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Membrane Glycoproteins of Enveloped Viruses

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I. MEMBRANES OF LIPID-CONTAINING VIRUSES

Lipid-containing animal viruses, including members of at least 10 different major groups, possess components in their envelopes which are similar to those of other biological membranes: lipids, proteins, and carbohydrates linked to specific subsets of lipids or proteins. Investigation of the fine structure, arrangement, and assembly of these viral membrane components is a field that has recently attracted increasing attention, both because of intrinsic interest in these viruses, many of which are of great public health importance, and because viral envelopes have several unique advantages in membrane re-

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search. The most important advantage is structural simplicity, in that lipid-containing viruses possess a small number of virus-coded polypeptides and glycoproteins as membrane components. Sufficient quantities of many enveloped viruses can be purified for detailed biochemical analysis, including primary structure studies of viral membrane proteins like those now underway. Purification procedures are rapid and simple, and a fairly homogeneous population of particles can be obtained free of contaminating cellular membranes. For the simpler lipid-containing viruses, the possibility therefore exists for complete structural determination of a biological membrane, and such information should contribute greatly to an understanding of the molecular details of membrane structure and interactions among membrane components in general.

Other advantages of enveloped viruses in studies of membrane structure and biogenesis include the ease of biosynthetic labeling of viruses grown in cell culture with specific radioactive precursors and the availability of mutants in defined gene products, some of which are proving to be useful in the analysis of viral membrane assembly. Since envelope components are integral parts of cellular membranes during the assembly of virus particles (Compans *et al.*, 1966; Compans and Choppin, 1971), studies of the synthesis and mechanism of insertion of these components into membranes should provide useful information pertaining to the biogenesis of cellular membranes. Many viral systems offer the additional advantage that host cell biosynthesis is inhibited during infection, so that it is possible to analyze the synthesis of membrane components in a cell in which only a small number of virus-coded membrane components are being produced and to follow the intracellular migration of specific membrane glycoproteins by radiolabeling procedures.

Finally, the ability to prepare viruses with specific modifications in either lipid or protein composition arises from the fact that viral envelope lipids are derived from the plasma membrane of the host cell, whereas viral proteins are entirely virus-coded. Thus by growing different virus types in the same cell it is possible to prepare a membrane in which the protein composition can be varied while the lipid composition remains constant. Similarly, by growing the same virus in a series of different cells, viral membranes are obtained which possess the same set of proteins but vary in lipid composition. These approaches have been useful in studies of lipid–protein interactions in viral membranes (Landsberger *et al.*, 1973).

Several recent reviews are available concerning both the experimental and theoretical aspects of viral envelope structure and assem-

TABLE I

THE ENVELOPED VIRUSES OF VERTEBRATES

Family	Virion properties			Cellular membrane site of assembly	Common members
	Shape	Size (nm)	Capsid structure		
RNA viruses					
Myxovirus	Spherical or filamentous	90–110	Helical	Plasma	Influenza
Paramyxovirus	Spherical or filamentous	120–150	Helical	Plasma	Mumps, measles, Sendai
Rhabdovirus	Bullet-shaped	70 × 175	Helical	Plasma or intracytoplasmic	Rabies, VSV
Togavirus	Spherical	50–60	Icosahedral	Plasma	Sindbis, SFV
Arenavirus	Spherical or pleomorphic	80–150	Strandlike	Plasma	Lymphocytic choriomeningitis
Bunyavirus	Spherical	90–150	Strandlike	Intracytoplasmic	Bunyamwera
Coronavirus	Spherical	80–120	?	Intracytoplasmic	Mouse hepatitis
Oncornavirus	Spherical	100–120	Icosahedral(?)	Plasma	Rous sarcoma, murine leukemia
DNA viruses					
Herpes virus	Spherical	150	Icosahedral	Nuclear	Herpes simplex
Pox virus	Oblong	300 × 200	—	*De novo* formation	Vaccinia

bly (Lenard and Compans, 1974; Blough and Tiffany, 1975; Wagner, 1975; Choppin and Compans, 1975; Compans and Choppin, 1975; Klenk, 1974). In this chapter, we emphasize recent information on the glycoprotein components of enveloped viruses and endeavor to point out specific findings on viral envelopes which we believe to be of broad significance.

In Table I are listed the 10 established major groups, or families, of lipid-containing viruses of vertebrates, and examples of the best studied members of certain groups. For several families, only limited information is available on viral envelope structure, whereas other groups have been the object of intensive study. We concentrate our discussion on the glycoproteins of the most intensively studied virus groups, which are the first four groups of RNA viruses listed in Table I: myxoviruses, paramyxoviruses, rhabdoviruses, and togaviruses. We also discuss selected findings for other virus groups where these have revealed unusual or important aspects of viral membrane structure or assembly.

II. COMPONENTS OF VIRAL MEMBRANES

Although enveloped viruses of different major groups vary in size and shape, as well as in the MWs of their structural polypeptides, there are general similarities in the types of polypeptide components present in virions. The types of structural components found in viral membranes are summarized briefly in the following discussion, and further detailed information is available in the recent reviews cited above.

A. Glycoproteins

All the enveloped viruses studied to date possess one or more glycoprotein species. The presence of carbohydrate covalently linked to proteins has usually been demonstrated by the incorporation of a radioactive precursor such as glucosamine or fucose into viral polypeptides, which is resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The specificity of labeling by these precursors is shown by the lack of their incorporation into carbohydrate-free polypeptides of the virion (Strauss *et al.*, 1970). The number of distinct glycoprotein species present in the virion varies for various virus groups. As shown in Table II, there is also marked variation in the glycoprotein content of virions when expressed as a percent of

TABLE II

PROTEIN COMPOSITION OF LIPID-CONTAINING VIRUSES

		Percent of total protein			
			Carbohydrate-free proteins		
Virus type	Number of glycoproteins	Glycoproteins	M Protein	Others	Source of data
Toga	3	76	—	24	Garoff *et al.* (1974)
Bunya	2	64	—	36	Obijeski *et al.* (1976)
Oncorna, B type	3	58.4	—	41.6	Yagi and Compans (1977)
Corona[a]	4	58	—	42	Hierholzer *et al.* (1972)
Paramyxo	2	39.5	14.0	46.5	Lamb and Mahy (1975)
Rhabdo	1	34.2	28.8	37	Bishop and Roy (1972)
Myxo	2	33.4	36	30.6	Compans and Choppin (1975)
Arena[b]	1–2	27.5	—	72.5	Vezza *et al.* (1977)
Herpes[c]	13	19.7	—	80.3	Heine *et al.* (1974)
Oncorna, C type[d]	1–2	7	—	93	Famulari *et al.* (1976)

[a] Classification of glycoproteins was stated to be only tentative.

[b] A single glycoprotein was detected in the arenaviruses Tacaribe and Tamiami (Gard *et al.*, 1977), whereas the data given are for Pichinde virions which contain two glycoproteins.

[c] The values represent our calculations from the autoradiograph scan data presented in the reference.

[d] The values given represent our calculations from the data presented in the reference.

total viral protein. As discussed in Section V, these data suggest that glycoproteins of the various virus groups may play a greater or lesser role in assembly and maintenance of the viral envelope structure. However, glycoproteins are essential components for viral infectivity even where they constitute only a minor fraction of the mass of the virion, since they are necessary for the initial events in the viral replication cycle. The fine structure, biological functions, and organization

of glycoproteins in the viral envelope are discussed in detail in Section IV.

The carbohydrates of viral glycoproteins are specified in large part by host cell transferases, whereas the amino acid sequences are coded by the viral genome. Glycoproteins of the same virus may exhibit host cell-dependent differences in electrophoretic mobility, which are due to differences in the carbohydrate components (Haslam *et al.*, 1970; Compans *et al.*, 1970; Schulze, 1970). Thus viral glycoproteins may be useful probes to detect differences in glycosylation among various cell types. An important exception to the general finding of host cell-specified carbohydrates is found in myxoviruses and paramyxoviruses. Glycoproteins of these viruses lack sialic acid (Klenk *et al.*, 1970a,b), which is believed to be a result of the virus-specified neuraminidase incorporated as a structural component of these virions.

B. Lipid Bilayer

All enveloped viruses contain lipid as a major structural component, and available evidence indicates that all the lipid is contained in a single bilayer structure which forms the matrix of the limiting membrane of the virion. Investigations using biophysical methods including x-ray diffraction (Harrison *et al.*, 1971) and electron spin resonance (Landsberger *et al.*, 1971, 1973; Landsberger and Compans, 1976) indicate that viral lipids are arranged in a bilayer. With electron microscopy, a well-defined unit membrane is observed at the location of the bilayer.

The virus derives its lipids from the host cell membrane where virus maturation occurs, which is the plasma membrane in most instances. Therefore a given virus can exhibit marked variation in lipid composition when grown in different cell types, which reflects the composition of the host cell plasma membrane in each instance (Klenk and Choppin, 1969, 1970; Quigley *et al.*, 1971). Apart from minor differences in carbohydrates of glycoproteins, virion proteins are indistinguishable when the virus is propagated in a variety of cells; therefore there appears to be little or no determining influence of viral proteins on the composition of the lipid bilayer. Since the viral envelope is continuous with the host cell membrane during morphogenesis, and membrane lipids are characterized by a high rate of lateral diffusion in the plane of the membrane, the similarity in lipid composition between viral envelopes and plasma membranes is not surprising.

The distribution of various lipid classes on the internal and external

sides of the bilayer has recently been investigated in influenza virions using phospholipase digestion and phospholipid exchange proteins (Tsai and Lenard, 1975; Rothman *et al.*, 1976). The results indicate that lipids are distributed asymmetrically, with the majority of the phosphatidylinositol and about half of the phosphatidylcholine in the external half of the bilayer and most of the phosphatidylethanolamine and phosphatidylserine in the inner half. These distributions are thought to reflect a similar distribution in the host cell plasma membrane. Since less than half of the total phospholipids were accessible by either approach, it was suggested that glycolipids constitute a significant part of the outer monolayer of the viral membrane. The presence of glycolipids in various enveloped viruses has been demonstrated by chemical analyses (Klenk and Choppin, 1970), and the types of glycolipids present also reflect those of the host cell. Myxo- and paramyxoviruses lack neuraminic acid residues in their glycolipids, which again is a likely result of their neuraminidase activity (Klenk and Choppin, 1970; Klenk *et al.*, 1970b). The reactivity of viral glycolipids toward specific lectins demonstrates that their carbohydrates are exposed on the external surface of the bilayer (Klenk *et al.*, 1972a).

C. Carbohydrate-Free Proteins

All polypeptides located internal to the viral lipid bilayer are devoid of carbohydrate, and they may be subdivided into several types according to their structural location and functions. All enveloped viruses possess at least one polypeptide species closely associated with the nucleic acid to form a nucleocapsid or core structure, which is termed the nucleocapsid protein. In addition, viruses with helical nucleocapsids possess another major internal protein, termed the membrane protein, that appears to be associated with the internal surface of the lipid bilayer. Most nucleocapsid proteins are not directly involved in interaction with the lipid bilayer, although the nucleocapsids of togaviruses are roughly spherical structures that appear to be closely apposed to the inner surface of the bilayer. The internal membrane (M) proteins of myxo-, paramyxo-, and rhabdoviruses occupy a similar location in the viral envelope. It appears that these proteins are primarily responsible for conferring considerable rigidity on the viral envelope, as compared to that of host cell membranes of similar lipid composition (Stoeffel and Bister, 1975; Landsberger and Compans, 1976; Lenard *et al.*, 1976).

More complex viruses possess additional internal polypeptides as

major components, but their precise locations in the virion have not been established. In addition, minor internal protein components are frequently observed in virions which possess enzymic functions, such as transcriptase activity. Such minor polypeptides are not thought to play a direct role in the structure or assembly of the viral envelope.

D. Carbohydrates

It is well established that carbohydrate chains are covalently linked to glycoproteins and glycolipids of the viral envelope, and both of these components were described above. However, it is uncertain whether or not other complex carbohydrates serve a function in viral envelope structure. The presence of sulfated mucopolysaccharides in highly purified virus preparations of several major groups was recently observed by radiolabeling virions with sulfate (Compans and Pinter, 1975; Pinter and Compans, 1975). These sulfated components are derived from the host cell, since they can be labeled selectively by the growth of cells in $^{35}SO_4$-containing medium prior to virus infection. In the case of influenza virus, it is possible that these components represent a host cell antigen described to be associated with purified virus particles (Knight, 1944). Labeled sulfated mucopolysaccharides can be removed from virus preparations by digestion with hyaluronidase or trypsin without any effect on viral infectivity, suggesting that these components are not essential (Pinter and Compans, 1975). It is likely that they associate with the external surface of the virion during the process of maturation.

III. ARRANGEMENT OF VIRAL ENVELOPE COMPONENTS

Enveloped viruses share many common features in the organization of their structural components, as indicated by several approaches which include electron microscopy, surface-labeling and proteolytic digestion experiments, and isolation of subviral components. Much of the evidence pertaining to the arrangement of membrane components has been discussed in other reviews cited above and is only briefly summarized in this section.

The organization of the viral membrane corresponds in many respects to the fluid mosaic membrane model (Singer and Nicolson, 1972). Viral glycoproteins appear to be integral membrane proteins which are exposed on the external surface of a lipid bilayer. The external location of the glycoproteins is indicated by their sensitivity to

proteolytic digestion and reactivity toward surface-labeling reagents (see review in Lenard and Compans, 1974). Partial penetration of the glycoproteins into the bilayer is suggested by the fact that segments of the glycoproteins of several virus groups are found to be resistant to protease (Gahmberg *et al.*, 1972; Mudd, 1974, Schloemer and Wagner, 1975b; Lenard *et al.*, 1976).

In contrast to the glycoproteins, the carbohydrate-free polypeptides of enveloped viruses are located internally to the lipid bilayer, as indicated by their resistance to protease treatment and lack of reactivity toward surface-labeling reagents. These internal proteins are also not reactive toward specific antibodies without disruption of the viral envelope. The best examples of nonglycosylated proteins which appear to be intimately associated with the internal surface of the envelope are the M proteins of myxo-, paramyxo-, and rhabdoviruses. However, whether these polypeptides associate with the bilayer as integral membrane proteins, or as peripheral membrane proteins such as spectrin in erythrocyte membranes, has not been clearly established.

In Fig. 1 the schematic cross section of an influenza virion shows the general arrangement of its structural components. The extent of penetration of the glycoproteins into the bilayer is not indicated but,

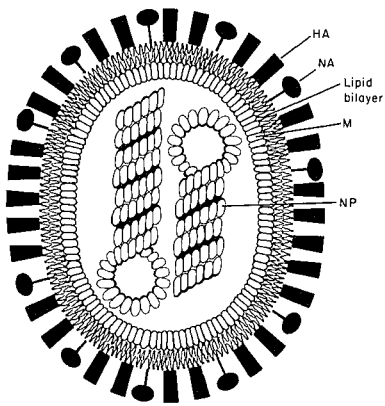


FIG. 1. Schematic cross section of an influenza virion, depicting the arrangement of the major structural polypeptides. The rodlike HA spikes and the more complex NA spikes are composed of HA and NA polypeptides, respectively. These glycoproteins are exposed at the external surface of the lipid bilayer, and segments may penetrate the bilayer (not shown). The internal membrane-associated M protein appears to form a closely packed layer beneath the bilayer. The helical nucleocapsids are found as multiple discrete segments, each containing an RNA molecule coated with a major nucleocapsid protein, (NP). The nucleocapsids probably interact directly with the M protein during virion assembly. (From Compans and Choppin, 1975.)

as discussed in Section IV, it is likely that a hydrophobic segment penetrates into the bilayer and may even traverse it. A closely packed layer of M protein is depicted on the internal surface of the bilayer in accordance with available information, and the helical nucleocapsids are depicted as twisted hairpinlike structures (Compans *et al.*, 1972). It is likely that the nucleocapsids interact with the M proteins during assembly and in so doing participate in determining virion size and shape. Little is known about these interactions, and they are not indicated in Fig. 1.

IV. STRUCTURE AND FUNCTION OF VIRAL GLYCOPROTEINS

A. Fine Structure of Envelope Glycoproteins

In this section we summarize the available information on the detailed structure of the glycoproteins of four virus groups. The information obtained includes the size and shape of viral glycoproteins, the number of polypeptide chains in the complete glycoprotein structure, and compositional data on the polypeptide and oligosaccharide portions of the molecules. Detailed structural information on glycoproteins has been obtained for only a few virus types and is obviously incomplete even for the best studied viral systems.

1. Influenza Virus Glycoproteins

Influenza viruses possess two glycoproteins with distinct biochemical and morphological properties: hemagglutinin (HA) and neuraminidase (NA). The HA of influenza virus is one of the best studied viral glycoproteins. Electron microscope studies of the isolated spike structure have shown that it is a rod-shaped triangular prism approximately 14 nm long and 5 nm wide (Laver and Valentine, 1969). The HA spike projects radially from the virion envelope, and it is probably a trimer having a MW of about 225,000 daltons (Schulze, 1975; Wiley *et al.*, 1977). HA glycoproteins can exist in one of two alternative forms: a single polypeptide with a MW of 75,000 daltons designated HA, or two polypeptides with MWs of approximately 50,000 and 25,000 daltons designated HA_1 and HA_2, respectively. HA_1 and HA_2 are proteolytic cleavage products of HA, which remain cross-linked by disulfide bonds (Lazarowitz *et al.*, 1971; Laver, 1971). The extent of cleavage of HA to HA_1 and HA_2 may vary from none to complete cleavage of all HA polypeptides (Lazarowitz *et al.*, 1973). The extent of cleavage de-

pends upon several factors including the host cell, the virus strain, and the presence or absence of proteases in the culture medium. The cleavage of HA may not be a requirement for infectivity of all virus strains, and spike morphology is not altered detectably by cleavage of HA to HA_1 and HA_2, but recent studies have shown that cleavage enhances the infectivity of some influenza virus strains (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975).

The tryptic peptide maps of HA_1 and HA_2 are distinct, and the only notable difference in the amino acid composition of the two polypeptides is the proline content (Laver, 1971). The proline content of HA_1 is at least six times higher than that of HA_2. HA_1 is tightly folded on itself, probably reflecting the high proline content. A portion of the HA spike is released by protease treatment as a soluble protein which can be crystallized (Brand and Skehel, 1972).

Functional HA spikes have been isolated from several influenza strains by treating virions with ionic or nonionic detergents. After removal of the detergent, the spikes aggregate, forming rosettelike structures, suggesting that the base of the HA spike is hydrophobic in nature (Laver and Valentine, 1969). Available evidence indicates that the HA spike is amphipathic and that a portion of it is buried within the lipid bilayer of the viral envelope. This is indicated by the finding that protease-treated virions retained HA_2 or portions thereof while having no identifiable spikes (Compans *et al.*, 1970; Lenard *et al.*, 1976).

The HA_2 glycoprotein therefore contains the hydrophobic end of the spike. This glycoprotein aggregates even in the presence of guanidine hydrochloride (Laver, 1971). The composition of a 50-residue peptide associated with the lipid bilayer, which is removed from the carboxyl terminus of HA_2 by bromelain, has been estimated, and 21 of the amino acids are hydrophobic in nature, whereas 14 may be charged (Skehel and Waterfield, 1975). The carboxyl terminus of this peptide is thought to be buried within the lipid bilayer. The amino-terminal end of HA_1 seems to extend radially from the virion surface, and a sequence of 10 residues from the amino-terminal end of HA_2 is highly conserved in both type-A and type-B influenza virions (Skehel and Waterfield, 1975). The hydrophobicity of HA_2 suggests that it is similar to other membrane-associated glycoprotein segments (Segrest *et al.*, 1972; Nakashima *et al.*, 1975), but freeze-fracture studies of the influenza virion indicate that extensive portions of HA and NA do not penetrate through the lipid bilayer (Bächi *et al.*, 1969). Circular dichroism studies of HA spikes isolated intact and those cleaved from the virion surface may be useful in further defining the secondary structure of the hydrophobic regions.

Until recently, little information was available concerning the carbohydrate portion of the HA glycoprotein. About 80% of the carbohydrate attached to viral proteins was shown to be linked to HA; the glycosyl moieties were shown to be comprised of glucosamine, mannose, galactose and fucose at a molar ratio of 6:4:1:1, respectively, and the total carbohydrate of HA, determined by chemical means, was estimated to be approximately 12,000 daltons (Laver, 1971). It was also found that HA_2 of influenza A virions contains fucose, whereas HA_2 of influenza B (GL1760) does not (Choppin *et al.*, 1975). Recently the glycopeptides obtained by pronase digestion of influenza A viruses have been characterized (Schwarz *et al.*, 1977; Nakamura and Compans, 1978b). Both groups showed that HA possesses type I (oligosaccharide side-chain comprised of glucosamine, mannose, galactose, fucose and sialic acid) and type II (oligosaccharides comprised of glucosamine and mannose) glycopeptides, but the distribution of each type on HA_1 and HA_2 was shown to depend upon the virus strain. Schwarz *et al.* (1977) showed that HA_1 of two avian influenza viruses possessed only type I glycopeptides, whereas both type I and II glycopeptides were shown to be present on HA_2. However, Nakamura and Compans (1978b) using the WSN strain of influenza showed that HA_1 possessed both type I and II glycopeptides whereas HA_2 contained only type I glycopeptides. Since it is likely that HA_2 occupies a similar structural location in WSN and avian hemagglutinin proteins, these results suggest that specific amino acid sequences determine whether a type I or type II oligosaccharide is added at a particular site on the glycoprotein.

The host cell type may also determine the type of oligosaccharide chains added to a given viral glycoprotein. Thus, although HA_2 of WSN strain influenza virions grown in MDBK cells contained only type I glycopeptides, both type I and II glycopeptides were found in HA_2 when grown in CEF cells (Nakamura and Compans, 1978c).

Schwarz *et al.* (1977) showed that the type I HA glycopeptides of fowl plague and N influenza strains grown in chicken embryo fibroblasts (CEF) had a molecular weight of approximately 2,600 daltons, whereas type II glycopeptides from the same source has a molecular weight of approximately 2,000 daltons. The molecular weights of type I and II glycopeptides of the WSN strain of influenza virus were also found to have approximately this size (Nakamura and Compans, 1978b). HA glycopeptides of virus grown in MDBK cells were slightly larger in size than those of virus grown in CEF cells. Based upon the estimated carbohydrate content (12,000 daltons) of the HA glycoprotein obtained by Laver (1971) and Schwarz and Klenk (1974) and the

size estimates of the type I and II glycopeptides of influenza virus grown in MDBK cells, it was estimated that HA_2 contains a single type I glycopeptide whereas HA_1 possesses two type I and one or two type II oligosaccharide side-chains for the WSN strain (Nakamura and Compans, 1978b).

The NA spike, as shown in Fig. 1, also projects radially from the lipid bilayer of the influenza virion but is morphologically distinguishable from the HA spike (Laver and Valentine, 1969). The NA spike is elongated and has a square knoblike structure at one end. The oblong head is approximately 5 × 8.5 nm, and it is attached to a fiber approximately 10 nm long. The NA spike has a MW of approximately 240,000 daltons and is comprised of four NA polypeptides each having a MW of approximately 58,000 daltons; two NA polypeptides appear to be linked by disulfide bonds to form dimers, which are thought in turn to aggregate by noncovalent bonds to form the tetrameric spike (Bucher and Kilbourne, 1972; Lazdins *et al.*, 1972). The amino acid composition of the NA has been determined, and it has a cysteine content significantly higher than that of the other viral polypeptides (Laver and Baker, 1972). Influenza NA is in some ways morphologically similar to enzymes involved in sugar metabolism in the gut, as discussed by Forstner and Riordan in this volume.

After trypsin treatment a tetramer of four coplaner subunits of 4 × 4 × 4 nm was isolated (Wrigley *et al.*, 1973). This structure appears to correspond to the knob, and it is enzymically active. The knob structure can no longer aggregate with itself or with HA molecules, indicating that the hydrophobic regions of the molecule have been removed by proteolysis. The MW of the portion of the NA monomer which remains associated with the envelope after trypsin treatment was estimated to be 7000 daltons by Lazdins *et al.* (1972) and 12,000 daltons by Wrigley *et al.* (1973). Presumably, this portion functions to attach the NA molecule to the virion and plays no role in the enzymic properties. Furthermore, the portion of the spike which remains associated with the viral envelope is more highly glycosylated than the knob-shaped portion of the NA spike with enzymic activity (Lazdins *et al.*, 1972).

NA is present in influenza virions in smaller amounts than HA, in a ratio of about three to four HA polypeptides for each NA polypeptide. Little information is available on the carbohydrate components of the NA.

Recently, both the HA and NA of influenza virions were shown to be sulfated glycoproteins (Compans and Pinter, 1975). The sulfate appears to be covalently linked to the oligosaccharide chains of viral gly-

coproteins (Nakamura and Compans, 1977, 1978a). Glycoproteins of enveloped viruses of all the other major groups studied also are sulfated, whereas carbohydrate-free polypeptides are not (Pinter and Compans, 1975; Kaplan and Ben-Porat, 1976).

2. Rhabdovirus G Protein

Rhabdoviruses are covered with closely spaced glycoprotein spikes approximately 10 nm in length as shown by electron microscope studies. Rhabdoviruses possess only a single glycoprotein species termed the G protein, and for vesicular stomatitis virus (VSV) it has a MW of approximately 69,000 daltons (Wagner, 1975). Cartwright *et al.* (1972) have postulated that only a single glycoprotein molecule constitutes the spike structure.

The G proteins of rhabdoviruses are amphipathic, like the glycoproteins of influenza virions. After protease treatment of VSV a fragment of the G protein was demonstrated to be associated with the intact virion (Mudd, 1974). More recently, Schloemer and Wagner (1975a) isolated a small nonglycosylated portion of the G protein from the envelope of protease-treated VSV virions. The fragment was found to have a MW of 5200 daltons, approximately equivalent to 50 amino acids. This is similar to the estimated size of the portion of the HA_2 protein of the influenza virion thought to be buried within the lipid bilayer. Amino acid analysis of the G-protein fragment showed that it contained a preponderance of hydrophobic amino acids. As is the case for the membrane-associated portions of influenza HA, the hydrophobic fragment of the G protein is long enough to penetrate the lipid bilayer. Conclusive evidence for such penetration has not been obtained, but cross-linking experiments with glutaraldehyde suggest that interactions may occur between G proteins and internal M proteins (Brown *et al.*, 1974).

The carbohydrates linked to the G protein of VSV have been studied in detail. The glycoprotein is 9–10% carbohydrate by weight and contains mannose, galactose, *N*-acetylglucosamine, and neuraminic acid as the major sugar components, with lesser amounts of *N*-acetylgalactosamine and fucose (McSharry and Wagner, 1971; Burge and Huang, 1970. Etchison and Holland, 1974a). The size and composition of the carbohydrate moieties per VSV glycoprotein are variable, depending upon the cell type in which the virions are grown (Burge and Huang, 1970; Etchison and Holland, 1974a). Likewise, the sequence of the carbohydrates within the glycosyl side-chains may exhibit cell depen-

dence (Moyer and Summers, 1974). The monosaccharide composition of the oligosaccharide side-chains is similar to that of influenza virus (Etchison and Holland, 1974b), with the exception that sialic acid is present on the termini. Klenk *et al.* (1970b) demonstrated the presence of sialic acid on the envelope of VS virions by treating the virus with colloidal iron hydroxide which stains sialic acid residues. The stain was shown to bind to the envelope of VSV, whereas it did not bind to influenza virions or paramyxoviruses as shown by electron microscopy. More recent studies have shown that the glycosyl moieties are not terminated by sialic acid when VSV is grown in mosquito cells, because these cells lack sialyl transferase (Schloemer and Wagner, 1975b).

Preliminary studies indicated that from 3–5 cyanogen bromide peptides of the VSV G-protein may be glycosylated (Wagner, 1975). However, Etchison and Holland (1974a,b) have calculated that there are only 2 glycopeptides of 3000–3400 daltons in each G protein molecule. More recent studies by Etchison *et al.* (1977) have indicated that the VSV G-protein possesses two identical glycopeptides. The number average molecular weight of the glycopeptides was estimated to be 3150 by gel filtration analysis and 3450 based on composition of the amino acid and sugar residues. Additional information concerning the structure of the glycopeptide was obtained by sequential chemical and enzymatic degradation. These results indicate that the glycopeptides are acidic type I glycopeptides with two or three mannose branches terminating in sialic acid. Moyer and co-workers (1976) have obtained evidence that the glycosyl residues are covalently linked to an asparagine residue of the G protein, and the sequence of the monosaccharides that constitute the oligosaccharide side-chains has been determined (Hunt and Summers, 1976b).

3. Togavirus Glycoproteins

Togaviruses are small, spherical enveloped viruses 50–60 nm in diameter with a core structure that appears to be icosahedral. Although there are many members of the togavirus group, the best studied are Sindbis virus and Semliki Forest virus (SFV). Sindbis virus possesses two glycoproteins designated E_1 and E_2; both have a MW of approximately 50,000 daltons, and they are not linked by disulfide bonds because they can be separated under nonreducing conditions and without alkylation (Schlesinger *et al.*, 1972). SFV is similar to Sindbis virus in that it also has 2 glycoproteins of similar molecular weight designated E_1 and E_2, but a third glycoprotein designated E_3 has also been

detected. The molecular weights of E_1, E_2, and E_3 were estimated to be 49,000, 52,000 and 10,000 daltons, respectively (Garoff *et al.*, 1974). E_2 and E_3 are synthesized as a common precursor protein (NVP68) that is cleaved to yield E_2 and E_3. E_1, E_2, and E_3 of SFV are present in equimolar ratios as are E_1 and E_2 of Sindbis virus.

The arrangement and relationship of the glycoproteins within the spike structure of the Sindbis and SFV virion are yet to be resolved. Garoff and Simons (1974) and Garoff (1974) have used the cross-linking agent dimethyl suberimidate to study the interrelationships of the glycoproteins of SFV. E_1 and E_2 were most readily cross-linked, but steric hindrance may have prevented the cross-linking of E_1, E_2, and E_3. Recent studies by Jones *et al.* (1977) have suggested that E_1 and E_2 of Sindbis virus, like E_1, E_2, and E_3 of SFV, probably constitute the glycoprotein spike, since the cleavage of PE_2, a precursor of E_2, does not occur in temperature sensitive mutants of complementation groups including that thought to represent E_1. Furthermore, cleavage is inhibited by antibodies directed against either E_1 or E_2. These data suggest that PE_2 and E_1 may exist as a complex in the membrane of the infected cell, and presumably also that E_1 and E_2 remain as a complex in the viral envelope.

Protease treatment of SFV cleaves the glycoproteins, E_1 and E_2, and residual segments can be isolated from the envelope of spikeless particles (Utermann and Simons, 1974). The amino acid composition of each of these peptides demonstrates that they are enriched in hydrophobic amino acids. The residual peptides have a MW of approximately 5000 daltons; hence they are comprised of about 50 amino acids. Thus, like the glycoproteins of influenza virus and VSV, togavirus glycoproteins are amphipathic in nature and appear to possess a peptide of similar size embedded within the viral envelope. When SFV was treated with high concentrations of dimethyl suberimidate, both the tail fragments of E_1 and E_2 were cross-linked with the nucleocapsid protein, which supports the conclusion that the hydrophobic segments, of E_1 and E_2 may penetrate through the lipid bilayer and interact with the nucleocapsid (Garoff and Simons, 1974).

Carbohydrate analysis of SFV showed that E_1 contains about 18 moles of monosaccharide, E_2 about 28 moles and E_3 about 22 moles (Garoff *et al.*, 1974). E_2 seemed to be particularly rich in mannose. It has recently been reported that the SFV glycoproteins E_1, E_2, and E_3 are differentially glycosylated (Mattila *et al.*, 1976). E_1 and E_3 were shown to contain, on the average, one type A glycosyl side chain. E_2 was shown to contain one type A glycosyl side chain and possibly one or two B-type glycosyl side chains. A-type oligosaccharides are complex structures containing fucose, galactose, mannose, and *N*-acetyl-

glucosamine, whereas B-type oligosaccharides contain only mannose and *N*-acetylglucosamine (Johnson and Clamp, 1971). The apparent MWs of the A-type units of E_1 and E_3 were 3400 and 4000 daltons, respectively. The B-type side chains of E_2 had an apparent MW of 2000 daltons, and the A-type units an approximate MW of 3100 daltons. Similar studies have been performed with Sindbis virus, and Keegstra *et al.* (1975) showed that one type-A and one type-B residue were attached to each of the two different glycoproteins. The extent of completion of the glycosyl side chains was shown to be dependent in part upon the cell in which the virus was grown (Burge and Huang, 1970; Keegstra *et al.*, 1975). BHK-21 cells were able to complete type-A side chains (i.e., to add galactose and terminal sialic acid), whereas chicken embryo fibroblasts were quite inefficient in adding sialic acid (Keegstra *et al.*, 1975).

Both SFV and Sindbis virus possess hemagglutinating activity. Recent studies by Dalrymple *et al.* (1976) localized this activity to the E_1 glycoprotein of Sindbis virus, whereas the E_2 glycoprotein possesses the antigenic determinants which react with neutralizing antibody.

4. Paramyxovirus Glycoproteins

The virions of paramyxoviruses are covered with glycoprotein surface projections approximately 10 nm in length. Two functionally distinct types of glycoproteins have been isolated from several members of the paramyxovirus group (Scheid *et al.*, 1972; Scheid and Choppin, 1973; 1974). In contrast to the situation in influenza viruses, which possess hemagglutinating and neuraminidase activities on distinct glycoprotein molecules, both of these activities are associated with the larger of the two glycoprotein species in paramyxoviruses, which is designated the HN glycoprotein. The smaller glycoprotein component in paramyxoviruses is associated with cell fusion and hemolysis activities and is designated the F glycoprotein.

Purified glycoproteins of SV5 form rosettelike clusters in the absence of detergents, suggesting that the spikes have hydrophobic bases (Scheid *et al.*, 1972). The aggregates formed are morphologically distinguishable, the HN protein forming berrylike aggregates while the F protein forms distinct rosettelike clusters consisting of radiating spikes 10–13 nm in length with distinct terminal knobs.

In the three best studied paramyxoviruses, SV5, Newcastle disease virus (NDV), and Sendai virus, the HN glycoprotein has a MW range of 65,000–74,000 daltons, and the HN spike solubilized by detergent treatment sediments at 8.9S (Scheid *et al.*, 1972). Although the exact size and fine structure of the HN spike remain to be determined, it has

been suggested that each morphological spike contains at least two glycoprotein monomers (Choppin and Compans, 1975). In some strains of NDV, an 82,000-dalton precursor of the HN glycoprotein designated HN_0 is incorporated into virions (Nagai and Klenk, 1977). These particles have reduced hemagglutinating and neuraminidase activities which are activated upon proteolytic cleavage, which produces the 74,000 dalton HN molecule.

In SV5, NDV, and Sendai virions, the F glycoprotein has a MW of ~56,000 daltons, and the F spike that is solubilized by treatment of SV5 virions with Triton X-100 has a sedimentation coefficient of approximately 6.7S (Scheid *et al.*, 1972). In Sendai virions (Homma and Ohuchi, 1973; Scheid and Choppin, 1974) and certain strains of NDV (Nagai *et al.*, 1976) grown in some cell types, the F glycoprotein is not found, and a larger precursor molecule designated F_0 is observed.

Recent studies have indicated that proteolytic cleavage of the F_0 glycoprotein yields two cleavage products, which have been designated F_1 and F_2 (Shimizu *et al.*, 1974; Nagai *et al.*, 1976; Scheid and Choppin, 1977). The F_2 cleavage product is more highly glycosylated than F_1, and appears to be located on the distal end of the spike; it contains a blocked N-terminal as is also found in the uncleaved F_0 glycoprotein (Scheid and Choppin, 1977). Thus the cleavage of F_0 generates a free N-terminal on the F_1 segment of the glycoprotein, and this appears to expose a new hydrophobic region of the molecule which may be important for virus-induced cell fusion.

The F_0 glycoprotein is inactive in cell fusion and hemolysis and can be converted into the active F glycoprotein by proteolytic cleavage, with concomitant activation of cell fusion and hemolysis. It is of particular interest that virions containing the F_0 precursor are not infective and gain infectivity upon such proteolytic cleavage.

B. Functions of Viral Glycoproteins

1. Adsorption to Receptors

In the case of enveloped viruses, glycoproteins located on the surface of the virion are the components involved in adsorption to cellular receptors, which may or may not be host cell glycoproteins. Hemagglutination by influenza virus has been studied as a model system for the adsorption of a virus to receptors, and considerable information has been obtained. Adsorption to the erythrocyte occurs by the HA spike binding to sialic acid-containing components on the cell surface. The receptor molecule has been isolated from chick red blood cells, and it is a major glycoprotein containing M and N blood group anti-

gens. A detailed summary of the properties of this receptor is given by Schulze (1975), as well as by Tanner in this volume. Removal of sialic acid from the receptor molecule by NA prevents the agglutination of erythrocytes by influenza virus. Hemagglutination can also be inhibited by pretreating the virus with specific antibodies.

While the model for adsorption of influenza virus to erythrocytes seems straightforward, its applicability to the adsorption of viruses to other cell types is uncertain, and little information is available on the nature of receptors for viruses. Liposomes containing gangliosides may be capable of acting as receptors for Sendai virus, a paramyxovirus (Haywood, 1974, 1975). Sialyoglycoproteins inserted in liposomes can also act as receptors, but the fact that gangliosides can act as receptors raises the possibility that glycolipids may be involved in viral attachment.

Further evidence that viral glycoproteins specify virus–host cell interactions has been obtained from the studies of Bishop *et al.* (1975). They produced spikeless particles of the Indiana serotype of VSV by treating the virus with bromelain or pronase. These particles were shown to be noninfectious but, when they were reconstituted with purified G protein isolated from the same strain or from the New Jersey serotype, infectivity was restored. Antibody directed against the homologous G protein used for reconstitution effectively neutralized the virus, but antibody directed against the serotype of the spikeless particle was ineffective in neutralization when glycoproteins from a different serotype were used for reconstitution.

The mechanism of adsorption of other enveloped viruses has not been studied to the same degree as that of ortho- and paramyxoviruses. Herpes viruses do not exhibit hemagglutinating activity, but they possess at least 13 glycoproteins which are asymmetrically located on the external surface of the envelope (Roizman and Furlong, 1974; O'Callaghan and Randall, 1976). Removal of the envelope by nonionic detergents irreversibly alters the infectivity of these viruses (Abodeely *et al.*, 1970).

Oncornavirus glycoproteins react specifically with host cell receptors and in the case of avian leukosis viruses they define the host range as well as the classification into subgroups based on interference and neutralization properties (Vogt and Ishizaki, 1966; Ishizaki and Vogt, 1966; Duff and Vogt, 1969). In addition, Tozawa *et al.* (1970) showed that the viral glycoproteins absorbed homologous neutralizing antibody but not antisera prepared against heterologous virions. The purified glycoproteins were shown to interfere with the early steps in infection by the homologous virus. The determination of host range by the glycoproteins of avian leukosis viruses was further demonstrated

by phenotypic mixing experiments. Defective Rous sarcoma virions that lack the envelope gene cannot infect cells. However, when grown in the presence of an avian leukosis virus, they acquire the glycoproteins of that virus, and the host range of the sarcoma virus reflects that of the leukosis virus (Hanafusa, 1965; Vogt, 1965; Kawai and Hanafusa, 1973). The major glycoprotein of Rauscher murine leukemia virus (gp71) binds specifically to receptor molecules found on murine cells but not on other mammalian cells (DeLarco and Todaro, 1976).

2. Cell Fusion

The phenomenon of cell fusion may be caused by members of several groups of enveloped viruses of which paramyxoviruses are the best studied. The properties of hemolysis and cell fusion are associated with the F glycoprotein as described above. Virus-induced cell fusion may occur in the absence of virus replication, in the presence of high concentrations of virus particles. Both infectious and noninfectious viruses are equally adept at causing such cell fusion which sometimes has been termed fusion from without (Bratt and Gallaher, 1972). In contrast, fusion with low multiplicities of virus has been called fusion from within and is dependent upon replication of the virus. It is likely that the F glycoprotein is involved in both types of fusion phenomena.

The process of fusion through the direct action of concentrated virus has been studied intensively since it was first observed by Okada (1958). Sendai virus-induced fusion involves several separate identifiable steps, as indicated by Maeda *et al.* (1977): (1) adsorption of the virus to the cell, which seems to occur at the tips of the spikes located on the virion surface; (2) aggregation of cells; (3) fusion of the viral envelope with the cell membrane and finally fusion of the cells. These studies suggest that the envelope bilayer of the virion and the membrane of the target cell are brought into contact by the action of the viral HN glycoprotein. The F protein then causes destabilization of the lipids, and an intermixing of lipids occurs. The mechanism of action of the F protein remains to be determined.

3. Neuraminidase Activity

A neuraminidase activity has been shown to be associated with orthomyxoviruses and paramyxoviruses. The functional role of this enzyme was uncertain for many years, and there was even doubt at some point that it was a viral gene product, since similar enzyme ac-

tivity is present in normal cells (White, 1974); however, these doubts have been resolved by studies of the biochemical, genetic, and antigenic properties of viral neuraminidase (see review in Bucher and Palese, 1975). Several distinct functions have been proposed for this enzyme. It is postulated that neuraminidase-containing virus lodges in the upper respiratory tract and binds to mucin via HA. The glycosyl residues of muchin are terminated by sialic acid (*N*-acetylneuraminic acid) to which HA binds, and one role of the neuraminidase may be to cleave the sialoglycoprotein bond, freeing the bound virion. It is presumed that in this way the virus is released, and that underlying cell receptors are exposed to which HA can attach (Davenport, 1976).

It has also been postulated that neuraminidase is involved in an early event such as penetration, but this is unlikely because virions remain infectious after inhibition of neuraminidase activity by specific antibody (Bucher and Palese, 1975). However, the possibility that neuraminidase participates in release of the budding virion from the infected cell surface has gained support from several types of experiments. By using influenza strains with different levels of enzyme activity, it was shown that virus strains having low activity were released from cells more slowly than strains with higher enzyme activity (Palese and Schulman, 1974). Further, in the presence of antibody to viral neuraminidase, which inhibited enzyme activity, virions were formed but release of virus into culture media was inhibited (Seto and Rott, 1966; Compans *et al.*, 1969; Webster, 1970). When bacterial neuraminidase was added to the cultures, infectious virus was released from the cells. These studies, however, are complicated by the fact that bivalent antibody can cause cross-linking of virions to viral antigens on cell surfaces, and Becht *et al.* (1971) reported that monovalent Fab fragments inhibited neuraminidase activity without affecting virus release.

More conclusive information on the function of the enzyme has been obtained with temperature-sensitive mutants of influenza virus which are defective in neuraminidase activity (Palese *et al.*, 1974). At the nonpermissive temperature, no neuraminidase activity is detected; virus particles are produced by cells, despite the fact that infectivity titers are markedly reduced. However, the virus particles form large aggregates, and in contrast to wild-type virions these particles contain sialic acid as shown by colloidal iron hydroxide staining. Since influenza virions bind to sialic acid residues, these results indicate that the mutant virus particles aggregate to each other, because sialic acid is added to viral carbohydrates, and that the essential function of viral neuraminidase is to remove or prevent the addition of such sialic acid. In support of this conclusion, the addition of bacterial neur-

aminidase to cells infected with these mutants cause a marked enhancement of virus release.

Further evidence supporting this role for the neuraminidase was obtained with a neuraminidase inhibitor, 2-deoxy-2,3-dehydro-*N*-trifluoracetylneuraminic acid (FANA). Influenza virus grown in the presence of FANA contains neuraminic acid on its envelope, and the particles undergo extensive aggregation (Palese and Compans, 1976). A marked reduction in virus yield is observed because of this aggregation, and treatment with purified neuraminidase results in a marked enhancement in progeny virus yields, apparently through disaggregation of virus. Thus viral neuraminidase is not required for assembly of progeny virions but appears to be essential for the removal of sialic acid from the surface of the virion itself.

4. Glycoproteins as Antigens

From the studies described above it is evident that the membranes of enveloped viruses are asymmetrically constructed, with the glycoproteins that comprise the spikes or surface projections physically located on the exterior of the viral envelope. Thus the glycoproteins are exposed to the immune surveillance system of the host and, being good immunogens, may elicit humoral and cellular immune responses (Evans, 1976). The classification of virus isolates into specific strains depends largely upon serological procedures, and for enveloped viruses, surface glycoproteins are usually the relevant antigens in such tests. Neutralizing antibodies are usually directed against the viral proteins involved in attachment to receptors, e.g., HA glycoprotein of influenza virus (Webster and Laver, 1975), G protein of VSV (Wagner, 1975), gp69/71 of murine leukemia viruses (Fischinger *et al.*, 1976; Strand and August, 1976), gp85 of avian leukosis viruses (Bolognesi, 1974), and E_2 of Sindbis virus (Dalrymple *et al.*, 1976). High concentrations of antibody can prevent attachment of the virus to receptors, but low concentrations can also neutralize infectivity by a mechanism that is not understood.

The specific determinants of viral glycoproteins recognized by virus-neutralizing antibodies have not been chemically characterized. It has been postulated that antibody molecules may recognize only determinants on the tip of the HA spike of the influenza virion (White, 1974). In reaching these conclusions, it has been assumed that the size of the antibody molecule precludes the possibility that it could make contact with any other part of the spike, since the spaces between the spikes are too small for the immunoglobulin to interact with other regions. Electron microscopic observations (Lafferty and

Oertilis, 1963) indicate that antibody molecules interact with the tips of surface spikes, and analysis of the tryptic peptides of HA molecules isolated from closely related influenza strains has shown that they rarely differ by more than one or two peptides (Laver and Webster, 1972; Webster and Laver, 1972), suggesting that the strain differences are indeed restricted to small regions or determinants of the HA spike.

The antigenic character of many viral glycoproteins is stable, but the determinants exhibited by the glycoproteins of influenza virions are characteristically variable, as evidenced by the many different strains of type-A and -B influenza. The HA and NA glycoproteins of influenza viruses are antigenically distinct, and they undergo antigenic changes independently of each other. The antigenic changes that occur may be gradual, in which case the different virus strains are clearly related to each other with respect to both surface antigens. Antigenic changes of this nature are termed antigenic drift, and they result from the interplay of viral mutability and immunological selection (Webster and Laver, 1975). The presence of antibody of low avidity may select for single-step mutants, which have an altered amino acid in the key area of the antigenic determinant, giving rise to a new viral strain.

At intervals of 10–15 years sudden and complete changes in the determinants of type-A influenza glycoproteins occur; the changes are such that the viruses that arise possess glycoproteins that appear completely distinct on peptide mapping (Laver and Webster, 1972). Dramatic changes in the antigenic determinants of viral glycoproteins are termed antigenic shifts, and it is these new viruses that cause worldwide influenza pandemics. It has been postulated that antigenic shift may occur because type-A strains of human origin may undergo recombination with type-A strains of avian and animal origin. The antigenic determinants of the new HA or NA are sufficiently different that the human host does not possess immunity, and the virus gains a selective advantage. Interestingly type-B influenza viruses do not undergo antigenic shift. The reason for this may lie in the fact that type-B influenza viruses have not been isolated from other animal species; thus it is probable that the type-B viruses can not undergo similar recombination events (Webster and Laver, 1975).

C. Function of Carbohydrates in Viral Glycoproteins

Viral glycoproteins provide excellent systems for analysis of the function of carbohydrates in membrane glycoproteins. Several approaches have been used to modify the carbohydrates, including treatment of virions with specific glycosidases, growth of virus in cells

with specific sugar transferase defects, and treatment of virus-infected cells with inhibitors of glycosylation.

Removal of sialic acid from the G protein of VSV has been reported to reduce the infectivity of VSV virions (Schloemer and Wagner, 1974), whereas the infectivity of SFV (Kennedy, 1974) and Friend leukemia virus (Schäfer *et al.*, 1977) were reportedly unaltered by such treatment. Moreover, enzymic addition of sialic acid to the glycoproteins of Sindbis virus did not alter the infectivity of this virus (Stollar *et al.*, 1976), while enhancing the infectivity of influenza virus (Schulze, 1975) and restoring infectivity to neuraminidase-treated VSV (Schloemer and Wagner, 1974).

Treatment of influenza virions with glycosidases alter hemagglutinating activity, but neuraminidase activity was unaffected; however, similar treatment of NDV, a paramyxovirus, resulted in no alteration in hemagglutinating or neuraminidase activities (Bikel and Knight, 1972). Schäfer *et al.* (1977) showed that the indirect hemagglutinating activity of Friend leukemia virus was inhibited by glycosidase treatment but that viral infectivity and determinants involved in viral interference and absorption of neutralizing antibody were unaltered.

Schlesinger *et al.* (1976) utilized a cell line deficient in the enzyme *N*-acetylglucosaminyltransferase to study the effects of alterations in carbohydrates of enveloped viruses. This enzyme deficiency results in the synthesis of membrane glycoproteins with decreased amounts of *N*-acetylglucosamine, galactose, and sialic acid. When VSV and Sindbis virus were grown in these cells, the apparent MWs of their glycoproteins were lower. The infectivity of VSV and Sindbis virus grown in these cells was not altered from that of fully glycosylated virions. However, glycosidase treatment of SFV was shown to decrease the infectivity of this virus (Kennedy, 1974). The apparent difference between these results remains to be resolved. It is possible that the core of the glycosyl side chain is added to the Sindbis virus glycoproteins in the enzyme-deficient cells, whereas the glycosidase treatment used by Kennedy may have removed more of the glycosyl moieties from the SFV glycoproteins.

Inhibitors of glycosylation have also been used to assess the role of glycosyl side chains of glycoproteins. Primarily, three different inhibitors have been used for this purpose: 2-deoxy-D-glucose (2-dG), an analog of glucose which substitutes for mannose, preventing the further addition of monosaccharides to the glycosyl side chain; D-glucosamine, which is thought to inhibit glycosylation at high concentrations by decreasing UTP pools in the cell and subsequent activation of other sugars (Scholtissek, 1971); and tunicamycin (TM), a glucosamine-containing antibiotic that inhibits the formation of *N*-acetyl-

glucosamine–lipid intermediates which serve as donors for the synthesis of the oligosaccharide side chains of glycoproteins (Tkacz and Lampen, 1975).

Kilbourne (1959) and Kaluza *et al.* (1972) were among the first investigators to utilize inhibitors of glycosylation to study the role of carbohydrates in influenza virions. They showed that 2-dG and D-glucosamine inhibited the biosynthesis of active HA, NA, and mature infectious influenza virions. Subsequent biochemical studies revealed that high concentrations of 2-dG or D-glucosamine prevented the synthesis of influenza virus glycoproteins (Klenk *et al.*, 1972b, 1974; Compans *et al.*, 1974; Nakamura and Compans, 1978a). Instead, an unglycosylated or incompletely glycosylated hemagglutinin precursor HA_0 was detected. Once synthesized, HA_0 was found associated with cytoplasmic membranes, as is the case with the normal glycoprotein. HA_0 glycoprotein in cells infected with the fowl plague strain (FPV) was cleaved by cellular proteases to yield a heterogeneous product (Klenk *et al.*, 1974). However, the comparable protein synthesized in cells infected with the WSN strain was processed and incorporated into virions (Nakamura and Compans, 1978a).

Because 2-dG and D-glucosamine interfere with metabolic reactions other than the glycosylation of glycoproteins, they may cause side effects that can affect virus replication. Therefore TM, a compound that seems to affect only the glycosylation of glycoproteins, has been employed to extend these studies. TM inhibited virion formation in FPV-infected cells and the unglycosylated glycoprotein HA_0 appeared to be degraded by cellular proteases (R. T. Schwarz *et al.*, 1976); HA_0 of the WSN strain synthesized in the presence of TM also appeared to be completely unglycosylated, whereas even at high concentrations of 2-dG and D-glucosamine some glycosylation occurred (Nakamura and Compans, 1978a). Nonetheless, TM did not inhibit virion formation to the same extent as 2-dG (Nakamura and Compans, 1978). The surface spike layer of WSN virions produced in the presence of 2-dG, D-glucosamine, and TM was altered morphologically, and the hemagglutinating activity of the virions was significantly reduced. These results suggest that glycosylation of virion glycoproteins is not required for influenza virion formation but is needed for biological activity of viral glycoproteins.

These glycosylation inhibitors have also been used with several other enveloped viruses. When herpes viruses are grown in the presence of 2-dG, the infectious virus yield is decreased by greater than 95%, but the yield of viral particles is not reduced (Courtney *et al.*, 1973). The reduction in infectivity was attributed to an inability of the virions to attach to the host cell and penetrate, implying that oligosac-

charide residues of herpes virus glycoproteins play a role in the attachment and recognition of host cell receptors.

The glycoproteins of VSV, Sindbis virus, and SFV are not glycosylated in the presence of TM (R. T. Schwarz *et al.*, 1976; Leavitt *et al.*, 1977). Mature VSV and Sindbis virions are not released from TM-treated cells, but nonglycosylated precursors are synthesized and seem to be stable within the cell (Leavitt *et al.*, 1977). Similarly, R. T. Schwarz *et al.* (1976) showed that unglycosylated SFV glycoprotein precursors were synthesized in TM-treated cells. Moreover, they were not degraded by host cell proteases and virion assembly was completely inhibited. It is interesting that, as noted above, HA_0 of FPV is degraded when it is grown in TM-treated chicken embryo fibroblasts, but the glycoprotein precursors of SFV synthesized in the same cells are stable. These results may be due to greater release of protease by FPV infection or, alternatively, the polypeptide backbone of SFV glycoproteins may be less susceptible to proteolytic degradation.

High concentrations of glucosamine rapidly shut off the production of infectious avian sarcoma virus particles (Hunter *et al.*, 1974). However, avian sarcoma virus particles were assembled and released at about 60% of the control level in TM-treated cells, and such particles appeared to lack glycoproteins (R. T. Schwarz *et al.*, 1976). The infectivity titer of the virus produced in TM-treated cells decreased by only 15%.

These results indicate that the effects of glycosylation inhibitors may vary with the virus and host cell, as well as with the specific inhibitor used. Side effects of some inhibitors may have marked effects on virus replication. However, the fact that in some systems virus particles are produced with unglycosylated or incompletely glycosylated glycoproteins clearly demonstrates that the complete glycosylation process is not essential for intracellular migration of glycoproteins or their incorporation into the plasma membrane and subsequently into virus particles. The biological activities of some glycoproteins such as those of influenza virus and SFV appear to require glycosyl moieties, whereas for other viruses, such as murine leukemia virus, this may not be the case.

D. Effects of Viral Proteins on Lipid Bilayer Structure

The membranes of enveloped viruses, particularly those of single-stranded RNA viruses, are unique in their simplicity of construction

and are useful systems for studying the interactions of specific proteins with the lipid bilayer. Hence comparative studies of the effects of viral proteins of the lipid bilayer structure have been made using the techniques of electron spin resonance (ESR), nuclear magnetic resonance (NMR), and fluorescence polarization (FP). ESR studies have provided evidence that the lipids of influenza virus, SV5, Rauscher leukemia virus, VSV, and SFV (Landsberger *et al.*, 1971, 1972, 1973; Sefton and Gaffney, 1974) are bilayer structures with fluid lipid phases similar to those observed for other biological membranes, but all viral membranes were shown to be substantially more rigid than the corresponding host cell plasma membrane. Proteolytic removal of the glycoprotein spikes from the surface of SV5 and influenza virions did not appreciably alter the fluidity of the lipid bilayer of these viruses. Lenard *et al.* (1976) have provided further evidence that the glycoproteins of the influenza virion contribute little to the rigidity of the lipid bilayer. The phospholipid composition of standard influenza particles, and "incomplete" virus produced upon serial undiluted passage, were compared and found to be indistinguishable, as were the ESR spectra of the two types of particles. The incomplete particles were shown to contain approximately twice the amount of glycoproteins relative to the complete particles. Thus it was concluded that the rigidity of viral membranes may be determined by the M protein and not by the viral glycoproteins. This may not be entirely the case for VSV and SFV, since Sefton and Gaffney (1974) and Landsberger and Compans (1976) observed that, when the glycoproteins of these viruses were removed by proteases, the envelope became more fluid, indicating that the viral glycoproteins may contribute to the rigidity of the envelope. However, Landsberger and Compans (1976) postulated that the major effect on VSV bilayer fluidity was exerted by the M protein, since the fluidity of the lipid bilayer was altered only slightly when the G protein was removed by protease, whereas vesicles prepared from extracted viral lipids were much more fluid than lipids in virions.

Using the technique of fluorescence depolarization, Moore *et al.* (1976) and Barenholz *et al.* (1976) showed that the envelope of SFV, Sindbis virus, and VSV has a higher microviscosity than that of the plasma membranes from which the virions budded. The increased microviscosity was attributed in part to insertion of the hydrophobic regions of the glycoproteins into the envelope bilayer. Stoffel and Bister (1975), using NMR spectra of ^{13}C-labeled lipids, also demonstrated that the envelope lipids of VSV are highly rigid, as a result of either lipid–lipid or lipid–protein interactions. In general it may be concluded therefore that the lipid bilayer of enveloped virions is more

rigid than the host cell membrane from which the virion buds. While virion glycoproteins do interact with the lipid bilayer and may affect the rigidity of the membrane to some extent, the internal membrane protein(s) may also be of equal or greater importance in determining membrane rigidity.

V. ASSEMBLY OF VIRAL MEMBRANES

A. Cellular Sites of Maturation

Most enveloped viruses form by a process of budding at the plasma membrane, with little or no participation of other membrane structures in the final steps of maturation. However, there are important exceptions in the case of certain virus groups. The capsids of herpes viruses are assembled in the nucleoplasm and are observed to bud through the inner nuclear membrane, acquiring their envelopes in the process (Roizman and Furlong, 1974; O'Callaghan and Randall, 1976). They appear to be transported as enveloped particles through cytoplasmic channels to the cell surface. Bunyaviruses (Murphy *et al.*, 1973) and coronaviruses (Oshiro, 1973) appear to form primarily by budding into cytoplasmic cisternae, and extracellular virions are observed associated with the plasma membrane. For the rhabdovirus group, several modes of maturation have been reported. VSV, the most widely studied member, usually forms by budding at the cell surface, but maturation at intracellular membranes has been observed. The New Jersey strain of VSV was observed to bud primarily from plasma membranes of L or Vero cells, and almost entirely at intracytoplasmic membranes of pig kidney cells (Zee *et al.*, 1970). These reports indicate that the site of maturation of a specific virus type may vary depending on the host cell. Rabies virus, which resembles VSV morphologically and biochemically, is exceptional in that assembly of the virion appears to occur through a process involving *de novo* formation of membranes in the cytoplasmic matrix (Hummeler *et al.*, 1967). *De novo* formation of membranes is the usual process of assembly for members of the pox virus group (Dales and Mosbach, 1968).

The appearance of viral proteins on the cell surface, as well as the cellular site of virus assembly, can be modified by external agents including antibodies and lectins. The phenomenon of antigenic modulation involves altering the expression of cell surface antigens by specific antibody, and such antigens may be of viral origin (Lampert *et al.*,

1975). This undoubtedly has an effect on viral maturation, although the precise effects have not been determined. Exposure of influenza virus-infected cells to concanavalin A appears to alter the site of virus maturation (Stitz *et al.*, 1977). In the presence of this lectin, normal maturation at the plasma membrane is not observed, but instead large numbers of virions are observed in intracellular vacuoles.

It is evident therefore that the maturation site for enveloped viruses can vary with the virus type as well as the host cell, and can be altered in response to specific stimuli. The process is likely to be determined as a result of interactions between virus-specific proteins and host cell membranes. It is remarkable that in most instances only a single type of cellular membrane is selected as the site of virus assembly in a particular virus-infected cell. An understanding of the mechanisms which govern the selection of the assembly site may provide new insights into the assembly of cellular membrane components and organelles, since similar interactions are likely to be involved in determining the location of subsets of cellular proteins in specific cellular organelles.

B. Synthesis and Intracellular Localization of Envelope Proteins

The synthesis and assembly of viral membrane components have been analyzed in cells in which host cell synthesis is inhibited as a result of virus infection. Similar conclusions have been made for several virus types. Viral glycoproteins appear to be synthesized on membrane-bound polyribosomes and remain associated with various cellular membranes (Spear and Roizman, 1970; Compans, 1973a,b; Stanley *et al.*, 1973; Klenk *et al.*, 1974; David, 1973; Hay, 1974; Atkinson *et al.*, 1976; Nagai *et al.*, 1976; Knipe *et al.*, 1977). Glycosylation occurs in association with cytoplasmic membranes. Glycoproteins appear to migrate from rough endoplasmic reticulum to smooth or Golgi complex membranes to the cell surface and are then incorporated into virions; at no time are they found as "soluble" cytoplasmic components. This general scheme for the synthesis and migration of proteins through intracellular membranes to the cell surface has been termed membrane flow. Although the precise intracellular location of viral glycoproteins remains to be established, a scheme may be envisaged in which these components remain associated with the cisternal side of membranes of the endoplasmic reticulum and Golgi complex after synthesis. Incorporation into the plasma membrane by a process of vesicle fusion would then result in the correct orientation on the cell surface for viral as-

sembly. A similar scheme has been suggested for the incorporation of glycoproteins into the plasma membrane, based on the distribution of oligosaccharides in different membrane fractions (Hirano *et al.*, 1972). Viral systems allowed investigators to follow the intracellular migration and glycosylation of specific glycoprotein species for the first time, whereas previous studies with cellular glycoproteins had dealt with cell fractions essentially uncharacterized as to the specific proteins present.

Recently, Katz *et al.* (1977) and Rothman and Lodish (1977) have described a new model system for studies of the incorporation of viral glycoproteins into membranes. Katz *et al.* (1977) have shown that the mRNA for VSV G-protein translated *in vitro* by wheat germ extracts in the presence of dog pancreas rough endoplasmic reticulum is associated with the membrane. Furthermore, the newly synthesized G protein was shown to span the membrane with the amino-terminal asymmetrically located. In addition, the polypeptide portion which penetrated the membrane was shown to be glycosylated. Glycosylation did not occur in the absence of membranes. These studies were extended by Rothman and Lodish (1977), who showed that the insertion of G-protein into the membrane begins when 80 or fewer amino acid residues are polymerized. Evidence was also obtained that the nascent chain is glycosylated while still attached to the ribosomes on the cytoplasmic side of the endoplasmic reticulum vesicle. Since the mechanism by which viral glycoproteins are inserted into membranes is in all probability similar to that of the insertion of host cell glycoproteins, further studies using viral systems may provide more insight into the mechanism of insertion of glycoproteins into membranes.

The process of glycosylation does not appear to play an important role in determining the intracellular migration of glycoproteins. Although this has been suggested as a possible function for carbohydrate components of glycoproteins, the available data using inhibitors of glycosylation suggest that it is possible to inhibit or extensively modify the glycosylation process without preventing the migration of viral glycoproteins to the cell surface (Courtney *et al.*, 1973; Compans *et al.*, 1974; Nakamura and Compans, 1978a).

The incorporation of carbohydrate-free M proteins into membranes appears to involve a distinct pathway in which cytoplasmic synthesis is followed by rapid association with the plasma membrane (Lazarowitz *et al.*, 1971; Meier-Ewert and Compans, 1974; Hay, 1974; Nagai *et al.*, 1976; Knipe *et al.*, 1977). No evidence for migration through cytoplasmic membrane structures has been obtained for these components, and it has been suggested that they are inserted directly

into membranes after synthesis. Cytoplasmic synthesis followed by direct insertion into membranes has also been proposed for some classes of cellular membrane proteins (Lodish and Small, 1975).

C. Sequence of Events in Viral Assembly

Although there are several unanswered questions concerning the precise steps in assembly even for the best studied enveloped viruses, the available information from electron microscope studies as well as the biochemical approaches described above suggest a scheme like that depicted in Fig. 2 for influenza virus. Glycoproteins appear to be inserted into the plasma membrane as the first step in assembly, after migration through cytoplasmic membranes. After they are inserted into the plasma membrane, initial random distribution may occur in which the proteins are free to undergo lateral diffusion in the plane of the membrane. Such random distribution is illustrated for the glycoproteins of parainfluenza virus in Fig. 3, and similar observations have been reported for other viruses (Birdwell and Strauss, 1974; H. Schwarz *et al.*, 1976). Random distribution of viral antigens (Fig. 3) is observed only when ferritin–antibody conjugates are applied to cells after glutaraldehyde fixation. In previous studies of the distribution of antigen, in which unfixed cells were used (Compans and Choppin, 1971), antigens were observed in discrete patches. It is likely that under these conditions they undergo lateral redistribution and aggregation into patches as a result of bivalent antibody.

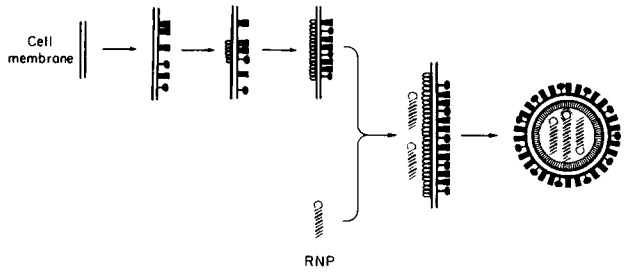
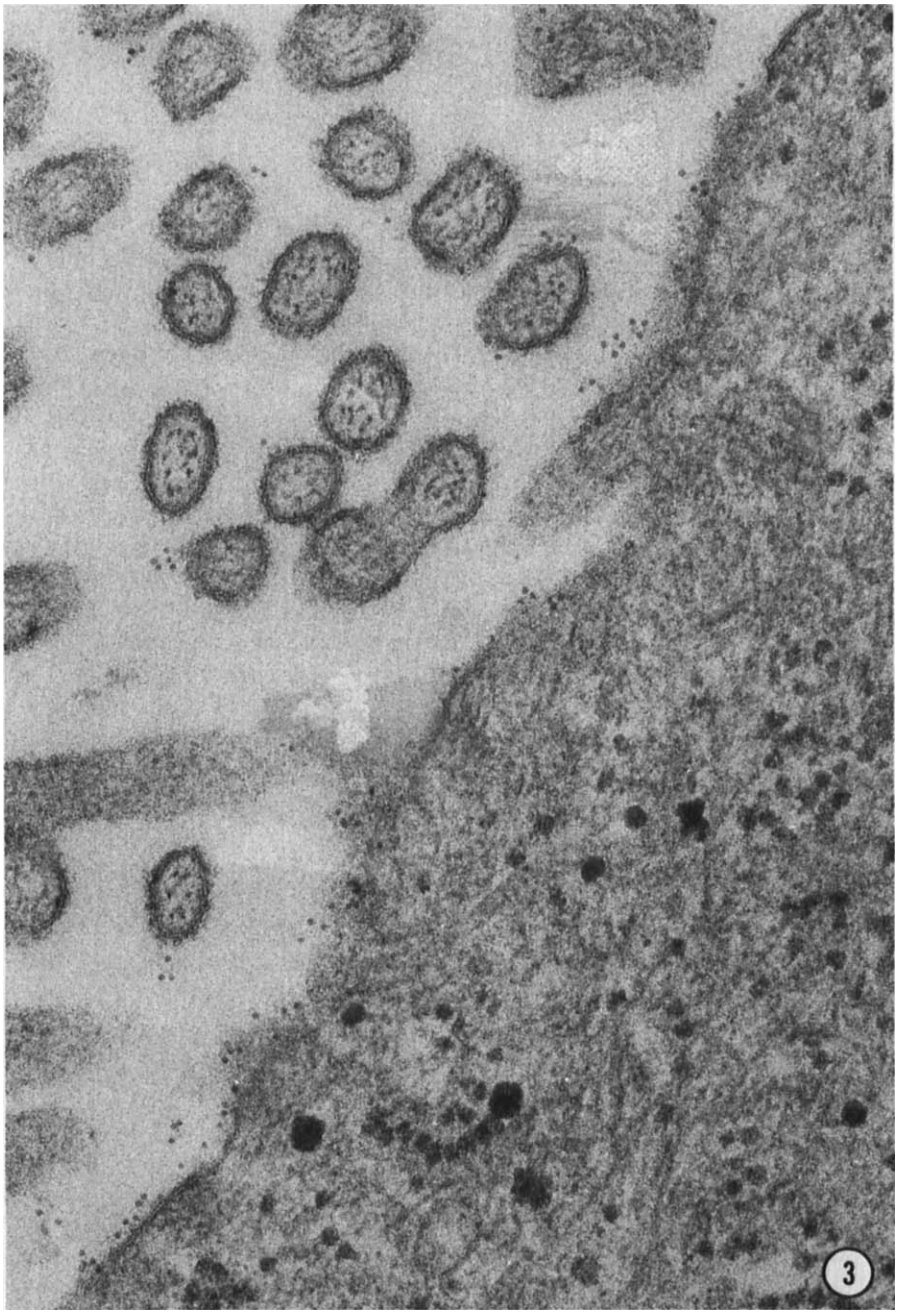


FIG. 2. Schematic diagram of the assembly process of an influenza virion. Glycoproteins are thought to be inserted into the plasma membrane by a process called membrane flow and are initially found randomly dispersed in the membrane. Following insertion of the M protein, glycoproteins are thought to accumulate in discrete regions from which host cell membrane proteins are excluded. The association of the ribonucleoprotein (RNP) with such regions of modified membrane is followed by budding and release of the completed virion.



FIG, 3. Labeling of the surface of an infected cell with ferritin-conjugated antibody to the parainfluenza virus SV5. The cell was fixed with glutaraldehyde prior to the application of antibody. Under these conditions, ferritin molecules appear randomly dispersed on the cell surface. × 100,000.

In the case of influenza virus, the M protein may form a domain on the internal surface of the plasma membrane, stabilized by protein–protein interactions. Specific recognition of the M protein by the glycoproteins could then produce an accumulation of glycoproteins in a circumscribed region of the cell surface. Alternatively, it is possible that M protein monomers associate with glycoprotein monomers at the plasma membrane, and that these complexes undergo lateral diffusion and aggregation into domains. In either case the lack of host cell membrane proteins in the viral envelope indicates that cellular proteins are efficiently excluded from the region of the plasma membrane which becomes the viral envelope.

Association of the nucleocapsid with regions of the cell surface containing viral envelope protein may stimulate the process of budding. The formation of virions involves outfolding of the cell membrane and envelopment of the nucleocapsid by the modified membrane. Some examples of the final stages in assembly of the parainfluenza virus SV5 are depicted in Figs. 4 and 5. Regions of the cell membrane, with underlying helical nucleocapsids, are shown in Fig. 4. The presence of virus-specific glycoproteins on the external surface is indicated by tagging with ferritin-conjugated antibody. In Fig. 5, the emerging virus particles tagged with ferritin–antibody are shown at higher magnifica-

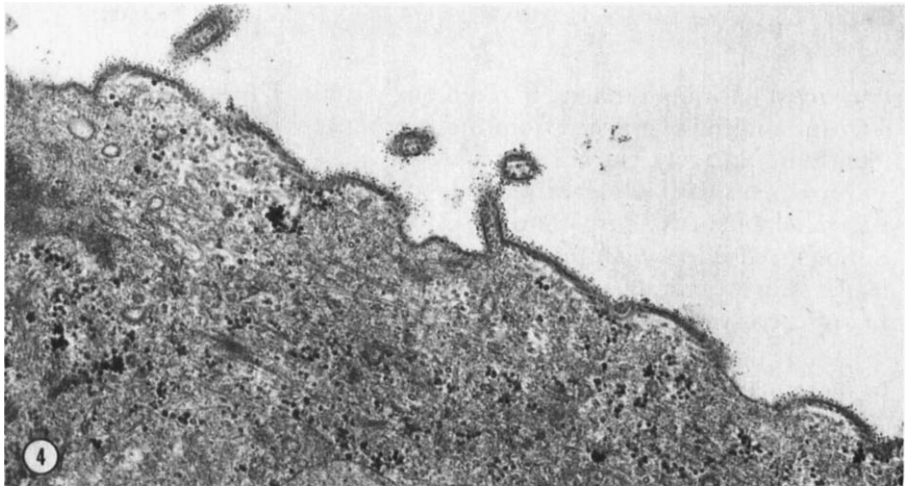


FIG. 4. A region of the surface of an MDBK bovine kidney cell infected with the parainfluenza virus SV5. The helical nucleocapsids of the virus are aligned under the cell membrane, and the external surface of the cell in these regions is tagged with ferritin-labeled antiviral antibody. ×35,000.

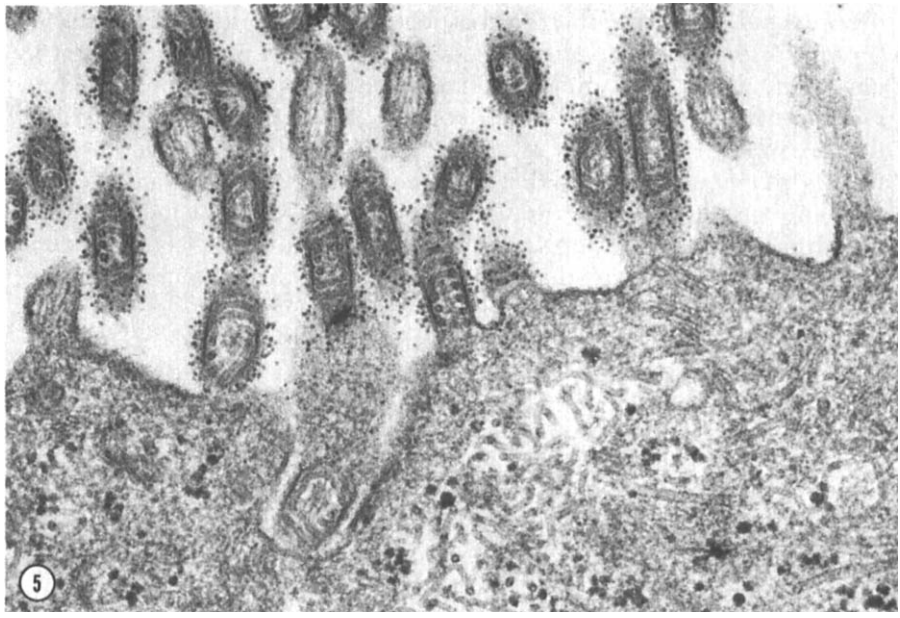


FIG. 5. Filamentous SV5 virions emerging from the surface of an infected MDBK cell. The virions are clearly tagged with ferritin–antibody, whereas adjacent areas of the cell surface are devoid of viral antigen. The helical nucleocapsids, in cross section or longitudinal section, are seen clearly in the emerging virus particles. ×80,000.

tion; most particles are long filamentous virions. The specificity of the ferritin labeling is evident from the tagging of virions and absence of ferritin on adjacent cell membranes.

For viruses with icosahedral nucleocapsids and no M protein, a similar mechanism for virus assembly has been proposed (Garoff and Simons, 1974) in which the nucleocapsid itself binds to glycoprotein-containing membranes, with subsequent lateral diffusion and clustering of the glycoproteins in this region.

D. Macromolecular Interactions in Membrane Assembly

Although information is accumulating about the pathways by which viral membrane proteins are incorporated into plasma membranes, there is little direct evidence concerning the precise interactions which lead to the formation of domains on the cell surface which contain virus-specific proteins and lack host cell proteins. The lack of significant amounts of host cell protein in the virion indicates that such

domains must be intermediates in assembly. Further, the available data on viral protein composition (Table II) and morphology suggest that assembly interactions may differ for various virus groups. In all cases it is likely that protein–protein interaction serves to create a patch of virus-specific proteins in a cellular membrane, but this interaction may occur on the external or internal surface of the lipid bilayer. As discussed above, for viruses that contain M proteins or well-defined icosahedral nucleocapsids, some evidence has been obtained for the initial random distribution of glycoproteins on the cell surface, which may be followed by lateral diffusion and accumulation of glycoproteins in juxtaposition to the M protein or icosahedral nucleocapsid, with transmembrane interactions between the external and internal proteins serving to anchor the viral glycoproteins in place. With other virus types that contain a large amount of glycoprotein and no obvious M protein or well-defined icosahedral capsid, it is possible that lateral interactions between the glycoproteins are important in assembly. The glycoproteins of togaviruses and bunyaviruses have been observed in a regular surface arrangement (von Bonsdorff and Harrison, 1975; von Bonsdorff and Pettersson, 1975). The latter viruses lack an M protein or an icosahedral internal component, and it has been suggested that direct interactions between glycoproteins may be involved in assembly and maintenance of the viral structure (von Bonsdorff and Pettersson, 1975). Similar interactions may be important in other virus groups in which glycoproteins are major protein constituents of the virion and there is no known internal membrane protein, such as B-type oncornaviruses and coronaviruses.

Pox viruses are unique in that formation of their lipid-containing membrane occurs *de novo* in the cytoplasm, rather than on a preexisting membrane structure. These viruses thus provide an unusual system for investigation of the molecular interactions involved in the formation of a highly organized structure within the cytoplasmic matrix (Dales and Mosbach, 1968). However, these viruses are structurally very complex, and limited information has been obtained on their molecular organization.

Shape and size determination may also be controlled by viral proteins at various levels; nucleocapsids, M proteins, or glycoproteins could be the determining factor for different virus groups. The phenomenon of phenotypic mixing of envelope glycoproteins in cells doubly infected with VSV and the parainfluenza virus SV5 clearly demonstrates that the internal proteins and not the glycoproteins determine the particle size and shape of rhabdoviruses (Choppin and Compans, 1970; McSharry *et al.*, 1971). This conclusion is supported

by the observation that particles with the internal proteins of VSV possess the characteristic bullet shape of VSV while containing mixtures of envelope glycoproteins derived from VSV and SV5. A similar analysis of phenotypically mixed particles produced by dual infections with viruses of other major groups may provide further insights into the macromolecular interactions involved in virion assembly.

The fixed shape and size of many lipid-containing viruses stands in contrast to the situation in membranous cellular organelles, which generally exhibit pleomorphism. Similar pleomorphism is observed in some enveloped viruses. The assembly of such membranes of organelles and pleomorphic viruses may be regulated more by the production of materials than by precise constraints imposed by macromolecular interactions.

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