysis of the cross-linked material showed that the only detectable oligomers were homo-oligomers of gp71. Dubovi and Wagner[329] examined the protein interactions of VS virus with a variety of cross-linking agents. Cross-linking of the nucleocapsid to the membrane protein was readily detected, but only small amounts of the possible glycoprotein-membrane (G-M) protein complex were detected. Again, VS virus treated with high concentrations of cross-linker could not be disrupted with Triton® X-100 in 0.4 *M* NaCl, a system that completely solubilizes the G and M proteins.[327] The reason for this resistance to detergent solubilization is unknown. Of considerable interest was evidence[329] that the VS viral nucleocapsid N protein lies in close enough proximity to the M protein to form N-M heterodimers. These data support the hypothesis that M protein complexed with N protein can form the recognition site for maturation with G protein in the cell membrane.

One rather surprising result from cross-linking studies was a report of the cross-linking of the HN glycoprotein of Newcastle disease virus to the nucleocapsid protein.[330] This report did not give sufficient detail to critically assess the identity of the proteins in the isolated oligomers. Should these data be confirmed, a reassessment of the possible roles of the virus proteins in morphogenesis may be needed. However, these data conflict with other findings.

Using a membrane reconstitution technique, Yoshida et al.[205] found that the glycoproteins of Sendai virus could not reassociate with the nucleocapsid unless the membrane proteins were also present.

IV. VIRUS-CELL MEMBRANE INTERACTIONS

A fundamental property of viral membranes is their capacity to interact with cellular membranes. These interactions are dependent on both the proteins and lipids of the viral membrane and occur in several well-defined ways. Hemagglutination was initially observed with influenza viruses, but has since been described in other virus systems as well.[331] Under characteristic conditions, viruses will agglutinate erythrocytes mainly through electrostatic bonds.[332] Furthermore, the myxoviruses and paramyxoviruses are capable of eluting from the cell surface through the action of viral neuraminidases, which remove the cellular neuraminic acid receptors.

Additional interactions between paramyxoviruses and cell membranes involve hemolysis and cell fusion, both of which appear to reqire incorporation or fusion of the viral membrane with the cell plasma membrane. In general, membrane fusion is one of the most ubiquitious cellular events. It is involved in such seemingly diverse processes as secretion, phagocytosis, intracellular degradation of foreign materials by ly-

sosomes, addition of newly synthesized patches of membrane to the plasma membrane, and virus infection. Even though the outcomes of these events are quite different, a similar fusion mechanism may be operating in each case,[333] although the details of this mechanism are still somewhat obscure.[334] Some investigators have found that lipid vesicles containing acidic phospholipids in the presence of divalent cations will fuse mammalian cells,[335] whereas others found that lysolecithin[336] or lysolecithin in conjunction with positively charged lipids[135] will induce cell fusion. To further complicate matters, two groups have shown that lipid vesicles composed of only neutral phosphatidylcholine will fuse with mammalian cells.[337,338] Since paramyxoviruses are widely used as cell-fusing agents, the properties which determine their interaction with cell membranes may be generally applicable to membrane fusion events.

Additional data obtained by Martin and MacDonald[134-136] indicate that lipid vesicles of very simple composition can mimic the hemagglutinin, hemolysin, and fusion activities of paramyxoviruses. Phosphatidylcholine vesicles containing only a positively charged lipid (stearylamine) attached to erythrocytes and mimicked the HA activity of paramyxoviruses, possibly indicating the requirement for a positively charged group for attachment to cells. An additional lipid, lysolecithin, was required for both fusion and hemolytic activities by vesicles, but lysolecthin does not appear necessary for the equivalent activities of virions. Possibly, lysolecithin in lipid vesicles produces the same membrane instability that other disruptive agents do in virions, thereby resulting in a similar mechanism for hemaglobin leakage. Cell fusion produced by paramyxoviruses, however, does not require any membrane instability but occurs with intact viral membranes.[339] This apparent inconsistency may result from two different mechanisms operating for viruses and lipid vesicles during fusion. In the case of true paramyxoviruses, a functional F protein is apparently always required for fusion.

Cellular requirements for fusion or hemolysis are less clearly defined. On the basis of differing susceptibility to fusion by SV5 virions, Klenk and Choppin[46] and Choppin et al.[340] proposed that the lipid composition of the cell plasma membrane might determine its fusability. Specifically, Klenk and Choppin[46] correlated a high cholesterol/phospholipid ratio with reduced fusion, and Choppin et al.[340] suggested a direct correlation between ganglioside-associated neuraminic acid and susceptibility to fusion. In a later, more comprehensive comparision of fusion of several cell lines with a variety of viruses, Poste et al.[341] found no correlation between cholesterol content and level of fusion. In fact, no consistent difference was observed between the level of fusion of any of the cell lines.

The attachment of Sendai virus to liposomes was seen prevously to depend on the presence of phosphatidylcholine, cholesterol, and gangliosides.[342] Additional studies revealed that liposomes of the same composition will adsorb Sendai virions at 4°C and envelope them upon subsequent incubation at 37°C in a process similar to phogacytosis.[343] Under these conditions, the viral and liposomal membranes do not fuse. The addition of sphingomyelin, phosphatidylserine, and phosphatidylethanolamine was necessary for fusion of the two membranes at 37°C.[344] It appears that gangliosides alone can serve as receptors for Sendai virions, although a more complete lipid composition is required for fusion of the viral and liposomal membranes. Additional support for these conclusions can be derived from a study of hemolysis of horse erythrocytes, which contain *N*-glycosyl-neuraminic acid rather than *N*-acetyl-neuraminic acid and are not hemolyzed by Sendai virus.[345] If concanavalin A is first bound to the horse erythrocytes, the Sendai virions will adsorb to the concanavalin A, resulting in the hemolysis of the horse erythrocytes.[345] This finding suggests that the specific receptor *N*-acetyl-neuraminic acid is required for virion attachment to initiate the hemolysis of erythrocytes.

The physical alterations of cell membranes caused by virus-induced hemagglutina-

tion, hemolysis, and cell fusion have been investigated in detail using ESR spectroscopy. Spin-labeled fatty acids and phosopholipids were employed to observe changes in the cell membrane fluidity and transfer of phosopholipids between viral and cell membranes during these events. Chicken erythrocytes, spin-labeled with fatty acids and subsequently agglutinated with either Sendai or influenza viruses, exhibited an increased membrane fluidity of similar magnitude to the increase caused by agglutination with the lectins, concanavalin A and wheat germ agglutinin.[346] Lectins have been shown to cause lateral movement of proteins and formation of "caps" in certain cells, both of which are dependent on the underlying microtubule and microfilament systems of the cell. It was suggested that the fluidization might be caused by microtubule-dependent lateral movement of membrane proteins, since human erythrocytes lacking microtubules (which are arranged in a marginal band beneath the plasma membrane) exhibited no fluidity change upon virus or lectin attachment.[346] A fluidity increase upon virus attachment was also observed using fluorescence depolarization of DPH-labeled BHK-21 or 3T3 cells.[347] Attachment of the lipid-enveloped West Nile virus (togavirus), the nonenveloped encephalomyocarditis virus, or polyoma virus resulted in a temperature-dependent fluidity increase. Rearrangement of the cell plasma membrane constituents was suggested as a possible cause of the increase, although a virus-dependent internalization of DPH into intracellular membranes could also cause a fluidity increase.

Spin-labeled phospholipids incorporated into Sendai viral membranes were employed to detect the intermixing of lipid molecules between virions and erythrocytes during attachment and/or subsequent fusion of the viral membrane to the cell.[348,349] Under conditions in which only attachment of virions occurred, i.e., with nonfusing influenza virus, Sendai virus at low temperature or Sendai virus with an inactive (Fo) fusion glycoprotein, little mixing of viral and cellular phosphatidylcholine molecules was detected. In the presence of active F glycoprotein, rapid intermixing of spin-labeled phosphatidylcholine molecules was observed. An active F glycoprotein in unlabeled Sendai virus was also necessary to catalyze the transfer of spin-labeled phosphatidylcholine between labeled influenza virions and unlabeled erythrocyte membranes or between two populations of erythorcytes, one labeled and one unlabeled.[349] Rapid lipid intermixing only in the presence of an active F glycoprotein suggests that membrane fusion is required for this to occur. In addition, Sendai virus can apparently induce influenza virions to fuse with erythrocyte membranes.

Erythrocytes spin-labeled with various phospholipids exhibit different fluidities for each phospholipid observed.[350-352] This heterogeneity can be abolished by osmotic hemolysis,[350,352] complement-induced hemolysis,[351] or Sendai-induced hemolysis,[352] presumably due to reorganization of the phospholipids upon lysis. It is not clear whether hemolysis is necessary for homogenization of the erythrocyte membrane fluidity or if cell fusion will suffice. In one study, early harvest virus grown in eggs, which contains fusing activity but little hemolyzing activity, caused no detectable change in the erythrocyte membrane fluidity,[352] whereas a second group did observe a change with early harvest virus.[349] The virions of the second study resulted in 15 to 20% hemolysis, which possibly is sufficient to detect a loss in heterogeneity of these spin-labeled phospholipids. In either case, hemolysis of erythrocytes appears to cause major structural reorganization of their membrane phospholipids.

No clear relationship has been established between fusion of virion membranes with cell membranes and the mechanism by which enveloped viruses invade cells, particularly those viruses devoid of fusion factor. A long-standing controversy has existed as to whether viruses, such as vesicular stomatitis, penetrate target cells by phagocytosis[353] or by fusion of virion membrane with cell cytoplasmic surface membrane.[354] Much of the controversy probably stems from the use of electron microscopic techniques which

defy quantitation. However, biochemical techniques have revealed some evidence for fusion of VS virus and cell membrane by identifying viral membrane proteins on the penetrated cell membrane.[355] In all likelihood, the primary event in viral penetration can be either virion fusion with plasma membrane or phagocytosis of virions followed by fusion with internal cell membranes and release of the virion nucleocapsid in the cytoplasm.

ACKNOWLEDGMENTS

The research by the authors described here was supported by Grant PCM77 00494 from the National Science Foundation, Grant VC88 from the American Cancer Society, and Grant AI-11112 from the National Institute of Allergy and Infectious Diseases. E. J. Patzer was a Predoctoral Trainee of the National Cancer Institute and E. J. Dubovi a Postdoctoral Fellow of the National Institute of Allergy and Infectious Diseases.

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