Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Biochimica et Biophysica Acta, 344 (1974) 51–94
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 85134

THE MEMBRANE STRUCTURE OF LIPID-CONTAINING VIRUSES

JOHN LENARD[a] and RICHARD W. COMPANS[b]

[a]*Department of Physiology, CMDNJ-Rutgers Medical School, Piscataway, N.J. 08854, and* [b]*The Rockefeller University, New York, N.Y. 10021 (U.S.A.)*

(Received September 26th, 1973)

CONTENTS

I. Introduction 52
II. The enveloped viruses 53
  A. Classification into major groups 53
  B. Brief descriptions of the major groups of enveloped viruses 53
  C. Modes of virus replication 56
  D. Chemical composition 57
III. Virion polypeptides 58
  A. Nucleocapsid proteins 60
  B. Glycoproteins 60
  C. Non-glycosylated polypeptides 64
  D. Absence of host cell proteins 65
  E. Methods for isolation of viral proteins 66
IV. Virion lipids 67
  A. The viral bilayer 68
  B. Origin of viral lipids 70
V. Lipid–protein interactions 75
VI. Organization of lipid and proteins in enveloped viruses: A summary 78
VII. Assembly of viral membranes 79
  A. Morphological studies 79
  B. Modes of assembly of patches of viral membrane proteins 80
  C. Synthesis and intracellular location of viral envelope proteins 83
  D. Glycosylation of viral glycoproteins 85
  E. De novo assembly of viral membranes 86
VIII. Structural relationships of enveloped viruses to other viruses 87
IX. Viral membranes and the fluid mosaic model 88
Acknowledgments 89
References 89

Abbreviations: *Viruses:* SFV, Semliki Forest virus; NDV, Newcastle disease virus; SV5, simian virus 5; VSV, vesicular stomatitis virus; RSV, Rous sarcoma virus. *Cells:* CEF, chick embryo fibroblasts; BHK, BHK21 line of baby hamster kidney cells; MDBK, Madin–Darby bovine kidney cell line; MK, primary rhesus monkey kidney cells; HaK, hamster kidney cell line; RK, primary rabbit kidney cells.

I. INTRODUCTION

A variety of animal viruses contain lipid as a major structural component of the virus particle. In addition, it has recently become apparent that lipid-containing viruses occur in plants and bacteria as well, although their known occurrence is not as frequent as in the case of viruses which infect animal cells. The lipid is generally present in a limiting membrane structure termed the viral envelope; hence these viruses are referred to as enveloped viruses. Although the presence of lipid serves to distinguish enveloped from non-enveloped viruses, lipid-containing viruses are a diverse group of agents which can be classified into several major groups on the basis of the structural properties of the virus particle. Fortunately, viruses which were originally classified together on the basis of structure have turned out almost invariably to be very similar in the biochemical processes of their replication, and to some extent in their biological properties.

In this paper we will review recent studies on the structure and composition of lipid-containing viruses, which indicate that there are many basic similarities in their membrane structure. Particular emphasis will be placed on those groups of viruses which form by a process of budding at a cellular membrane. These viruses are excellent systems for the study of the structure and biosynthesis of membranes for a variety of reasons, which will be discussed at length in this paper. The viruses can be isolated in a high degree of purity and contain a limited number of structural protein components, all of which appear to be coded by the viral genome. The lipids of the membranes of many of these viruses closely reflect those of the plasma membrane of the host cell of origin; thus it is possible to obtain viruses with the same proteins but different lipids by growing a given virus in two different cell types. Similarly, one can obtain viral membranes with similar lipid composition but no protein in common by growing two different viruses in the same cell type. For studies of membrane biogenesis, it is significant that the envelope proteins of many of these viruses become an integral part of the cell surface membrane prior to formation of virus by a process of budding. In addition, it is often the case that host cell biosynthesis is markedly inhibited during virus infection, and it is therefore possible to analyse a system in which only a small number of identifiable proteins are produced by a cell, and to follow the pathway by which they are incorporated into the cellular plasma membrane.

It is not our purpose to review the very extensive literature on the replication and biology of the various membrane-containing viruses. However, as a background for the discussion of viral membrane structure, we will present in the following section a brief description of the major groups of lipid-containing viruses, their biological properties, morphology, and overall chemical composition. Several sources are available for more extensive general information on virus classification, biological properties and composition [1–3].

II. THE ENVELOPED VIRUSES

IIA. Classification into major groups

Viruses are placed into major groups on the basis of the following structural properties: (1) Type of nucleic acid (RNA or DNA, single- or double-stranded, molecular weight); (2) symmetry and organization of the nucleocapsid, i.e. the symmetrical protein shell which encloses the nucleic acid; and (3) presence or absence of an envelope enclosing the nucleocapsid.

TABLE I

MAJOR GROUPS OF LIPID-CONTAINING VIRUSES

DS, double-stranded; SS, single-stranded.

Group	Nucleic acid		Virion		
	Type	Molecular weight ($\times 10^{-6}$)	Shape	Size (Å)	Capsid structure
Pox virus	DS DNA	160–200	Brick shaped	3000 × 2000	Complex
Herpes virus	DS DNA	Approx. 100	Spherical	1200	Icosahedral
PM2 phage	DS DNA	6	Spherical	600	Icosahedral
Togavirus	SS RNA	2–3	Spherical	700	?
Myxovirus	SS RNA	3–4	Spherical or filamentous	1000	Helical
Paramyxovirus	SS RNA	6–7	Spherical or filamentous	1200–2000	Helical
Rhabdovirus	SS RNA	4–6	Bullet shaped	700 × 1750	Helical
RNA tumor virus	SS RNA	10–12	Spherical	1200	?
Arenovirus	SS RNA	?	Spherical	600–1200	?
Coronavirus	? RNA	?	Spherical	800–1200	?

The major groups of lipid-containing viruses are listed in Table I. Although this table serves primarily to classify the animal viruses, a number of plant viruses may also fit into some of these groups. The lipid-containing bacteriophage PM2 has no structural counterpart among animal virus and is therefore listed as a distinct virus group.

IIB. Brief descriptions of the major groups of enveloped viruses

The morphology of some of the best-studied enveloped viruses is shown in Fig. 1. There is considerable variation in size, shape and capsid structure among the viruses illustrated. However, there is a basic similarity in that all are surrounded by a membrane with a layer of spikes or projections on the outer surface.

Pox virus group. These are the largest and most complex of any of the animal viruses. The virions are roughly rectangular and have a complex morphology with an internal core enclosed by a membrane, two lateral bodies, and an outer covering of filamentous material. They are the only DNA-containing viruses known to replicate in the cytoplasm. The best-studied member of the group is vaccinia virus.

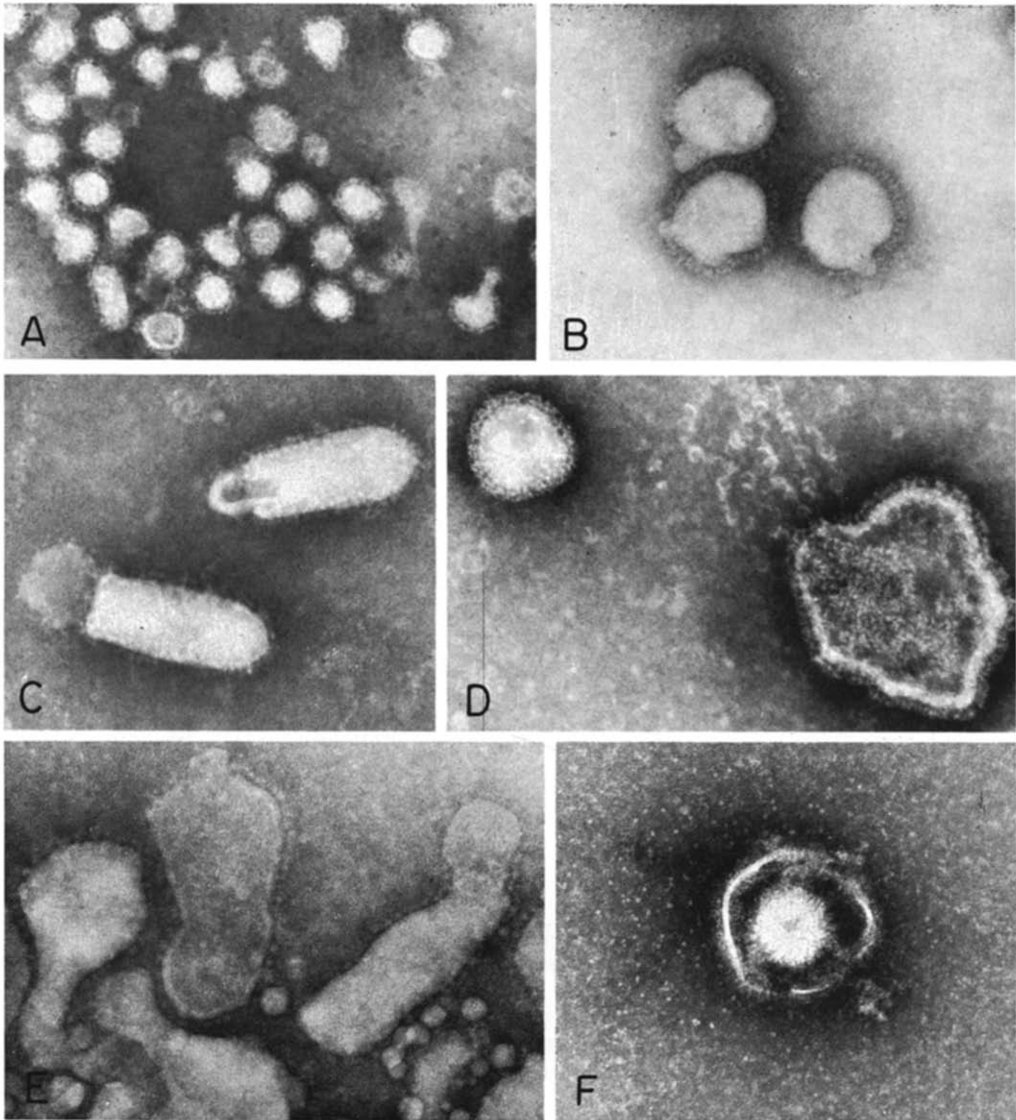


Fig. 1. Electron micrographs of enveloped virus particles, negatively stained with sodium phosphotungstate. A. Sindbis virus (× 100 000). B. Influenza virus (× 140 000). C. Vesicular stomatitis virus (× 140 000). D. Parainfluenza virus SV5 (× 100 000). E. Rous sarcoma virus (× 140 000). F. Herpes simplex virus (× 100 000).

Other members of this group cause diseases in a variety of animal species; often a skin rash occurs during infection.

Herpes virus group. The herpes viruses are another group of large DNA-containing viruses. The icosahedral nucleocapsids are assembled in the nucleus and acquire a membrane during maturation, which occurs at the nuclear envelope. The

Herpes simplex virus of man and pseudorabies virus of pigs are the two viruses which have been studied in greatest detail. Recently evidence has been obtained that Herpes simplex virus may be an oncogenic virus [4].

Bacteriophage PM2. This is a small icosahedral DNA phage which possesses lipid as a structural component of its capsid-like shell. It differs in structure from any animal or plant virus group.

Togavirus group. These are the smallest of the RNA-containing enveloped viruses and the simplest in terms of composition. The name indicates the presence of an outer coat or envelope. Most members of the group were originally classified as arboviruses, a name derived from the fact that they are "arthropod-borne". The arboviruses consist of over 200 different agents, many of which are causes of encephalitis. There are two main antigenic subgroups, A and B, with spherical particles 600–700 Å in diameter. However, other arboviruses differ in morphology; hence the name togavirus has been adopted for the group of agents which have the structure of the arboviruses in groups A and B. Some viruses such as rubella, which are not arthropod-borne, are also included in the togavirus group because they possess similar structural features. The envelope encloses a nucleocapsid which may be icosahedral. Sindbis virus and Semliki Forest virus (SFV) have been studied in greatest detail.

Myxovirus group. This group includes the influenza viruses of man and animals, and was named on the basis of the affinity of these viruses for "certain mucins", i.e. neuraminic acid-containing mucoproteins [5]. The virus particles may be roughly spherical, approx. 1000 Å in diameter, or filamentous particles of the same diameter but up to several micrometres in length. They possess a spike-covered envelope which encloses an internal component composed of multiple short ribonucleoprotein strands about 100 Å in diameter, each of which contains a molecule of single-stranded RNA.

Paramyxovirus group. These viruses were originally classified as myxoviruses because of a similar affinity for mucins, but have since been placed in a distinct group because of marked differences in structure and replication. The virions are larger and possess a helical nucleocapsid, 180 Å in diameter, which contains a single RNA molecule. A number of these viruses cause respiratory infections, whereas others such as mumps and measles produce generalized disease. Some of the best-studied paramyxoviruses are Newcastle disease virus (NDV), simian virus 5 (SV5) and Sendai viruses.

Rhabdovirus group. This group is characterized by a distinct bullet-shaped morphology. The internal component is formed by a strand which is coiled into a cylinder-like structure, and is contained by a membrane closely wrapped around it. Vesicular stomatitis virus (VSV) is the best-studied member of the group. Rabies virus, several arthropod-borne viruses, and some plant viruses also have the characteristic morphology of the rhabdovirus group.

RNA tumor virus group. RNA viruses from several animal species have been shown to be causes of leukemias or solid tumors. They possess very similar biochem-

ical and morphological properties and are therefore classified as a distinct virus group, although no name has been universally adopted. Rous sarcoma virus (RSV) is probably the best studied of these agents.

Arenovirus group. This group includes lymphocytic choriomeningitis virus and several arthropod-borne viruses. They have a limiting membrane which encloses dense internal granules which may be ribosomes. Information on their composition and replication is limited.

Coronavirus group. This is another group with similar morphological properties, but there is limited information on their structure, composition and replication. Mouse hepatitis, avian infectious bronchitis, and some unnamed human respiratory viruses are members.

IIC. Modes of virus replication

The processes of replication of enveloped viruses will not be described in detail, and we will merely point out that it has recently become apparent that a variety of pathways are utilized in the replication of viruses of different major groups. This is particularly true for viruses with RNA genomes, as summarized in Table II.

TABLE II

MODES OF REPLICATION OF ENVELOPED RNA VIRUSES

Major group	State of RNA in virion*	Relation of genome to mRNA	Template for synthesis of genome
Togavirus	Single molecule	Genome is mRNA	RNA
Myxovirus	Segmented	mRNA is complementary to genome (tentative)	RNA
Paramyxovirus	Single molecule	mRNA is complementary to genome	RNA
Rhabdovirus	Single molecule	mRNA is complementary to genome	RNA
RNA tumor virus	Segmented	?	DNA
Arenovirus	Probably segmented	?	?

* All groups contain single-stranded RNA genomes.

RNA genomes occur either as a single molecule or as multiple segments. In many instances the viral RNA does not appear to be the messenger for protein synthesis, but molecules complementary to the genome serve as messenger RNA. Furthermore, the synthesis of progeny genomic RNA may either occur on an RNA or DNA template. Viruses in which messenger RNA is complementary to virion RNA contain a polymerase in the virion, which catalyses the synthesis of the complementary RNA. RNA viruses which replicate through a DNA intermediate contain an enzymatic activity in the virion which catalyses the synthesis of DNA which is complementary to viral RNA.

DNA viruses, in contrast, all appear to contain single molecules of nucleic acid as their genetic material, and the events in their replication probably show less diversity than the RNA viruses.

Viruses also differ in the process of protein synthesis. In some cases a single large polypeptide is the initial product of synthesis directed by a single messenger RNA, and is subsequently cleaved into multiple polypeptide species. With other viruses the final polypeptide products are coded directly by distinct messenger RNAs.

In no instance does the mode of replication appear to determine the structural properties of the virus particle. Thus it will be seen that viruses which differ greatly in their replicative mechanisms show marked similarities in their membrane structure. Those components involved in the enzymatic activities necessary for virus replication may be relatively minor components which do not play an important role as structural components of the virion.

IID. Chemical composition

Data on the overall chemical composition of virions in each of the major groups of lipid-containing viruses are summarized in Table III. All these viruses contain protein, lipid and carbohydrate in addition to their nucleic acid. The nucleic acid comprises a small part of the mass of the RNA viruses but a considerable percentage of the mass of the large DNA-containing viruses. The overall proportions of the remaining chemical constituents are fairly similar for most major groups. Pox viruses are exceptional in possessing a much lower lipid content, as percent of dry mass, than any of the other viruses.

The carbohydrate of enveloped viruses appears to be found both as glycolipid and glycoprotein in the viral envelope. No purely polysaccharide compound has been identified in these viruses. Of the total carbohydrate in the paramyxovirus SV5 grown in Madin–Darby bovine kidney (MDBK) cells, 65% was estimated to be in the form of glycoprotein and 35% glycolipid [15]. The total carbohydrate composition of VSV was found to differ substantially from that of isolated glyco-

TABLE III

CHEMICAL COMPOSITION OF LIPID-CONTAINING VIRUSES

Group	Composition (%)				References
	Nucleic Acid	Protein	Lipid	Carbohydrate	
DNA viruses:					
Pox virus	5.6	85–90	5.8	2.8	6
PM2 phage	approx. 10	75	approx. 15	trace	7
RNA viruses:					
Togavirus	5–6	approx. 60	29	7	8,9
Myxovirus	0.9	60–70	approx. 25	5–8	10,11
Paramyxovirus	0.9	73	20	6	12
Rhabdovirus	3	65	20	13	13
RNA tumor virus	1.9	64	31	6	14

protein [16]. In another study it was shown that 87 % of the sialic acid of VSV grown in baby hamster kidney (BHK21) cells is bound to protein and 13 % to lipid [17].

III. VIRION POLYPEPTIDES

Early information on protein components of enveloped viruses was obtained primarily from immunological identification of viral antigens, and from biological assays for certain viral proteins. The use of the procedures of classical protein chemistry, as applied to soluble proteins, has had very limited success with viral proteins because they are particularly difficult to obtain in a monodisperse solution unless strong dissociating conditions are used. The introduction of the technique of polyacrylamide gel electrophoresis in buffers containing sodium dodecylsulfate by Maizel [18,19] provided the methodology for a systematic analysis of viral polypeptides, as well as a simple method for evaluating the molecular weights of the polypeptide chains [20]. All virus particles which have been studied are dissociated into their individual polypeptide chains when heated in the presence of sodium dodecylsulfate and a reducing agent.

The polypeptide composition of some representative enveloped viruses is summarized in Table IV. The toga-, myxo-, paramyxo- and rhabdovirus groups have a small number of polypeptide components, and the patterns obtained from different viruses in each group are very similar with some differences in minor components. Because of their relatively simple polypeptide composition, the most detailed information on the arrangement of structural components is available for these viruses. The herpes, pox and RNA tumor viruses are much more complex in structure, and much remains to be done before a complete picture of the arrangement of their polypeptide components is achieved. However, there is no evidence to suggest that the basic membrane structure of herpes or RNA tumor viruses, both of which form by budding at cell membranes, is different in principle from that of the simpler viruses. The pox viruses, which will not be discussed in detail, differ in that their membrane forms de novo in the cytoplasm (Section VIIE). They have a much more complex structure, about which many questions remain to be answered.

Several general conclusions have emerged from the extensive studies which have been carried out on proteins of enveloped viruses: (1) Most viruses contain a single polypeptide which is closely associated with the viral nucleic acid in a symmetrical nucleocapsid structure. (2) All enveloped viruses contain one or more species of glycoproteins which are located on the external surface of the viral envelope, forming the layer of projections which are visible by electron microscopy. (3) All carbohydrate-free polypeptides appear to be located in the interior of the virus particle. In many viruses, one of these non-glycosylated polypeptides is a major component which appears to be associated with the inner surface of the viral membranes. (4) The viral polypeptides are coded by the viral genome. This is particularly clear with the major structural polypeptides, and even with minor components there

TABLE IV

POLYPEPTIDE COMPOSITION OF SOME ENVELOPED VIRUSES

Group		Virus	Type of polypeptide*				References***
			Nucleocapsid subunit	Glycoproteins	Other major polypeptides**	Minor polypeptides**	
I.	Togavirus	Sindbis	32 000	53 000 47 000	–	–	21
II.	Myxovirus	Influenza Ao/WSN	60 000	75 000 or 50 000 + 25 000 55 000	26 000	1–2 species 80 000–100 000	22–24
III.	Paramyxovirus	SV5, NDV, Sendai	60 000	65 000–75 000 55 000	40 000	1–4 species 50 000–75 000	25
IV.	Rhabdovirus	VSV	52 000	67 000	25 000	4 species 40 000–175 000	26
V.	RNA tumor virus	RSV	?	3–4 species 30 000–110 000	4–5 species 11 000–26 000	Several species	27–29
VI.	Arenovirus	Pichinde	?	72 000 34 000	72 000	?	30
VII.	Herpes virus	Herpes simplex	approx. 11 species	Several species	Several species	Over 10 species	31,32
VIII.	Pox virus	Vaccinia, Fowl pox	–	39 000 41 000	5–6 species 11 000–63 000	Over 20 species	33,34

* The approximate molecular weight of each major structural polypeptide is indicated. If a small number of components of the same type (e.g. glycoprotein) are present, the molecular weight of each species is given. For minor components or large numbers of components of the same type, the number of species present and their range of molecular weights are given.

** In virus groups II–IV, "major" polypeptides comprise more than 20% of the total protein content. For groups V, VII, and VIII, which contain a large number of polypeptides, "major" polypeptides comprise more than 5% of the total protein content.

*** These references were selected because they describe the largest number of polypeptides and glycoprotein species which appear to be virion components. Numerous other reports exist which describe fewer polypeptide components in many of the major groups of viruses.

is no instance in which it has been established that a polypeptide derived from the host cell is an essential viral component.

IIIA. Nucleocapsid proteins

When enveloped virus particles are treated with certain non-ionic detergents or the weakly ionic detergent sodium deoxycholate, the envelope is disrupted. In many instances it is possible to isolate the viral nucleocapsid, i.e. a symmetrical structure composed of the viral nucleic acid and a protein shell showing either helical or icosahedral symmetry. The myxo-, paramyxo-, and rhabdoviruses all have nucleocapsids with helical symmetry which contain a single major polypeptide species [35-38]. The togavirus nucleocapsid may be icosahedral [39,40], and also contains a single polypeptide species [41,42]. A single polypeptide also appears to be closely associated with the RNA of the RNA tumor viruses [43,44] but no symmetry was evident in the ribonucleoproteins of these viruses [43] so that the term nucleocapsid may not apply. The herpes virus particle possesses a large icosahedral nucleocapsid which, unlike that of the various RNA-containing viruses, appears to contain many different species of polypeptides [32].

The major structural proteins which are not present in viral nucleocapsids are presumed to be components of the viral envelope. Two types of components may be distinguished, glycoproteins and proteins free of carbohydrate.

IIIB. Glycoproteins

Carbohydrates are universally present in enveloped viruses, and evidence has been obtained for linkage of carbohydrate to specific proteins of enveloped viruses in all major groups (see Table IV). Only one protein of the rhabdovirus particle is glycosylated, and two or more species are present in the other virus groups. The myxoviruses contain four identifiable glycoprotein species, but it has been established that the largest glycoprotein is cleaved into two smaller components [45,46]; therefore only two of these four species represent primary gene products. It has not been established whether similar cleavage events take place in other viruses which contain many glycoprotein species, e.g. herpes and RNA tumor viruses. Thus it remains to be established with the more complex viruses that each component that can be identified in a polyacrylamide gel is a distinct gene product with a unique amino acid sequence.

There is extensive evidence that the glycoproteins are located on the surface of the viral envelope, and that they form the projections or spikes seen by negative staining. This evidence includes experiments in which it has been possible to remove the surface projections by treatment with proteolytic enzymes, and the glycoproteins are found to be digested by this treatment [22,23,28,47–51]. The resulting spikeless particles are non-infectious and have lost the biological activities which reside in the surface glycoproteins, such as the neuraminidase and hemagglutinating activities of myxo- and paramyxoviruses. It has been possible to isolate the glycoproteins from many enveloped viruses, and it is apparent that they have the morphology of the

surface projections [52-55]. Procedures for selectively labeling polypeptides on the outer surfaces of membranes, such as iodination with lactoperoxidase [56–58], reduction of Schiff's bases formed by reaction with pyridoxal phosphate [59], and reaction with formylmethionylsulfone methylphosphate [60] all have been shown to specifically label the glycoproteins of enveloped virus particles.

Intact influenza virus particles can be agglutinated with the plant lectin concanavalin A [51]. The receptor for this agglutinin resides in the glycoproteins on the viral surface, since protease-treated particles devoid of spikes are not agglutinated.

Although the information contained in the glycoprotein polypeptide sequence is known to originate in the viral genome, the origin of the information in the carbohydrate sequence(s) has not been determined. Strauss et al. [9] have shown that glucosamine, mannose, galactose, fucose and sialic acid are all covalently bound in the glycoprotein of Sindbis virus. The glycopeptides obtained from Sindbis virus after Pronase digestion were analyzed by gel filtration [61]. Three glycopeptide fractions, differing in carbohydrate molecular weight and composition, were recognized. The smallest contained only mannose and glucosamine, suggesting, by analogy with some mammalian glycopeptides, that this was a "core" glycopeptide from which the larger ones were obtained by the further action of specific glycosyltransferases. Since the viral genome is insufficiently large to specify the minimum of five glycosyltransferases required for incorporation of five different monosaccharides, it was suggested that the incorporation occurred via host cell transferases. Grimes and Burge [62] showed that neither the specific activity, nor the acceptor specificity of two different cellular glycosyltransferases was altered by infection with Sindbis virus. Further, they showed that Sindbis glycoprotein can act as an acceptor for the sialyltransferase from uninfected cells. Since viral glycoprotein is the only glycoprotein being synthesized in the infected cell, the results indicate that host transferases are involved in viral glycoprotein synthesis [62].

It is not yet clear whether the carbohydrate sequence is completely or only partially specified by the host cell. When Sindbis virus was grown in two different host cells, quite different amounts of sialic acid were incorporated into the glycopeptides [9]. However, when Sindbis and VSV were grown in the same cell type, differences in sialic acid were also found [63]. A remarkably similar gel filtration profile of glycopeptides was found for Sindbis and VSV, each grown in two different cell types, after removal of sialic acid [63].

Viral specificity is clearly seen in one aspect of the carbohydrate composition of the myxo- and paramyxoviruses, i.e. those viruses which have associated neuraminidase activity. In these cases sialic acid is not detectable in the viral particle, in contrast to the normal amounts present in the host cells [15,134,170]. On the other hand, carbohydrate bound to the viral hemagglutinin has been shown to be a host cell specific antigen in influenza virions [64–66]. Thus, it appears that the extent of host cell specificity of the viral carbohydrate sequences will only be resolved by much more complete composition and sequence data than have yet been obtained.

A major function of the glycoprotein components is in the process of attachment

and penetration into the host cell. Antibody to the viral glycoproteins will generally serve to neutralize the infectivity of the virus, and antigenic differences between virus strains which are important in immunity to disease are usually a result of differences between their glycoproteins.

Recently several viral glycoproteins have been isolated and partially characterized. One of the best studied is the hemagglutinin protein of influenza virus. The designation of this protein derives from its ability to attach to neuraminic acid-containing glycoproteins on surfaces of cells, and it will attach and bridge erythrocytes causing agglutination [67]. This has proven to be a rapid, quantitative assay for influenza and other viruses. The hemagglutinin is the component to which neutralizing antibody is directed [68,69], and extensive antigenic variation in the hemagglutinin protein is the major reason for the frequent appearance of the new strains of influenza virus which give rise to pandemics of influenza. The hemagglutinin is one of the glycoproteins on the surface of the influenza virion, the other being the enzyme neuraminidase, which cleaves neuraminic acid from the same species of glycoproteins to which the hemagglutinin will attach. The neuraminidase appears to function in elution of virus from the cell surface [70,71].

The hemagglutinin protein of influenza virus is a rod-shaped spike structure approx. 140 Å long and 40 Å wide [52,53]. The isolated spikes form characteristic rosette-like aggregates, suggesting that the glycoproteins are amphipathic and aggregate at their hydrophobic ends [52,53]. The hemagglutinin polypeptides, which form the spike structures, may exist in one of two alternative forms: a single polypeptide of approx. 75 000 daltons which is designated HA, or two polypeptides of approx. 50 000 and approx. 25 000 daltons designated HA_1 and HA_2, respectively. Evidence has been obtained that the HA polypeptide can undergo proteolytic cleavage into the polypeptides HA_1 and HA_2, but that such cleavage is not essential for the formation of infectious, hemagglutinating virus particles [45,46]. The extent of cleavage depends on the virus strain and the host cell, and is correlated with the extent of cytopathic effects, suggesting that host cell enzymes are involved [46].

Laver [72] has isolated the polypeptides HA_1 and HA_2 from the Ao/BEL strain of influenza virus, from which the hemagglutinin can be recovered undenatured after dodecylsulfate treatment. In the cleaved form, the two polypeptides HA_1 and HA_2 are linked by disulfide bonds, and the intact spike appears to consist of two or three complexes of $HA_1 + HA_2$. The two chains have been separated preparatively on a gradient of guanidine. HCl and dithiothreitol, and their amino acid compositions have been determined. Polypeptide HA_1 contained about 9 times more proline and much more glucosamine than polypeptide HA_2, but otherwise the two chains had very similar amino acid composition. Thus amino acid analysis did not indicate that one of the two polypeptides had a marked hydrophobic composition. Other evidence suggests that polypeptide HA_2 may be located in the part of the spike attached to the viral envelope. Thus treatment of the WSN strain with the protease bromelain for a limited time removed the spike structures and abolished

the hemagglutinating activity, but the resulting particles still contained the HA_2 polypeptide [22].

In certain other strains of influenza virus, treatment with bromelain released proteins which could be recovered from the supernatant after removal of the spikeless particles [73]. These corresponded to viral polypeptide HA_1, and a polypeptide of slightly lower molecular weight than polypeptide HA_2, and possessed the antigenic characteristics of the hemagglutinin. Concentration of the material led to the formation of crystals consisting of the two glycoproteins. Thus it appears that bromelain removes a small segment of the HA_2 polypeptide which was involved in attachment of the hemagglutinin to the viral envelope, and that the remainder of the spike is resistant to proteolytic digestion. The modified spikes do not in fact cause agglutination of erythrocytes, indicating that the missing segment may be a hydrophobic region responsible for the ability of the spikes to aggregate into rosettes. Such rosettes from unmodified spikes are polyvalent and therefore cause hemagglutination.

The HA_1 polypeptide appears to contain the antigenic determinants which induce and bind to hemagglutination-inhibiting antibody. Eckert [74] isolated a polypeptide corresponding to the HA_1 polypeptide by extraction of virus with urea or guanidine in the presence of reducing agents, which induced neutralizing antibody in experimental animals. Chromatography of antiviral antiserum on affinity columns of this protein led to absorption of all hemagglutination-inhibiting antibodies.

The detailed morphology of the neuraminidase subunits of the B/Lee strain of influenza virus has been studied by Wrigley et al. [75]. After trypsin treatment, the subunits appeared to be tetramers consisting of four coplanar subunits of $40 \times 40 \times 40$ Å. This tetramer appears to correspond to the knob which is seen at the end of a stalk in neuraminidase subunits isolated by detergent treatment [52]. The intact neuraminidase polypeptide had an estimated molecular weight of approx. 60 000 in polyacrylamide gels, and after trypsin treatment a 48 000 mol. wt polypeptide was recovered [75,76]. Thus trypsin appears to remove a stalk by which the protease-resistant, enzymatically active tetramer is attached to the viral membrane.

In contrast to the influenza viruses which contain distinct hemagglutinin and neuraminidase components, the parainfluenza viruses contain one glycoprotein which possesses both of these activities, and a second glycoprotein with neither activity [54,77]. The proteins aggregate into rosette-like structures in the absence of detergent, and can be distinguished morphologically from each other.

Gahmberg et al. [78] have identified a hydrophobic segment of the glycoprotein of SFV which may anchor this protein to the viral lipid layer. After treatment of the virus with thermolysin, the glycoprotein surface projections were degraded and a low molecular weight degradation product, rich in hydrophobic amino acids, remained attached to the viral membrane. This provides chemical evidence for the amphipathic nature of viral glycoproteins, which was suggested previously [52] because of the characteristic aggregation observed with such proteins in the absence of detergents.

IIC. Non-glycosylated polypeptides

Most enveloped viruses contain at least one non-glycosylated polypeptide as a major component in addition to the protein subunit of the nucleocapsid. The single exception thus far is the togavirus group, which contains only two glycoproteins and the nucleocapsid protein [21]. The nucleocapsids of these viruses are essentially spherical structures, and are thus able to form a layer beneath the viral lipid [39,40]. The results of X-ray diffraction studies on Sindbis virus confirm that in fact the viral lipid is in a bilayer around the nucleocapsid [79].

In the myxo-, paramyxo- and rhabdovirus groups the polypeptide with lowest molecular weight is a major non-glycosylated polypeptide of the virion [22,23,25,26,48,50,86]. This component is not present in the nucleocapsid, and in each of these virus groups it is thought to be associated with the inner surface of the viral envelope.

The existence of such a protein in the envelope of influenza virus was postulated earlier because of the finding of an electron-dense layer immediately underneath the unit membrane structure in the viral envelope [80,81]. The finding of a major protein of about 26 000 molecular weight, which was not associated with the nucleocapsid and was completely resistant to proteolytic digestion, suggested that this protein was associated with the viral membrane, and it has been referred to as the M, or membrane protein [22,23,82]. Several additional observations support the idea that the M protein is located in a shell beneath the lipid bilayer. The presence of a shell or layer surrounding the nucleocapsid of influenza virus is revealed in glutaraldehyde-fixed particles which have been treated with Nonidet P-40, which removes all of the phospholipid [83]. Iodination of influenza virus proteins using chloramine T oxidation, under certain conditions, results in labeling of the membrane protein more rapidly than the nucleocapsid protein, indicating that the membrane protein is external to the nucleocapsid [56]. The membrane proteins of two strains of influenza virus have been isolated and their amino acid compositions determined, but no unusual distribution was found [84].

Paramyxoviruses contain a major non-glycosylated protein of about 40 000 molecular weight, which is not associated with the nucleocapsid [25,48,50]. In SV5 virus, this polypeptide was resistant to proteolytic digestion under conditions which removed the glycoproteins from the surface of the virus [48]. The spikeless particles of SV5 consist of two distinct structural components: a helical nucleocapsid, and a pleomorphic membrane structure which surrounds it [48]. Thus it is apparent that the approx. 40 000 mol. wt protein, which is not in the nucleocapsid, is associated with the viral membrane. Procedures have been described for isolation of the non-glycosylated membrane protein from SV5, and also from VSV, and the amino acid composition of the SV5 membrane protein has been determined [86].

The smallest polypeptide in VSV is resistant to treatment with proteolytic enzymes which remove the glycoproteins from the surface [49,50], and it was therefore concluded that this polypeptide may be an internal protein, associated with the viral membrane. Recently it has been reported that lactoperoxidase-catalysed iodination

results in labeling of this low molecular weight protein as well as the glycoprotein, but not of the nucleocapsid protein [85]. While these results might suggest that the low molecular weight protein is located on the outer surface, the findings are complicated by the fact that two other minor polypeptides, known to be associated with the nucleocapsid [26], are also labeled. Thus it remains to be demonstrated that the reaction conditions have not altered the integrity of the viral membrane.

The RNA tumor viruses contain two or more major non-glycosylated polypeptides which do not appear to be closely associated with the RNA; however, the internal structural organization has not been established for these viruses, and it is not certain whether these are envelope components or alternatively associated with the ribonucleoprotein but lost when the virus is disrupted [43].

The primary role of internal membrane proteins may be in stabilizing the envelope structure, as well as in the process of viral assembly. Thus a continuous layer of protein beneath the viral lipid may be essential to impart stability to the membrane. In contrast, the presence of the surface glycoproteins does not appear to greatly influence the organization of the influenza virus membrane, as discussed further in Section V. The process of viral assembly, described in Section VII, may involve the internal membrane protein as a recognition site for alignment of viral nucleocapsid beneath the cell membrane, which is then followed by the process of budding.

IIID. Absence of host cell proteins

During the process of budding at the cell surface, the envelope of an emerging virus particle contains a unit membrane which is continuous with, and morphologically identical to, the unit membrane at the surface of the host cell [80,81,87,88]. It therefore seemed likely that host cell components, in the form of constituents of the cell membrane, could become incorporated into the viral envelope. Host cell antigens in enveloped virus particles have been frequently reported [64,89–93]. It has now been found with a variety of viruses, however, that all of the major structural polypeptides appear to be coded by the viral genome. Possible mechanisms for viral membrane assembly are discussed in Section VIIB. As we have discussed elsewhere, host cell antigens may be present in virions in the form of carbohydrate components bound either to lipid (Section IVB) or to protein (Section IIB).

Several lines of evidence support the conclusion that all major proteins of enveloped viruses are virus coded. First, when a given virus is grown in a variety of host cell types, the same pattern of polypeptides is obtained [94,95]. With influenza virus, minor host-dependent differences were detected in the electrophoretic migration of glycoproteins, but not of carbohydrate-free proteins [22,23]. However, the glycoproteins are also known to be virus coded in that they are the strain-specific hemagglutinin and neuraminidase components [82]. By prelabeling the host cell protein, Holland and Kiehn [96] calculated that less than 1% of the protein of influenza virus could be host cell coded. It is also possible to grow different enveloped viruses in the same cell type, and demonstrate that they have no protein in common.

With more complex viruses it is more difficult to establish the presence or absence of host cell proteins. All 24 of the polypeptides detected in Herpes simplex virus appeared to be synthesized after infection and were thought to be virus specific [31]. In the RNA tumor viruses, the situation is further complicated by the finding that the genetic information for virus production may be present in cells which appear to be uninfected. It has been possible to induce the appearance of these viruses by physical or chemical treatment of a variety of virus-negative normal or transformed cells [97–100]. The induction of Epstein–Barr virus, a herpes-type virus, from virus-free human lymphoid cells has also been reported [101,102]. In these instances the genetic information for some viral proteins may reside in the cell as well as in the virus, but may not be expressed unless the process of virus formation is stimulated in the cell.

A variety of enzymes, presumably derived from the host cell, have been detected in RNA tumor virus preparations. An ATPase associated with avian myeloblastosis virus particles was the first such enzyme described [103]. More recently, several other enzymatic activities were found in purified virions of the Schmidt–Ruppin strain of RSV [104]. These include nucleotide kinase, phosphatase, hexokinase and lactate dehydrogenase activities. It was suggested [104] that these activities are included in viruses as a result of binding to virion proteins, and it remains to be established whether any such activity is essential for virus replication.

IIIE. Methods for isolation of viral proteins

While the insolubility of viral proteins has hindered studies using methods of classical protein chemistry, it is an advantage in permitting the fractionation of virus particles into subviral components on the basis of physical properties (usually sedimentation coefficient and buoyant density). In principle, a given viral polypeptide is associated with only one type of structural component. Thus separation of viral spikes, nucleocapsids, spikeless particles or isolated envelopes may yield isolated polypeptides, and in any event serves as a starting point for further subfractionation by other procedures.

Viral polypeptides may be obtained directly from sodium dodecylsulfate–polyacrylamide gels, but biological activities are lost and removal of dodecylsulfate may present a problem. With some strains of influenza virus, it is possible to isolate the two surface components, hemagglutinin and neuraminidase, after dodecylsulfate treatment at room temperature, and retain biological activity [52]. The neuraminidase of a dodecylsulfate-sensitive strain has been isolated by treatment with Nonidet P-40 and chromatography on DEAE-cellulose column [105].

Scheid and co-workers [54] described a procedure which separates each of the envelope polypeptides of the parainfluenza virus SV5 with full recovery of biological activity. Treatment with 2% Triton X-100 in the presence of 0.5 M KCl solubilizes the two viral glycoproteins as well as the non-glycosylated membrane protein. After dialysis against low ionic strength buffer, the non-glycosylated protein precipitates, leaving the glycoproteins in solution. The two glycoproteins can be separated by

sedimentation on a sucrose gradient containing 1 M KCl and 1% Triton. A similar procedure was used to separate the glycoproteins of NDV [77] and Triton treatment was also used to isolate the glycoprotein of VSV [86,106]. Thus this procedure may be generally useful in the isolation of viral glycoproteins. Triton is readily removed from the protein preparation by butanol extraction, and full recovery of hemagglutinating and neuraminidase activities was obtained with the SV5 and NDV glycoproteins.

Separation of detergent-solubilized proteins of VSV in a two-phase polyethyleneglycol–dextran sulfate system was used by Bishop and Roy [26] to isolate the glycoprotein, membrane protein, and a ribonucleoprotein complex from VSV. When treatment of virions with the detergent Triton N-101 was followed by polyethyleneglycol–dextran sulfate extraction, only the glycoprotein was present in the polyethyleneglycol phase. After addition of 1 M NaCl to the dextran phase and reextraction with polyethyleneglycol, all of the membrane protein was present in the new polyethyleneglycol phase. The remaining proteins, RNA, and viral RNA polymerase activity were recovered in the dextran phase.

Polypeptides of several enveloped viruses have also been isolated by dissociation with guanidine·HCl followed by gel filtration in the presence of guanidine·HCl [45,107].

IV. VIRION LIPIDS

Earlier investigations of the lipids of enveloped viruses have yielded several facts which appear to characterize enveloped viruses in general:

(1) A distinct envelope, visualized by electron microscopy, surrounds each virus particle.

(2) The virus particle forms by budding from the plasma membrane (or, in the case of herpes virus, from the nuclear membrane). The envelope of the budding virus is continuous with the unit membrane of the host cell as seen by thin-section electron microscopy.

(3) Lipid is a substantial portion of the dry weight of the virion (Table III).

(4) The virion incorporates preformed host cell lipids into its structure. This has been shown by studies on cells whose lipids were radioactively labeled prior to infection [108–110], and is clearly supported by many of the studies described below. This situation contrasts with that of the viral proteins which are all specified by the viral genome (Section IID).

The following questions can be asked: (1) How is the lipid arranged and organized in the viral particle? (2) From which part or organelle of the host cell does the lipid arise? (3) What is the role, if any, of the viral proteins in directing incorporation of lipid into the virion? These three questions, which are clearly interrelated, have provided the impetus for most of the recent work in this area.

IVA. The viral bilayer

Recent findings provide strong evidence that viral lipids are arranged in the form of a bilayer. X-ray diffraction studies of Sindbis virus by Harrison et al. [79] demonstrated a deep minimum in the electron density profile centered at 232 Å from the center of the virion. A unit membrane structure is seen in electron micrographs at approximately this position. The electron density at the center of the minimum is characteristic of a hydrocarbon phase, i.e. the center of a bilayer, and the width of the trough (48 Å) is similar to that observed for other bilayer structures. It was concluded that at least 90% of the structure at 232 Å was lipid bilayer, so that little if any protein can traverse it [79]. X-ray diffraction evidence has also been presented for the presence of a bilayer structure in the bacteriophage PM2, although it has been suggested that some protein may traverse the bilayer in this virus [7].

Spin labeling has provided evidence for a bilayer structure in influenza virus [111]. By incorporating into the virus stearic acid derivatives containing nitroxide groups attached to different carbons of the fatty acid chain, a "flexibility gradient" was demonstrated in the viral lipid phase. This "flexibility gradient", i.e. the tendency of the spin label attached close to the polar head group of the stearic acid to be in a highly rigid and restrictive environment, with a progressive increase in environmental fluidity at increasing distances down the fatty acid chain, is characteristic of the bilayer structure in a number of membranes and synthetic preparations [112]. The presence of a bilayer in Rauscher murine leukemia virus [113] and SV5 [114] was demonstrated in a similar fashion.

It thus seems likely that a lipid bilayer is generally present in enveloped viruses. Since bilayers have been shown to be ubiquitous structures in biological membranes, and since viral envelopes appear continuous with the host plasma membrane during budding, this is not an altogether unexpected finding. It suggests that gross reorganization of the lipid structure does not take place as the lipid leaves the plasma membrane and becomes part of the viral envelope.

Gahmberg et al. [60] have used a radioactive surface label, $[^{35}S]$-formylmethionylsulfone methylphosphate, in an attempt to learn the detailed arrangement of lipids in the bilayer of SFV. This compound reacts with free amino groups at elevated pH and was claimed not to penetrate the red cell membrane [115]. Observations made with this reagent suggested to Bretscher [116,117] that the red cell membrane bilayer is asymmetric, with the choline-containing phospholipids predominating in the outer monolayer, while phosphatidylethanolamine and phosphatidylserine, which contain free amino groups, are concentrated in the inner monolayer. When intact SFV is labeled with this reagent, the relative distribution of labeling of three phospholipids which contain free amino groups is the same as that in the total viral lipid. However, the total amount of labeling of these three lipids is increased 8-fold after the virus is disrupted by non-ionic detergent. This could arise from preferential localization of the phospholipids containing free amino groups on the inner surface of the bilayer. Alternatively, it might arise from shielding of the lipid by the viral

TABLE V

LIPID COMPOSITION OF SOME VIRUSES AND THEIR HOST CELL PLASMA MEMBRANES

Abbreviations: Chol, cholesterol; PL, phospholipid; Sph, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PM, plasma membrane; ND, not detected.

Sample	Host cell	Chol: PL ratio	Phospholipid (% of total)						References
			Sph	PC	PE	PS	PI	PA	
Sindbis	BHK		18.2±2.2	26.2±4.1	35.4±4.5	20.3±3.1	ND		127
PM	BHK	0.73	16.5±1.8	26.3±3.5	32.7±4.2	20.6±3.1	3.5±0.8		127
Sindbis	CEF		20.5±4.2	26.4±3.3	32.1±2.8	20.8±2.5	ND		127
PM	CEF	0.55	3.3±0.5	43.5±5.7	23.8±4.1	11.1±2.2	18.8±2.1		127
SFV	BHK21C13	0.97	21	34	26	12	0.9	4.1	129
PM	BHK21C13	0.56	17	40	21	7.1	3.2	5.7	129
SV5	MK cells	0.89	12.3	25.2	40.3	17.9	2.9*		122,132,133
PM	MK	0.81	11.8	32.1	38.8	17.2	ND*		122,132,133
SV5	BHK21F	0.64	30.0	38.5	15.6	5.2	10.5		122,132,133
PM	BHK21F	0.68	24.2	49.5	11.2	5.1	10.0		122,132,133
SV5	MDBK	0.84	27.3	23.8	40.9	2.0	5.2		122,132,133
PM	MDBK	0.75	22.8	44.5	27.2	2.2	2.9		122,132,133
SV5	HaK	0.60	25.8	43.8	17.1	5.0	8.5		122,132,133
PM	HaK	0.51	24.4	46.8	13.0	5.0	11.7		122,132,133
RSV	CEF	0.61	29±2	28±2	30±3	11±2***			14
NDV	CEF		26±2	26±3	33±3	10±2			14
Sendai	CEF		24	27	31	14			14
Sindbis	CEF		29	21	33	15			14
PM	CEF	0.51	20±2	35±3	31±3	13±2			14
VS_{Ind}	L	0.65	20.9	16.4	33.1	17.3	5.9		13
VS_{NJ}	L	0.96	22.0	15.9	31.3	15.4	4.4		13
PM	L	0.58	16.2	51.0	22.6	7.0	<0.1		13
Pseudorabies virus (Herpes)	RK		21.0	51.2**	21.0	3.5			141
Inner nuclear membrane	RK		20.8	50.5**	21.5	3.7			141

* MK cells, 11.4% PI reported.
** PC + PI
*** PS + PI

glycoprotein, or from some other difference in reactivity or accessibility of the phospholipids in the intact and disrupted states.

It is likely that lipids, and particularly glycolipids, may be distributed asymmetrically between the inner and outer halves of the bilayer in viral membranes. However, existing data (Table V) do not support a model [116,213] in which phosphatidylserine and phosphatidylethanolamine are always located only in the inner half, and sphingomyelin and phosphatidylcholine in the outer half, of viral membranes. Such a model might be expected to require a molar ratio of amine-containing to choline-containing phospholipids of 1. Alternatively, it has been suggested that protein can substitute for some of the lipids of the inner monolayer, permitting a molar ratio of less than 1 [116,213]. However, the ratio of amine-containing to choline-containing phospholipids in SV5 varies from 1.6:1 when grown in primary rhesus monkey kidney (MK) cells to 0.3:1 when grown in BHK21F cells [122]. It is important that these very different molaı ratios occur in viral membranes which contain the same set of proteins [214]. The possibility that protein may replace part of the lipid in the bilayer therefore cannot compensate for these differences in phospholipid composition.

It has been suggested that the distribution of stain after osmium fixation may reflect the content of amine-containing phospholipids [213]. Using osmium fixation and the same staining procedure, both sides of the membrane were clearly stained in the MK cell, but only the cytoplasmic side was stained in the BHK21F cell [87]. Assuming that the stain is in fact selective for amine-containing phospholipids, this would suggest that these phospholipids are localized in the inner surface when they are present in relatively small amounts, but are present in both surfaces when they are present in larger amounts, such as in MK plasma membranes. It seems that the presence of large amounts of amine-containing phospholipids in the outer half of a membrane such as that of the MK cell, or the MK-grown virus, should be experimentally demonstrable. Such a finding might be correlated with functional differences such as susceptibility to virus-induced cell fusion and immune cytolysis [133].

The role of the lipid bilayer in maintaining the stability of SFV was investigated by Friedman and Pastan [118]. They found that infectivity of the virus was not affected by hydrolysis of nearly 60% of the viral phospholipids by purified phospholipase C. However, thermal inactivation of the treated virus at 37°C was more rapid than inactivation of an untreated control. These results suggest that the integrity of the lipid bilayer serves to stabilize the viral structure, but that intact phospholipids are not required for infectivity.

IVB. Origin of viral lipids

Many experiments have been directed toward learning the subcellular origins of the viral lipids, and the degree to which viral proteins direct lipid incorporation. These experiments are based on comparative lipid analysis, of different viruses or virus strains grown under similar conditions in some cases, and of viruses and their host cells or host plasma membranes in others. Before discussing the results of these

studies it seems worthwhile to point out the limitations inherent in this experimental approach.

(1) Comparison of different viruses grown in ovo or in vivo presents the most difficult problems of interpretation. In this case virus multiplication proceeds through several cycles of infection in an organism composed of many different cell types. It seems most probable that not all cell types are equally involved in virus multiplication, and it is quite possible that different cell types are involved in the multiplication of different viruses. Also, various effects of virus infection upon cellular phospholipid synthesis have been reported. Thus, $^{32}PO_4^{3-}$ incorporation into phospholipids increased in several cell types after Sendai virus infection [119,120] while a decrease was found after Sindbis infection [110,121]. A decrease in sphingomyelin synthesis and an increase in synthesis of the major glycolipid, globoside, was observed in SV5-infected MDBK cells [215,216]. Such changes might affect cellular lipid composition in unknown but important ways after several cycles of infection. On the other hand, other systems which permit virus propagation without significant cytopathic effects possess obvious advantages for comparison of viral and cellular lipids [132].

(2) The purity of the viral and/or cellular populations being compared may not be comparable. In fact, the criteria for purity necessary to compare lipid compositions are not easy to establish. It must be remembered that bilayers in membranes are highly dynamic, non-covalently stabilized structures capable of complex interactions and exchanges with their environment. This has been strikingly demonstrated in a study in which plasma membranes isolated from the same cell line by two different procedures were found to have significantly different lipid compositions [13]. Similarly, many virus preparations may not be pure by the chemical criteria required for meaningful comparisons of lipid composition. It has been reported that lipid composition of viruses can be altered by introducing fatty acids [122], phospholipids [123] or steroids [124] into the growth medium. These exogenous lipids may be incorporated into some cellular lipid pool, or they may be intercalated directly into the existing viral or plasma membrane bilayer. Fatty acid derivatives can be introduced into the viral bilayer, for example, simply by incubating the purified virus with a spin-labeled or fluorescent stearic acid derivative [114,125,126].

Bilayer constituents can readily be removed from as well as added to the structure. Thus, fatty acid spin labels can be removed from the viral bilayer by incubating labeled virus with serum albumin [125]. It was recently found that influenza virus grown and purified by standard procedures contained tightly bound serum proteins, presumably arising from serum in the growth medium [59]. Impurities such as these in viral preparations would not be detected in electron micrographs, or in studies of viral proteins after incorporation of radioactive amino acid precursors, but they may influence the viral lipid composition.

(3) Problems associated with incomplete lipid extraction, losses on chromatography and inaccuracies in analytical methods could introduce substantial uncertainties into each individual determination.

Several of the comparisons of lipid composition of purified viruses and plasma membranes are listed in Table V. These and other studies are discussed below.

Togaviruses. David compared phospholipid and fatty acid compositions of Sindbis virus grown in chick embryo fibroblasts (CEF) and BHK cells [127]. The viral lipid composition was quite different from the total cellular lipids of either cell, and from the CEF plasma membrane (but not from the BHK plasma membrane). However, the viruses grown in the two cells had closely similar lipid contents, and it was suggested that the lipid composition reflected the lipid affinities of the viral proteins [127]. On the other hand, Heydrick et al. [128] reported that Venezuelan equine encephalitis virus had different lipids when grown in L cells as compared to the same virus grown in CEF. The lipid composition of SFV was found to be quite similar to the plasma membrane of the BHK21 cells in which it was grown, but to differ from whole cells or endoplasmic reticulum [129]. The virus contained much less free fatty acid and a little more cholesterol than the plasma membrane. The phospholipid composition of Uukuniemi virus grown in the same cells was virtually identical to that of Semliki Forest virus, suggesting that the plasma membrane composition rather than the lipid affinity of different viral proteins determined the viral lipid composition [130]. This argument was further elaborated by an analysis of the fatty acid chains in different phospholipid classes isolated from SFV, and plasma membranes and endoplasmic reticulum from BHK21 cells [131]. Each phospholipid class was found to contain a characteristic mixture of fatty acids which differed from that in all other classes. The fatty acids in each viral lipid class resembled that of its counterpart in both the host plasma membrane and endoplasmic reticulum [131].

Paramyxoviruses. In a thorough study, Klenk and Choppin [122,132,133] analysed the lipid composition of four different cell types, their plasma membranes and SV5 virus grown in each cell type. They found that the plasma membrane composition differed substantially from the whole cell, and that membranes from different cells differed from each other. The composition of the virus generally resembled that of its host cell plasma membrane fairly closely, although some differences in both phospholipid class and phospholipid fatty acid compositions were found. The authors concluded that "in general, the virions acquire the lipid pattern of the host cell plasma membrane" but that "within narrow limits, some selective rearrangement of membrane lipids incorporated into virions is possible in some cells" [122]. The neutral glycolipids characteristic of each cell type were incorporated into virions grown in that cell, while gangliosides were not, a finding which the authors attributed to the action of viral neuraminidase [134]. When VSV, a virus which lacks neuraminidase, was grown in the same cell type it contained the ganglioside found in the plasma membrane, thus supporting the concept that the viral lipids reflect the plasma membrane composition, and that the absence of gangliosides in SV5 is due to the neuraminidase of the virus [17].

Using spin labels, comparisons were made of the "flexibility gradient" of two different viruses (SV5 and influenza) grown in two different cell lines (MDBK and BHK) [114]. The characteristics of this gradient, which is influenced by lipid com-

position, was found to depend upon the cells in which the viruses were grown, but not upon the viruses themselves. These differences were tentatively correlated with the different cholesterol: phospholipid ratios previously reported (Table V) for the plasma membranes of the cell types [114].

Blough and Lawson [135] compared lipids of NDV and Sendai virus grown in ovo. While the overall lipid distribution, and the class distribution of phospholipids were similar in the two viruses, differences were observed in the distribution of phospholipid fatty acids. Several differences in fatty acid composition were also noted by Tiffany and Blough [136] in three strains of NDV propagated in ovo.

Hirano et al. [119] have reported a study in which CEF and MK cells were labeled with $^{32}PO_4^{3-}$ before Sendai virus infection, followed by labeling with [^3H]-acetate after infection. The $^3H/^{32}P$ ratio of lipids in the isolated virions was several times higher than that in the lipids of infected or uninfected cells, suggesting that newly synthesized fatty acids were preferentially incorporated into viral lipids [119].

Rhabdovirus. McSharry and Wagner [13] grew two antigenically distinct serotypes of VSV in L cells. They observed that the proportion of neutral lipids was higher in the New Jersey serotype than in the Indiana serotype. A less pronounced difference was found when these serotypes were grown in CEF, and the difference was limited to neutral lipids other than cholesterol [13]. The lipid composition of both viruses resembled the host cell plasma membrane more closely than the whole host cell. The authors concluded that while the viral lipid composition "primarily" reflected its membrane site of maturation, there was some evidence that the viral proteins could influence the proportions of phospholipid and neutral lipid incorporated into the virus [13]. Klenk and Choppin [17] found that a single ganglioside was present in BHK21 cells, and was also found as the only ganglioside in VSV grown in these cells. This was supported by the finding that the host cell antigen of VSV grown in BHK21 cells was glycolipid in nature [137]. The finding that L cells synthesize desmosterol and not cholesterol provided the basis for a study of sterol incorporation into VSV by Bates and Rothblat [124]. These workers showed that L cells with different ratios of cholesterol to desmosterol could be prepared by adding cholesterol to the growth medium. Viral infectivity and growth was independent of this ratio, but VSV was found to contain a consistently higher cholesterol:desmosterol ratio than the host cells [124]. It was not determined whether the viral ratio was closer to that of the host plasma membrane.

Myxovirus. Blough and co-workers [138] have reported differences in fatty acid composition of different strains of influenza virus grown in ovo. They have also observed different lipid composition in the same strain of influenza virus grown in eggs treated with vitamin A [139] or with branched chain fatty acids [140]. They interpret these results to indicate that lipid incorporation into the virus is directed by the viral protein.

Klenk et al. [51] have shown that the A-antigen of fowl plague virus, which is derived from the host cell, absorbs anti A-antibody only after proteolytic removal of

the surface glycoprotein spikes. This indicates that host cell glycolipid is present in the outer surface of the completed virion.

RNA tumor viruses. Quigley et al. [14] have compared the lipids of RSV with those of the plasma membranes of CEF in which they were grown. They found a viral lipid composition which "broadly resembles" that of the plasma membrane. However, a closer similarity was found between RSV and other budding viruses grown in the same cell (NDV, Sindbis and Sendai), suggesting that "the requirements of budding during maturation, or subsequent adsorption to host cells, may impose restrictions on the lipid composition of these otherwise different viruses." [14].

Herpes virus. Ben-Porat and Kaplan [141] showed that the phospholipid distribution in pseudorabies virus was identical to that in the inner nuclear membrane from which it buds, but quite different from that in the cytoplasm. Infection of primary rabbit kidney (RK) cells with this virus causes an increase in the amount of cellular phospholipid associated with the inner nuclear membrane, i.e. the membrane becomes enlarged [142]. The phospholipid which is incorporated into the inner nuclear membrane in response to viral infection appears in the mature virions in preference to the phospholipid originally present in the membrane [141]. Thus, although the viral membrane consists mainly of phospholipids preexisting in the cell at the time of infection, in common with the other enveloped viruses, these lipids are apparently organized into new nuclear membrane in response to infection. This new membrane is preferentially incorporated into budding virus.

Several generalizations can be made from these reports. The lipid composition of enveloped viruses which form by budding at the plasma membrane seems to resemble that of the plasma membrane more closely than that of any other cellular structure which has been studied. This is perhaps most clearly indicated by several findings that host cell glycolipid is incorporated into the virion. The major discrepancies between viral and membrane phospholipid composition appear to occur in cell systems derived from embryonated hen's eggs, which are clearly heterogeneous cell populations in which a small proportion of the cells may produce most of the virus. These differences have been emphasized by some workers and minimized by others, and are generally fairly limited in extent. They may arise from the problems of purity which have been discussed previously, or from selection by viral proteins, which is frequently proposed. However, a consideration of recent discoveries about bilayer and membrane structure suggest that alternative explanations are possible.

It has become apparent from recent studies that the lipid bilayer, whether synthetic or incorporated into a biological membrane, is a highly dynamic rather than a static structure. It was recently demonstrated that phospholipid spin labels show extremely rapid lateral diffusion within monolayers of a biological membrane [143]. A rate of lateral diffusion on the order of 5 μm/s has been estimated for the spin label. To the extent that this rate of diffusion is characteristic of membrane lipids in general, the lipid distribution within monolayers of a plasma membrane must be completely randomized every few seconds. Remarkably high rates of both lateral

and rotational diffusion of membrane proteins have also been observed in several membrane preparations [144–146].

The effect of membrane proteins on lateral diffusion of lipids is not yet known, but recent studies suggest that membrane proteins act to bind a portion of the lipid [147,148], thus presumably reducing their rates of diffusion. In the limit, the lipid diffusion would be identical to that of the protein to which it is bound, but other effects of protein on lipid motion can be imagined which are preferential rather than completely specific. Such an effect might reduce the average mobility of one class of lipids relative to the others to some degree intermediate between freely diffusing lipid and that tightly bound to protein. Such interactions in the plasma membrane may act to make a population of lipids available to the viral proteins which is not accurately reflected by the total lipid composition of the membrane.

The problem of the origin of viral lipids is further complicated by recent findings that envelope proteins are associated with intracellular membranes prior to the release of mature virions (see Section VIIC). The mechanism of their migration to the plasma membrane is not known, but it may well involve incorporation of an associated fragment of smooth endoplasmic reticulum into the plasma membrane. Such incorporation into the nuclear envelope is suggested by observations on herpes virus discussed above. Precise knowledge about the origin of viral lipids must thus await further insight regarding lipid–protein interactions in membranes as well as the mechanisms of viral assembly.

V. LIPID–PROTEIN INTERACTIONS

Several observations suggest extensive interactions between lipids and proteins in the mature virion. As discussed above, the surface projections or spikes which characterize the enveloped viruses are known to be glycoprotein in nature. The glycoprotein must be stabilized on the viral surface by interaction with lipid. These spikes behave as integral membrane proteins [53] in that they can only be released through the action of detergents, and are insoluble in the absence of detergent.

Despite these indications of hydrophobic stabilization of the glycoprotein spikes in the viral bilayer, the precise mode of interaction is not yet clear. The electron density profile of Sindbis virus obtained from X-ray diffraction does not show any perturbation of the apolar interior of the bilayer which might indicate penetration by protein [79] . Also, intramembranous particles are not observed in Sindbis or influenza virus by electron microscopy after freeze-cleaving [39, 88]. Proteolytic digestion of the intact virus results in complete removal of the glycoprotein in several different viruses, although a hydrophobic peptide derived from the glycoprotein and resistant to further proteolysis was found in SFV [78]. Complete proteolytic removal of the glycoprotein spikes on influenza virus did not result in any detectable change in the viral bilayer as monitored by several spin label probes [111]. This finding suggested

that the spikes do not penetrate deeply into the bilayer [111]. Recent results with vesicular stomatitis virus indicate, however, that the bilayer becomes detectably more fluid upon removal of spikes with protease, suggesting that spikes of VSV penetrate the bilayer to a greater extent [212].

It has recently been demonstrated that a glycoprotein fraction and lipids solubilized from Sendai virus using non-ionic detergents can be recombined by gradual removal of the detergent to yield functional lipoprotein particles [149, 150]. All of the functional properties of the viral surface – hemagglutinin, neuraminidase, hemolysis and cell fusion – were associated with the reconstituted particles. By electron microscopy large membranous particles of 500–3000 Å were seen, with spikes extending irregularly from both surfaces. Lipid extracts of Sendai virus, influenza virus and NDV were equally effective in reconstituting these structures. However, when glycoproteins from influenza virus were used, hemolysin and cell fusing activities were not present, although microscopically similar membrane fragments were produced [150]. This result demonstrates the specificity of the reconstituted membranes, since intact influenza virions also lack these activities.

In a further study, it was shown that purified phosphatidylethanolamine, sphingomyelin and phosphatidylcholine, but not phosphatidylserine, were effective in reconstituting the membrane activities with the same Sendai virus glycoprotein fraction [151]. Phosphatidylethanolamine was the most effective phospholipid, causing reconstitution at concentrations comparable to those required with the total viral phospholipid mixture. The addition of small amounts of cholesterol increased the effectiveness of phosphatidylethanolamine for reconstitution, while large amounts decreased it [151]. While the precise nature of the interaction between the glycoprotein and lipid cannot be deduced from these observations, the reconstituted system clearly offers a promising experimental approach for further insights in this area.

There are also indications that internal proteins interact with the viral bilayer. This might be expected from the X-ray studies of Sindbis virus, in which no separation was found between the high electron density of the polar portion of the bilayer and the high electron density of the protein and nucleic acid of the interior [79]. Any appreciable space between the two structures (as has been suggested by Nermut [152] to occur in influenza virus based on the finding of an electroluminescent space between the unit membrane and the underlying structure) should result in a trough in the electron density profile just internal to the lipid polar region. Further, recent studies have demonstrated transfer of fluorescence from internal proteins of influenza virions to a fluorescent probe, 12-(9-anthroyl)stearic acid, incorporated into the lipid bilayer. Since the maximum distance over which fluorescence transfer can occur in this donor–acceptor system is about 39 Å, this sets a maximum distance of approx. 11 Å between aromatic residues of the internal viral proteins and the lipid bilayer [126]. Preliminary observations suggest that alterations of bilayer structure affect the conformation of internal viral proteins, suggesting a very close relationship [126].

These observations provide some basis for the suggestion that viral proteins might direct the incorporation of lipids into the virion to some extent. Whether and how they actually do so, however, is part of the broader question of the nature and specificity of lipid interaction with membrane protein and the role of these interactions in membrane assembly.

VI. ORGANIZATION OF LIPID AND PROTEIN IN ENVELOPED VIRUS PARTICLES: A SUMMARY

Fig. 2 summarizes the structural information currently available for influenza virus, and will serve as a focus for discussion of the similarities and differences between the structures of the simpler enveloped viruses.

The outer surface of the virion is coated with spikes made up of glycoproteins. In Fig. 2, two types of spikes are depicted, corresponding to the hemagglutinin and neuraminidase which consist of different glycoprotein species. It has been suggested that spikes manifesting these different activities possess different structures on the basis of their appearance in the electron microscope after separation in detergents [52]. Although other viruses do not possess these two activities, the selective removal of glycoproteins with concomitant disappearance of spikes has been reported for all of the simpler enveloped viruses (Section IIB). Thus, the arrangement of glycoprotein spikes on the outer surface as shown in Fig. 2 can be considered a general structural feature of these particles. The amount of glycoprotein in influenza virus has been found to vary over an almost 2-fold range under different growth conditions [46], suggesting that protein–protein interactions between the glycoproteins is not required to maintain the viral structure.

As discussed above (Section V), the nature of the interaction between lipid and glycoprotein is not known. The solubility characteristics of the glycoproteins argue for some hydrophobic interaction with the lipid bilayer, while X-ray diffraction and spin label evidence suggests that the glycoproteins do not penetrate deeply; accordingly, their apparent attachment to the surface of the bilayer in Fig. 2 is purely a matter of artistic convenience.

The viral lipid is depicted in Fig. 2 as an uninterrupted bilayer, in accord with the X-ray diffraction data reported for Sindbis virus and the spin label studies on influenza and SV5 virions (Section IVB).

The internal membrane protein is depicted as a continuous shell immediately beneath the bilayer. Calculations of the number of M protein molecules required to form such a shell in influenza virions agree closely with the average number of molecules per particle actually found from the polypeptide composition [83].

It is in regard to the location and stabilization of the internal membrane protein that the greatest differences might be expected between the envelope structure of the different viruses. The togaviruses do not have such a protein; they possess a single internal protein which is associated with the RNA to form a nucleocapsid. This protein is a "hydrophilic" rather than a "lipophilic" one, using the criterion of binding

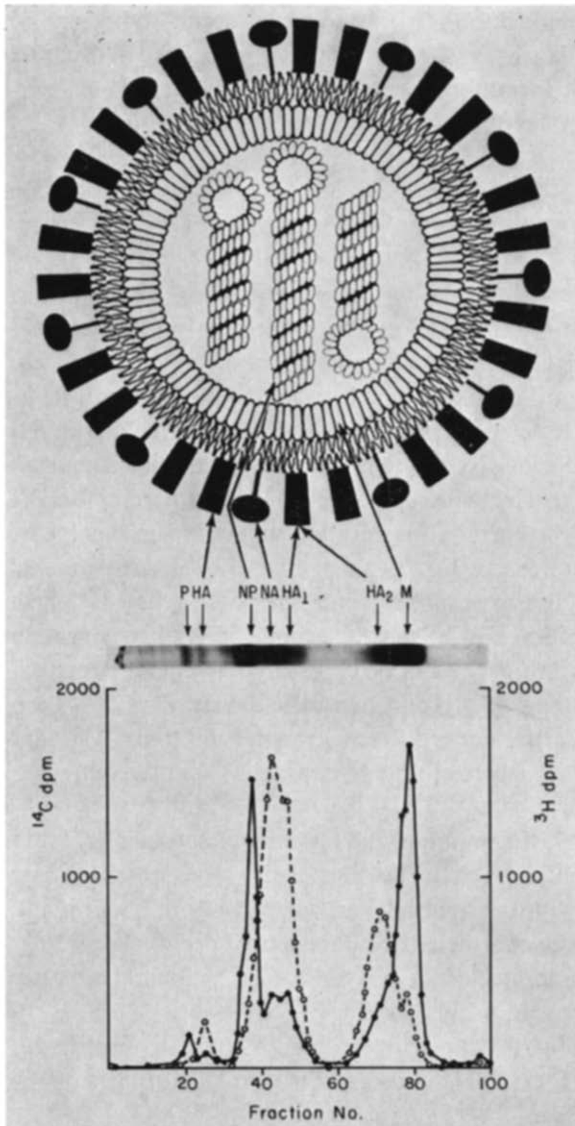


Fig. 2. Schematic diagram of an influenza virion in cross-section illustrating the structural components and their relation to polypeptides in a polyacrylamide gel. The bands visible by staining with Coomassie blue as well as the radioactivity profile of the same gel are shown. The virus was grown in the presence of [^{14}C]amino acids (●—●) and [^{3}H]glucosamine (○—○). Designations for viral polypeptides proposed at Influenza Workshop I [82] are used: P, high molecular weight polypeptide of unknown function; HA, uncleaved hemagglutinin polypeptide; NP, nucleocapsid subunit; NA, neuraminidase polypeptide; HA_1 and HA_2, cleaved hemagglutinin polypeptides; M, membrane polypeptide. The shapes of the hemagglutinin and neuraminidase subunits are adapted from Laver and Valentine [52]. The structure of the nucleocapsid is taken from Compans et al. [156].

of Triton X-100 [217]. It has been shown that water-soluble proteins generally bind negligible amounts of this detergent, while membrane proteins bind substantial quantities [153]. On the other hand, the M protein of influenza virions and corresponding proteins of other viruses are insoluble in the absence of detergents, and thus presumably fall into the "lipophilic" class.

Attempts to isolate "cores" from influenza virions, i.e. spherical lipid-free structures possessing all the internal proteins, have had questionable success. In one strain such structures were isolated, although they appeared in a sufficient variety of shapes and sizes as to suggest that their stability was marginal [154]. They could be prepared from another strain only after stabilization by cross-linking with glutaraldehyde [155]. The isolation of a stable core structure would require that protein–protein interaction between the M protein subunits are sufficient to hold the core together. The failure to isolate cores as stable structures implies that lipid–protein interactions play a larger role in stabilizing these particles. Each virus might have its own unique balance of protein–protein and protein–lipid interaction in stabilizing the shell of membrane protein. In cases where the protein–lipid interaction predominates, one might be more apt to find protein-directed selection of lipids from the membrane for incorporation into the viral bilayer.

The nucleoprotein structure is depicted in Fig. 2 as composed of several discrete ribonucleoprotein strands possessing a characteristic structure, which is in accordance with recent observations on isolated ribonucleoproteins [35,156]. It has alternatively been suggested that a single continuous nucleoprotein structure exists in the intact virion, although such putative structures have not been isolated [83]. In either case, no interaction between this structure and the M protein shell is specified in the figure because no relevant experimental data have yet been presented. However, such interactions may be expected to play a role in the process of viral assembly and may provide an additional means of stabilizing the completed virion.

VII. ASSEMBLY OF VIRAL MEMBRANES

VIIA. Morphological studies

The vast majority of lipid-containing viruses are assembled at a preexisting cellular membrane. With most groups of viruses, the plasma membrane is the site of virus maturation, whereas with the herpes virus group the inner nuclear membrane serves as the maturation site, and maturation of VSV has been reported to occur on either the plasma membrane or intracellular membranes depending on the host cell type [157]. Detailed electron microscopic studies have been carried out on the process of maturation of a variety of viruses which form by budding, including togaviruses [158,159], myxoviruses [80,88], paramyxoviruses [87,161], rhabdoviruses [157,160], RNA tumor viruses [162] and herpes viruses [163,164]. The paramyxoviruses provide a system in which viral components, because of their relatively large size, are easily recognizable by electron microscopy. The helical nucleocapsids of

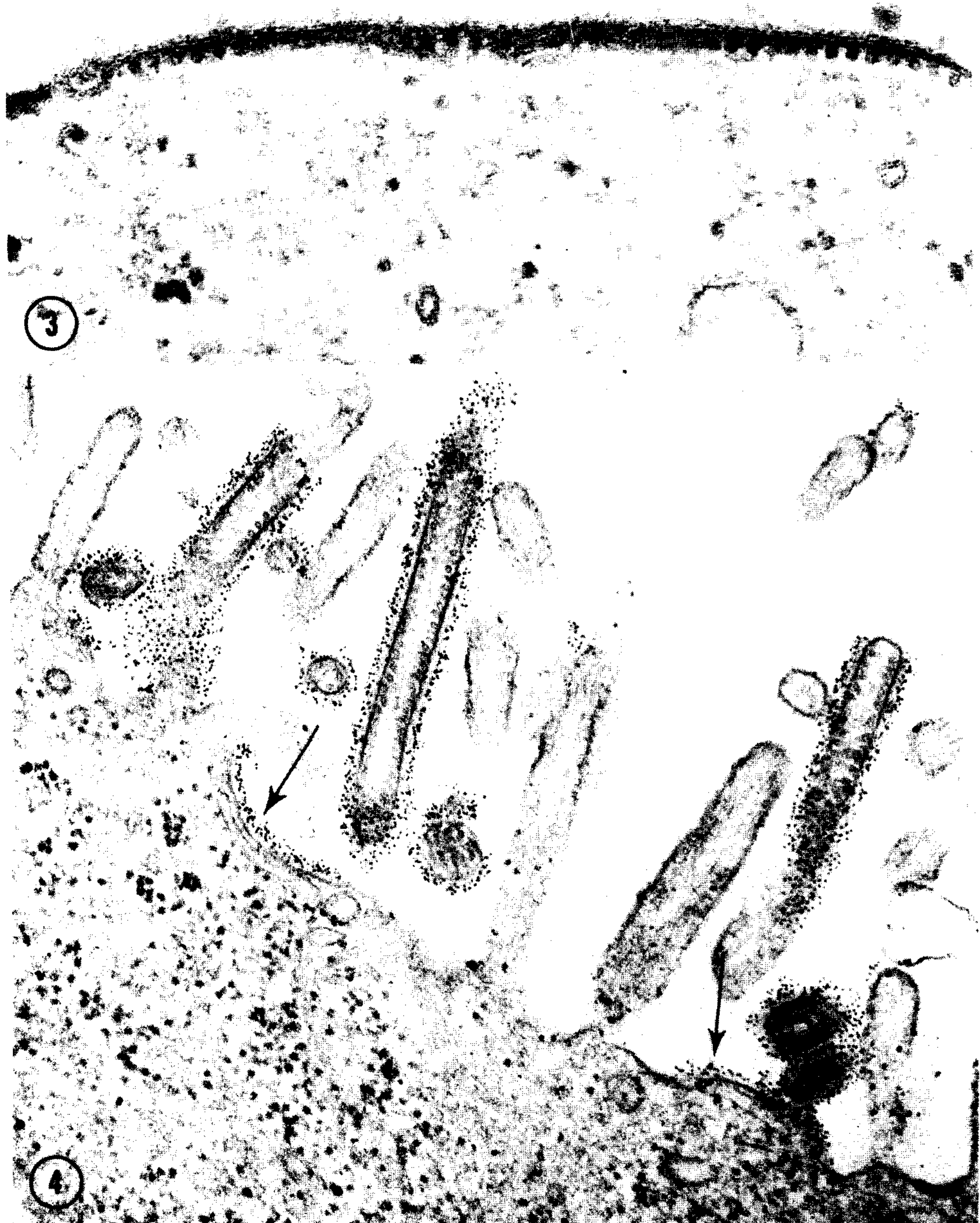
paramyxoviruses are assembled free in the cytoplasmic matrix of the infected cell. The nucleocapsids then align in a regular arrangement under regions of the plasma membrane, and a layer of surface projections is present on the outer surface of such areas of membrane (Fig. 3). Virion formation then occurs by a process of outfolding of such a region of modified membrane. During the process of budding, the emerging virus particle is seen to contain a unit membrane structure which is continuous with the unit membrane on the cell surface. The steps in the assembly of influenza virus appear to be generally similar, although the nucleocapsid is more difficult to visualize by electron microscopy [80,88].

Several observations suggest that the first viral components to arrive at the plasma membrane are envelope proteins, and that they are present in membranes which appear to be normal in morphology [81,168,216]. Specific adsorption of erythrocytes occurs to surfaces of cells infected with influenza and parainfluenza viruses, presumably to regions of membrane containing hemagglutinin polypeptides, yet no morphological change is detectable in regions of membrane showing such hemadsorption [80,165,168]. Using ferritin-labeled antiviral antibody, it is also possible to demonstrate antigens of influenza and parainfluenza viruses in membranes of normal morphology [168,169]. The antigens of parainfluenza viruses occur in discrete patches, as would be expected of a region of membrane containing entirely virus-coded polypeptides (Fig. 4). The highly ordered arrangement of SV5 nucleocapsid beneath the cell membrane also suggests that the nucleocapsid is aligning with specific components at the cell surface rather than associating with cell membranes at random. This is further indicated by the lack of association of nucleocapsid with intracellular membranes, and the fact that the large accumulations of nucleocapsids which frequently are found in paramyxovirus-infected cells occur free in the cytoplasmic matrix, rather than in association with cell membranes [87].

Despite the apparent continuity between the membrane of the cell and that of the emerging virus particle, several observations indicate that there is an abrupt transition from virus-specific to cell-specific components occurring at the base of a budding virus particle: (1) A layer of surface projections covers the virus particle, but is absent on the adjacent cell surface [87]; (2) Ferritin-labeled antiviral antibody binds to the entire surface of budding virions, but not the adjacent cell membrane [168]; (3) Neuraminic acid residues are present on cell surfaces, but are absent in influenza and parainfluenza virions, presumably as a result of viral neuraminidase [134,170]. Staining of surfaces of cells infected with influenza and parainfluenza viruses with

Fig. 3. Region of the plasma membrane of an SV5-infected MK cell showing SV5 nucleocapsid regularly aligned under the plasma membrane. In cross-section the helical nucleocapsid appears as electron-dense circular profiles. The membrane shows a layer of projections on the outer surface. From Compans et al. [87]. Magnification , × 105 000.

Fig. 4. Tagging of the surface of an SV5-infected cell with ferritin-labeled antiviral antibody. Filamentous SV5 virions in the process of budding at the cell surface are tagged, while normal cellular microvilli are untagged. Ferritin tagging also indicates the presence of viral antigen in two patches of the cell surface (arrows). Magnification, × 62 000.


3
4

colloidal iron hydroxide reveals that neuraminic acid residues are present on the entire surface except in the membranes of emerging virus particles [170]. These observations indicate that viral membranes are derived from discrete, localized regions of the cell surface which differ in composition from the regions of cell membranes which are immediately adjacent to them.

Observations on phenotypic mixing of envelope proteins of SV5 and VSV provide information about the specificity of protein–protein interaction in virus assembly [218,219]. Upon mixed infection with SV5 and VSV, it is possible to obtain virions with the typical bullet shape of VSV which contain the two SV5 glycoproteins, but neither the SV5 membrane nor nucleocapsid protein. This indicates that there are no stringent restrictions on the type of glycoprotein which may be incorporated into the envelope of VSV and suggests that viral spikes do not recognize a specific membrane protein, as might be expected if spikes penetrated the bilayer. However, the absence of the non-glycosylated SV5 membrane protein in the phenotypically mixed virions suggests that in the process of assembly, VSV nucleocapsid associates specifically with those areas of the cell membrane which contain the VSV membrane protein, and not with areas containing the SV5 membrane protein.

VIIB. Modes of assembly of patches of viral membrane proteins

The observations described in the preceding section strongly suggest that the immediate precursor to a viral envelope is a discrete patch of membrane containing virus-specific proteins. In addition to the morphological studies already mentioned, the absence of significant amounts of host cell polypeptides in viral envelopes indicates that they are derived from segments of the plasma membrane from which host cell polypeptides are excluded. One of the primary questions regarding the process of viral assembly is the exact mechanism by which such patches of viral membrane are generated. Alternative modes of generating discrete patches of virus-specific components are depicted in Fig. 5. The first three alternatives depict mechanisms involving insertion of individual molecules into the plasma membrane directly.

(1) Viral polypeptides may be added gradually to a defined domain of the cell surface. Host cell polypeptides could either be absent or progressively displaced from such regions.

(2) A viral polypeptide may be inserted into the membrane and act as a nucleation site for further insertion of polypeptides, leading to progressive growth of the patch.

(3) Viral polypeptides may be inserted into the plasma membrane at random, followed by lateral diffusion and aggregation into patches containing only viral proteins.

The last two possibilities depict mechanisms involving incorporation into the plasma membrane of preformed membranous structures containing viral polypeptides.

(4) A segment of membrane containing a preexisting patch of viral polypeptides

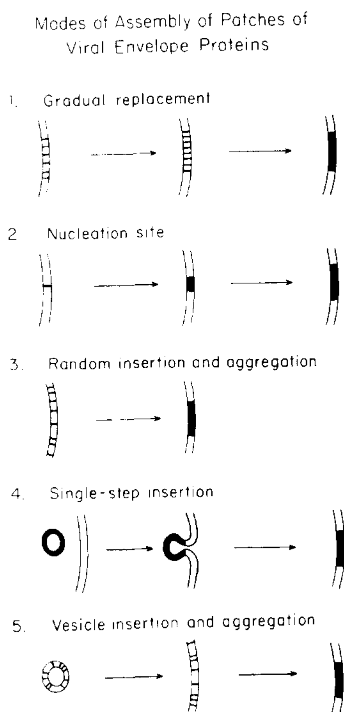


Fig. 5. Schematic diagram of possible modes of assembly of patches of viral envelope proteins. From Compans and Caliguiri [210].

may fuse with the plasma membrane, thus inserting a patch of viral protein in a single step. The presumptive "precursor" vesicles could be formed either by insertion of viral polypeptides into preexisting intracellular membranes, such as endoplasmic reticulum or Golgi membranes, or by de novo formation of membranes from viral proteins and lipid.

(5) A vesicle containing interspersed viral and cellular polypeptides may be inserted into the plasma membrane, followed by lateral diffusion and aggregation of the viral proteins into patches.

The situation is further complicated by the fact that different viral membrane proteins may arrive at the cell surface independently and by different pathways.

There is not yet sufficient information to permit us to distinguish with certainty between these alternative modes of incorporation of polypeptides into the plasma membrane, even for a single envelope component. However, studies on intracellular location of viral polypeptides, described below, suggest that intracellular membranes do play a role in the biogenesis of viral membranes.

VIIC. Synthesis and intracellular location of viral envelope proteins

In a variety of virus-cell systems, host cell protein synthesis is inhibited during infection, and it is possible to recognize virus-specific polypeptides clearly in poly-

acrylamide gel profiles when entire cells or subcellular fractions are examined. Such a system provides an excellent opportunity for study of the process of addition of viral polypeptides to membranes, a topic now under active investigation in many laboratories.

A general finding which has already emerged from cell fractionation studies is the association of viral envelope polypeptides with intracellular membrane fractions. Spear et al. [171] found herpes virus glycoproteins to be incorporated into several membrane fractions of HEp-2 cells, whereas other viral proteins were not associated with membranes. These glycoproteins bind to membranes during or after their synthesis and become glycosylated in association with membranes [172]. Herpes virus glycoproteins were not detected free in the cytoplasm [172]. Wagner et al. [173] found the glycoprotein and non-glycosylated membrane protein of VSV to be associated with various cytoplasmic membrane classes. Smooth cytoplasmic membranes from influenza virus-infected cells contained large amounts of the viral glycoproteins as well as a detectable peak of the non-glycosylated membrane polypeptide [174–176]. Rough endoplasmic reticulum fractions also contained the hemagglutinin polypeptide, and the results of pulse-chase experiments suggested that this glycoprotein migrates from rough to smooth membranes [174,176]. Virtually all of the hemagglutinin polypeptide is membrane associated, rather than free in the cytoplasm [96,174–177]. The envelope glycoproteins of Sindbis virus are also associated with a membrane-containing cell fraction [178].

The exact site of synthesis of viral envelope proteins has not been established. In particular, it is not known whether these proteins are synthesized on membrane-associated or on membrane-free polysomes. In the latter case, it would appear that insertion into membranes after synthesis is extremely rapid, since there are no pools of soluble envelope polypeptides.

Several investigations have been made of the association of envelope proteins with isolated plasma membranes, which is the cellular site of maturation for many groups of viruses. Plasma membranes of influenza virus-infected cells are found to contain all the polypeptides of the virion [45,175]. In short pulses, the envelope proteins predominate, whereas with longer labeling or chase periods all viral proteins are found, although the relative amount of the hemagglutinin polypeptide HA is significantly higher than in virions. Both the glycoprotein and membrane protein of VSV were found associated with plasma membranes after short pulses [179], whereas the nucleocapsid polypeptide was found in increasing amounts depending on the length of the chase period. However, the membrane protein was observed to associate with plasma membranes in vitro, so that it is uncertain whether in vivo incorporation of this protein into membrane occurs. These results with influenza and VSV are compatible with the conclusions, made from electron microscopic studies, that envelope proteins arrive at the plasma membrane first, and nucleocapsids associate subsequently with these modified regions of membrane.

Lactoperoxidase-catalysed iodination has been used to study the proteins exposed on the outer surface of Sindbis virus-infected cells [58]. Both glycoproteins

could be detected above the background of cellular polypeptides, indicating that the viral glycoproteins comprise a significant proportion of the proteins of the total cell which are exposed at the surface. A component which is the immediate precursor to one of the two envelope proteins was not detected, indicating that it is located intracellularly. Thus proteolytic cleavage of the precursor may accompany the appearance of this protein on the surface.

Purified plasma membranes of herpes virus-infected cells contain viral glycoproteins similar to those present in the purified virion [180], despite the fact that the virus is assembled at the nuclear envelope, rather than the plasma membrane. These glycoproteins may be responsible for altered immunological specificity and social behavior of infected cells [181]. When plasma membrane vesicles from infected cells are treated with antiviral antibody, they are found to band at a higher buoyant density [182]. The prelabeled cellular proteins in the plasma membrane also shift to a higher density, indicating that viral and cellular proteins are contiguous in the vesicles.

VIID. Glycosylation of viral glycoproteins

Synthesis of glycoproteins differs from synthesis of non-glycosylated polypeptides in that the attachment of saccharide residues is a separate process which may be temporally and topologically distinct from synthesis of the polypeptide backbone. Investigations of the synthesis of glycoproteins have been carried out on a variety of systems [183], but it is hazardous to generalize from one cell type or protein species to another. It is clear that the synthesis of complex carbohydrate occurs by stepwise attachment of individual monosaccharides by specific transferases. Evidence has also been obtained that different saccharide residues may be attached in distinct cellular compartments. Residues most proximal to the polypeptide chain, including glucosamine, appear to be attached to polypeptides in rough endoplasmic reticulum [184,185]. Other residues located nearer to the distal termini, including galactose and fucose, appear to be attached in smooth membrane elements [186].

Limited information is available on the sites of glycosylation of viral glycoproteins. By labeling influenza virus-infected cells with glucosamine, it was found that hemagglutinin polypeptides in both rough and smooth cytoplasmic membrane fractions were labeled [187]. In contrast, fucose labeled the hemagglutinin polypeptides in smooth membranes, but not in rough membranes. Stanley et al. [175] observed that fucose continued to be incorporated into the hemagglutinin polypeptide for 10–15 min after inhibition of protein synthesis by puromycin, whereas incorporation of glucosamine ceased rapidly. In pulse-chase experiments, the bulk of the HA polypeptide was found in rough membranes after a short pulse [174,176], but the amount in smooth membranes increased after chases [174–176]. These results all suggest that the HA polypeptide may be synthesized in association with rough endoplasmic reticulum, where glycosylation is initiated, but that glycosyl residues nearer to the distal termini of side chains are incorporated after the HA polypeptide has migrated to the smooth cytoplasmic membranes. The finding of a qualitative differ-

ence in carbohydrate content of HA polypeptides in smooth and rough cytoplasmic membranes supports the view that the components in rough membranes are precursors of the smooth membrane-associated polypeptides, and that both may represent essential stages in the process by which completed HA polypeptides arrive at the cell surface.

The synthesis of the HA glycoprotein of influenza virus is inhibited by relatively high concentrations of glucosamine or 2-deoxyglucose, whereas the synthesis of non-glycosylated viral polypeptides is unaffected [188–190]. Under these conditions a new protein is detected which is thought to be a non-glycosylated precursor of the HA polypeptide [190]. The yield of infectious virus and hemagglutinin in such cells is inhibited, indicating that glycosylation of the HA polypeptide is essential for correct assembly or activity of this polypeptide. The hemagglutinating activity of one strain of influenza virus, but not of another, was reported to be destroyed by a mixture of glycosidases free of demonstrable proteolytic activity [191].

Glucosamine was not found in nascent herpes virus peptides released from polyribosomes by puromycin treatment, but was incorporated into macromolecules associated with membranes [172]. Pulse-chase experiments with amino acids and glucosamine indicated that viral glycoproteins were inserted into membranes before they were extensively glycosylated and glycosylation of these polypeptides took place in membranes [172].

VIIE. De novo assembly of viral membranes

In contrast to the majority of enveloped viruses which acquire their membranes during a budding process, the envelopes of pox viruses are formed de novo in discrete cytoplasmic foci of viral material which have been termed factories [192]. In synchronously infected cells, viral membranes are the first identifiable morphological component of vaccinia virions to appear in such factories, and initially consist of short, 500–1000 Å segments each possessing a trilaminar membrane coated externally with a layer of 100 Å-long projections [192]. Later, the membranes appear as arcs or closed circles, indicating that in three dimensions they become envelopes of spherical particles. As the membranes grow, they appear to surround nascent DNA and protein which are present in the factories.

The attachment of the 100 Å layer of projections on the external surface of the membranes appears to determine the spherical conformation of the envelope. Thus treatment with actinomycin D produced membranes lacking projections in some areas, and in such regions the unit membrane remained more flexible, even rounding up into vesicles.

If glycoproteins of viruses which form by budding are glycosylated by transferases associated with cellular membranes, the question arises whether viral membranes which form de novo contain glycoproteins, and if so, what is the site of glycosylation? A glycoprotein of approx. 40 000 daltons is present in the envelope of vaccinia virus [193,194]. However, in contrast to glycoproteins of other enveloped viruses, this viral glycoprotein appears to contain glucosamine as the sole

sugar constituent; fucose, galactose, or manose could not be detected [194]. Whether the attachment of glucosamine is directed by a cellular or virus-coded transferase has not been established.

The glycoprotein of the vaccinia virion is present in relatively small amounts in infected cells, but several other glycoproteins appear to be virus-induced and present in large amounts in the cytoplasm [195]. These glycoproteins appear to be associated with cellular membranes, but their functional role remains to be established.

VIII STRUCTURAL RELATIONSHIPS OF ENVELOPED VIRUSES TO OTHER VIRUSES

Ever since it was first suggested by Crick and Watson [196] that small crystallizable viruses contained identical subunits packed together in a regular manner, and that this hypothesis placed certain constraints of symmetry upon the structure of such viruses, symmetry has held an important place among the concepts used to understand viral structure. The search for symmetry has been extended to the far more complex enveloped viruses, and symmetrical nucleoprotein structures (consisting of identical subunits regularly packed, in accord with the original hypothesis) have indeed been characterized. However, in our view, approaches to establishing symmetry in the viral envelope have been less successful.

One element of the viral envelope which might come within the limitations of Crick and Watson's original argument – that is, that identical subunits must associate together in a regular manner to yield the final structure – is the non-glycosylated, internal membrane protein. However, as discussed above (Section V) it is not yet clear whether this protein does in fact associate with itself in such a manner, or whether its interaction with lipid is comparable or even predominant in importance. Consequently, there is no reason to assume, a priori, that the viral envelope is characterized by symmetrical constraints. The characterization of phenotypically mixed viral particles containing the internal proteins of VSV and the glycoprotein spikes of SV5 [218,219] also argues against the existence of the specific interactions which are required to generate a symmetrical structure.

Nonetheless, symmetrical elements have been proposed in several viral envelopes, notably influenza [197] and VSV [198], largely on the basis of regular arrangements which have been observed by electron microscopy [197,199,200]. We do not find such arguments convincing. A regular arrangement of rhodopsin molecules in rod outer segment membranes was proposed on the basis of the appearance of a regular pattern of surface features in electron micrographs [201]. Subsequent investigations using X-ray diffraction [202] and other physical techniques [145,146] showed that the rhodopsin molecules in these membranes are actually characterized by a very high degree of both rotational and lateral diffusion, rendering the existence of a regular, packed array of these molecules in this membrane under physiological conditions highly unlikely.

Recent evidence from a variety of sources has concurred in demonstrating that

both lipids and proteins in biological membranes are characterized by a far higher degree of lateral diffusion than was previously thought to be the case [143–146]. This characteristically high diffusion implies that there is no a priori reason for long-range order to exist in a biological membrane. If such order does exist, then specific factors must be found to account for it [203].

As has been documented in Section IV, the lipids of enveloped viruses are arranged in a bilayer, and their composition bears a close resemblance to that of cellular membranes. There is thus no reason to suppose that the lipid phase provides a rigid matrix to hold the viral proteins in some regular array, at least at physiological temperature and conditions of hydration. Rather, a bilayer of this composition would be expected to provide a fluid matrix such as is present in the host cell plasma membrane, and such fluidity is demonstrated by spin-label studies [111,113,114]. The logical conclusion from these considerations is that viral envelopes should resemble biological membranes in their structural characteristics far more closely than they resemble the small crystallizable viruses.

IX VIRAL MEMBRANES AND THE FLUID MOSAIC MODEL

The general structural organization of the influenza virus envelope proposed in 1969 by Laver and Valentine [52] corresponds in many respects to the fluid mosaic model subsequently proposed for the arrangement of lipids and proteins in biological membranes, a model which is supported by much recent experimental evidence [203]. The subsequent studies which have been reviewed in this article have confirmed that there are indeed many similarities between the organization of viral membranes and other membranes. Structural asymmetry, with all carbohydrate-containing components located on the external surface, is a characteristic of both viral membranes and cellular surface membranes such as the erythrocyte ghost. A lipid matrix which interacts with amphipathic globular proteins is the basic structure proposed in the fluid mosaic model, and is clearly the most likely arrangement for spike interaction with the lipid bilayer. The degree of penetration of viral spike proteins into the bilayer appears to be more limited than that of some cellular proteins. One indication of this difference is the presence of particles seen by freeze–fracture electron microscopy in cellular membranes, which are thought to be proteins deeply penetrating the lipid phase. Such particles are not found in similar studies of viral membranes [39,88], indicating that viral proteins do not penetrate the membrane to this extent. However, it is possible that various cellular proteins may also be bound to the membrane in a manner similar to that of viral spikes. To cite just a single specific example, the glycocalyx of the intestinal brush border is a glycoprotein coat, often visualized as knobs, which can be removed by suitable treatment with proteases [204,205] or non-ionic detergents [206], conditions strongly reminiscent of those which are effective in removing the viral glycoprotein spikes.

In addition to the lipid matrix, most viral membranes appear to have, on their

internal surface, a protein matrix which is composed of the non-glycosylated membrane or matrix protein. It is possible that other membranes may also possess such a double matrix structure, at least in specialized regions. By a protein matrix, we mean a large two-dimensional aggregate stabilized by protein–protein interaction. Two specific candidates for such an organization in cellular membranes are the large two-dimensional aggregates of intramembranous particles observed in gap junctions [207], and the protein termed spectrin which is associated with the inner surface of the erythrocyte membrane [208].

The fluidity of the lipid phase of viral membranes, as demonstrated by spin-label studies, is similar to that observed in other membranes [111]. However, at least some of the proteins associated with cellular membranes are able to undergo lateral diffusion in the plane of the membrane [209], whereas such lateral diffusion must be restricted in the case of viral proteins incorporated into cellular plasma membranes. The main indication that such diffusion is restricted is the specific exclusion of cellular proteins from the viral envelope. The mechanism by which such lateral diffusion of viral proteins could be restricted is not clear, and could involve protein–protein interaction or lipid-mediated interactions. It is possible that some cellular membrane proteins are likewise restricted in lateral diffusion by similar mechanisms.

In conclusion, the available evidence suggests that the structure of viral membranes is similar to that of other biological membranes in many important respects, and may resemble certain specific cellular membranes even more closely. Thus enveloped viruses are interesting and pertinent systems for studies of membrane structure.

ACKNOWLEDGMENTS

Research by the authors was supported by research grant No. AI 10884 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and National Science Foundation grant GB-36789 and a grant from the Cystic Fibrosis Research Foundation.

REFERENCES

1 Andrewes, C. and Pereira, H. G. (1972) Viruses of Vertebrates, Williams and Wilkins, Baltimore, Md.
2 Maramorosch, K. and Kurstak, E. (1971) Comparative Virology, Academic Press, New York, N.Y.
3 Melnick, J. L. (1973) Progr. Med. Virol. 15, 380–384
4 Rapp, F. (1973) J. Natl Cancer Inst. 50, 825–832
5 Andrewes, C. H., Bang, F. B. and Burnet, F. M. (1955) Virology 1, 176–184
6 Smadel, J. E. and Hoagland, C. L. (1942) Bacteriol. Rev. 6, 79–110
7 Harrison, S. C., Caspar, D. L. D., Camerini-Otero, R. D. and Franklin, R. M. (1971) Nature New Biol. 229, 197–201

8 Pfefferkorn, E. R. and Hunter, H. S. (1963) Virology 20, 433–445
9 Strauss, J. H., Jr, Burge, B. W. and Darnell, J. E. (1970) J. Mol. Biol. 47, 437–448
10 Ada, G. L and Perry, B. T. (1954) Austral. J. Exp. Biol. Med. Sci. 32, 453–468
11 Frommhagen, L. H., Knight, C. A. and Freeman, N. K. (1959) Virology 8, 176–197
12 Klenk, H. D. and Choppin, P. W. (1969) Virology 37, 155–157
13 McSharry, J. J. and Wagner, R. R. (1971) J. Virol. 7, 59–70
14 Quigley, J. P., Rifkin, D. B. and Reich, E. (1971) Virology 46, 106–116
15 Klenk, H. D., Caliguiri, L. A. and Choppin, P. W. (1970) Virology 42, 473–481
16 McSharry, J. J. and Wagner, R. R. (1971) J. Virol. 7, 412–415
17 Klenk, H. D. and Choppin, P. W. (1971) J. Virol. 7, 416–417
18 Maizel, J. V., Jr (1963) Biochem. Biophys. Res. Commun. 13, 483–489
19 Maizel, J. V., Jr (1964) Fed. Proc. 23, 160
20 Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1967) Biochem. Biophys. Res. Commun. 28, 815–820
21 Schlesinger, M. J., Schlesinger, S. and Burge, B. W. (1972) Virology 47, 539–541
22 Compans, R. W., Klenk, H. D., Caliguiri, L. A. and Choppin, P. W. (1970) Virology 42, 880–889
23 Schulze, I. T. (1970) Virology 42, 890–904
24 Skehel, J. J. and Schild, G. C. (1971) Virology 44, 396–408
25 Mountcastle, W. E., Compans, R. W. and Choppin, P. W. (1971) J. Virol. 7, 47–52
26 Bishop, D. H. L. and Roy, P. (1972) J. Virol. 10, 234–243
27 Hung, P. P., Robinson, H. L. and Robinson, W. S. (1971) Virology 43, 251–266
28 Rifkin, D. B. and Compans, R. W. (1971) Virology 46, 485–489
29 Cheung, K., Smith, R. E., Stone, M. P. and Joklik, W. K. (1972) Virology 50, 851–864
30 Ramos, B. A., Courtney, R. J. and Rawls, W. E. (1972) J. Virol. 10, 661–667
31 Spear, P. G. and Roizman, B. (1972) J. Virol. 9, 143–159
32 Abodeely, R. A., Palmer, E., Lawson, L. A. and Randall, C. C. (1971) Virology 44, 146–152
33 Sarov, I. and Joklik, W. K. (1973) Virology 50, 579–592
34 Obijeski, J. F., Palmer, E. L., Gafford, L. G. and Randall, C. C. (1973) Virology 51, 512–516
35 Pons, M. W., Schulze, I. and Hirst, G. K. (1969) Virology 39, 250–259
36 Mountcastle, W. E., Compans, R. W., Caliguiri, L. A. and Choppin, P. W. (1970) J. Virol. 6, 677–684
37 Wagner, R. R., Schnaitman, T. C., Snyder, R. M. and Schnaitman, C. A. (1969) J. Virol. 3, 611–618
38 Kang, C. Y. and Prevec, L. (1969) J. Virol. 3, 404–413
39 Brown, D. T., Waite, M. R. F. and Pfefferkorn, E. R. (1972) J. Virol. 10, 524–536
40 Horzinek, M. and Mussgay, M. (1969) J. Virol. 4, 514–520
41 Acheson, N. H. and Tamm, I. (1967) Virology 41, 321–329
42 Strauss, J. H., Jr, Burge, B. W., Pfefferkorn, E. R. and Darnell, J. E., Jr (1968) Proc. Natl Acad. Sci. U.S. 59, 533–537
43 Quigley, J. P., Rifkin, D. B. and Compans, R. W. (1972) Virology 50, 65–75
44 Davis, N. L. and Rueckert, R. R. (1972) J. Virol. 10, 1010–1020
45 Lazarowitz, S. G., Compans, R. W. and Choppin, P. W. (1971) Virology 46, 830–843
46 Lazarowitz, S. G., Compans, R. W. and Choppin, P. W. (1973) Virology 52, 199–212
47 Compans, R. W. (1971) Nature New Biol. 229, 114–115
48 Chen, C., Compans, R. W. and Choppin, P. W. (1971) J. Gen. Virol. 11, 53–58
49 Cartwright, B., Talbot, P. and Brown, F. (1970) J. Gen. Virol. 7, 267–272
50 McSharry, J. J., Compans, R. W. and Choppin, P. W. (1971) J. Virol. 8, 722–729
51 Klenk, H. D., Rott, R. and Becht, H. (1972) Virology 47, 579–591
52 Laver, W. G. and Valentine, R. C. (1969) Virology 38, 105–119
53 Webster, R. G. and Darlington, R. W. (1969) J. Virol. 4, 182–187
54 Scheid, A., Caliguiri, L. A., Compans, R. W. and Choppin, P. W. (1972) Virology 50, 640–652
55 Bolognesi, D. P., Bauer, H., Gelderblom, H. and Huper, G. (1972) Virology 47, 551–566
56 Stanley, P. and Haslam, E. A. (1971) Virology 46, 764–773
57 Robinson, W. S. and Robinson, H. L. (1972) in Membrane Research (Fox, C. F., ed.), pp. 187–201, Academic Press, New York, N.Y.
58 Sefton, B. M., Wickus, G. G. and Burge, B. W. (1973) J. Virol. 11, 730–735

59 Rifkin, D. B., Compans, R. W. and Reich, E. (1972) J. Biol. Chem. 247, 6432–6437
60 Gahmberg, C. G., Simons, K., Renkonen, O. and Kääriainen, L. (1972) Virology 50, 259–262
61 Burge, B. W. and Strauss, J. H., Jr (1970) J. Mol. Biol. 47, 449–466
62 Grimes, W. J. and Burge, B. W. (1971) J. Virol. 7, 309–313
63 Burge, B. W. and Huang, A. S. (1970) J. Virol. 6, 176–182
64 Laver, W. G. and Webster, R. G. (1966) Virology 30, 104–115
65 Harboe, A. (1963) Acta Pathol. Microbiol. Scand. 57, 317–330
66 Harboe, A. (1963) Acta Pathol. Microbiol. Scand. 57, 488–492
67 Hirst, G. K. (1942) J. Exp. Med. 76, 195–209
68 Kilbourne, E. D., Laver, W. G., Schulman, J. L. and Webster, R. G. (1968) J. Virol. 2, 281–288
69 Webster, R. G., Laver, W. G. and Kilbourne, E. D. (1968) J. Gen. Virol. 3, 315–326
70 Webster, R. G. (1970) in The Biology of Large RNA Viruses (Barry, R. D. and Maby, B. W. J., eds), pp. 53–74, Academic Press, London
71 Compans, R. W., Dimmock, N. J. and Meier-Ewert, H. (1969) J. Virol. 4, 528–534
72 Laver, W. G. (1971) Virology 45, 275–288
73 Brand, C. M. and Skehel, J. J. (1972) Nature New Biol. 238, 145–147
74 Eckert, E. A. (1973) J. Virol. 11, 183–192
75 Wrigley, N. G., Skehel, J. J., Charlwood, P. A. and Brand, C. M. (1973) Virology 51, 525–529
76 Lazdins, I., Haslam, E. A. and White, D. O. (1972) Virology 49, 758–765
77 Scheid, A. and Choppin, P. W. (1973) J. Virol. 11, 263–271
78 Gahmberg, C. G., Utermann, G. and Simons, K. (1972) FEBS Lett. 28, 179–182
79 Harrison, S. C., David, A., Jumblatt, J. and Darnell, J. E. (1971) J. Mol. Biol. 60, 523–528
80 Compans, R. W. and Dimmock, N. J. (1969) Virology 39, 499–515
81 Compans, R. W., Dimmock, N. J. and Meier-Ewert, H. (1970) in The Biology of Large RNA Viruses (Barry, R. D. and Maby, B. W. J., eds), pp. 87–108, Academic Press, London
82 Kilbourne, E. D., Choppin, P. W., Schulze, I. T., Scholtissek, C. and Bucher, D. L. (1972) J. Infect. Dis. 125, 447–455
83 Schulte, I. T. (1972) Virology 47, 181–196
84 Laver, W. G. and Baker, N. (1972) J. Gen. Virol. 17, 61–67
85 Walter, G. and Mudd, J. A. (1973) Virology 52, 574–577
86 McSharry, J. J., Compans, R. W., Lackland, H. and Choppin, P. W. (1972) Abstr. Annu. Meeting Am. Soc. Microbiol., p. 215
87 Compans, R. W., Holmes, K. V., Dales, S. and Choppin, P. W. (1966) Virology 30, 411–426
88 Bachi, T., Gerhard, W., Lindenmann, J. and Mühlethaler, K. (1969) J. Virol. 4, 769–776
89 Knight, C. A. (1946) J. Exp. Med. 83, 281–294
90 Smith, W., Belyavin, G. and Sheffield, F. W. (1953) Nature 172, 669–670
91 Isacson, P. and Koch, A. E. (1965) Virology 27, 129–138
92 Haukenes, G., Harboe, A. and Mortonsson-Egnund, K. (1965) Acta Pathol. Microbiol. Scand. 64, 534–542
93 Drzenick, R., Saber, M. S. and Rott, R. (1966) Z. Naturforsch. 21b, 254–260
94 Wagner, R. R., Schnaitman, C. A. and Snyder, R. M. (1969) J. Virol. 3, 395–403
95 Caliguiri, L. A., Klenk, H. D. and Choppin, P. W. (1969) Virology 39, 460–466
96 Holland, J. J. and Kiehn, E. D. (1970) Science 167, 202–205
97 Lowy, D. R., Rowe, W. P., Teich, N. and Hartley, J. W. (1971) Science 174, 155–156
98 Rowe, W. P., Hartley, J. W., Lander, M. R., Pugh, W. E. and Teich, N. (1971) Virology 46, 866–876
99 Aaronson, S. A., Todaro, G. J. and Scolnick, E. M. (1971) Science 174, 157–158
100 Weiss, R. A., Friis, R. R., Katz, E. and Vogt, P. K. (1971) Virology 46, 920
101 Gerber, P. (1972) Proc. Natl Acad. Sci. U.S. 69, 83–85
102 Hampar, B., Derge, J. G., Martos, L. M. and Walker, J. L. (1972) Proc. Natl Acad. Sci. U.S. 69, 78–82
103 Green, I. and Beard, J. W. (1955) J. Natl Cancer Inst. 15, 1217–1229
104 Mizutani, S. and Temin, H. M. (1971) J. Virol. 8, 409–416
105 Gregoriades, A. (1973) Virology 49, 333–336
106 Kelley, J. M., Emerson, S. U. and Wagner, R. R. (1972) J. Virol. 10, 1231–1235
107 Fleissner, E. (1971) J. Virol. 8, 778–785
108 Wecker, E. (1957) Z. Naturforsch. 126, 208

109 Kates, M., Allison, A. C., Tyrell, D. A. and James, A. T. (1961) Biochim. Biophys. Acta 52, 455–466
110 Pfefferkorn, E. R. and Hunter, H. S. (1963) Virology 20, 446–456
111 Landsberger, F. R., Lenard, J., Paxton, J. and Compans, R. W. (1971) Proc. Natl Acad. Sci. U.S. 68, 2579–2583
112 McConnell, H. M. and McFarland, B. G. (1972) Ann. N.Y. Acad. Sci. 195,207–217
113 Landsberger, F. R., Compans, R. W., Paxton, J. and Lenard, J. (1972) J. Supramol. Struct. 1, 50–54
114 Landsberger, F. R., Compans, R. W., Choppin, P. W. and Lenard, J. (1973) Biochemistry 12, 4498–4502
115 Bretscher, M. (1971) J. Mol. Biol. 58, 775–781; however, compare with Schmidt-Ullrich, R., Knufermann, H. and Wallach, D. F. H. (1973) Biochim. Biophys. Acta 307, 353–365
116 Bretscher, M. (1972) Nature New Biol. 236, 11–12
117 Bretscher, M. (1972) J. Mol. Biol. 71, 523–528
118 Friedman, R. M. and Pastan, I. (1969) J. Mol. Biol. 40, 107–115
119 Hirano, K., Yamaura, K., Shiluta, H. and Matumoto, M. (1971) Jap. J. Microbiol. 15, 383–396
120 Blair, C. D. and Brennan, P. J. (1972) J. Virol. 9, 813–822
121 Waite, M. R. F. and Pfefferkorn, E. R. (1970) J. Virol. 6, 637–643
122 Klenk, H. D. and Choppin, P. W. (1970) Virology 40, 939–947
123 Simpson, R. W. and Hauser, R. E. (1966) Virology 30, 684–697
124 Bates, S. R. and Rothblat, G. H. (1972) J. Virol. 9, 883–890
125 Lenard, J. and Landsberger, F. R., unpublished observations
126 Lenard, J., Wong, C. Y. and Compans, R. W., Biochim. Biophys. Acta 332, 341–349
127 David, A. E. (1971) Virology 46, 711–720
128 Heydrick, F. P., Comer, J. F. and Wachter, R. F. (1971) J. Virol. 7, 642–645
129 Renkonen, O., Kääriainen, L., Simons, K. and Gahmberg, C. G. (1971) Virology 46, 318–326
130 Renkonen, O., Kääriainen, L., Petterson, R. and Oker-Blom, N. (1972) Virology 50, 899–901
131 Laine, R., Kettunen, M., Gahmberg, C. G., Kääriainen, L. and Renkonen, O. (1972) J. Virol. 10, 433–438
132 Klenk, H. D. and Choppin, P. W. (1969) Virology 38, 255–268
133 Choppin, P. W., Klenk, H. D., Compans, R. W. and Caliguiri, L. A. (1971) in Perspectives in Virology (Pollard, M., ed.), pp. 127–158, Academic Press, New York, N.Y.
134 Klenk, H. D. and Choppin, P. W. (1970) Proc. Natl Acad. Sci. U.S. 66, 57–64
135 Blough, H. A. and Lawson, D. E. M. (1968) Virology 36, 286–292
136 Tiffany, J. M. and Blough, H. A. (1969) Virology 37, 492–494
137 Cartwright, B. and Brown, F. (1972) J. Gen. Virol. 15, 243–245
138 Tiffany, J. M. and Blough, H. A. (1969) Science 163, 573–574
139 Blough, H. A., Weinstein, D. B., Lawson, D. E. M. and Kodicek, E. (1967) Virology 33, 459–466
140 Blough, H. A. and Tiffany, J. M. (1969) Proc. Natl Acad. Sci. U.S. 62, 242–247
141 Ben-Porat, T. and Kaplan, A. S. (1971) Virology 45, 252–264
142 Ben-Porat, T. and Kaplan, A. S. (1972) Nature 235, 165–166
143 Scandella, C. J., Deraux, P. and McConnell, H. M. (1972) Proc. Natl Acad. Sci. U.S. 69, 2056–2060
144 Frye, L. D. and Edidin, M. (1970) J. Cell Sci. 7, 319–335
145 Brown, P. K. (1972) Nature New Biol. 236, 35–38
146 Cone, R. A. (1972) Nature New Biol. 236, 39–43
147 Hong, K. and Hubbell, W. L. (1972) Proc. Natl Acad. Sci. U. S. 69, 2617–2621
148 Jost, P. C., Griffith, O. H., Capaldi, R. A. and Vanderkooi, G. (1973) Proc. Natl Acad. Sci. U.S. 70, 480–484
149 Shimizu, K., Hosaka, Y. and Shimizu, Y. K. (1972) J. Virol. 9, 842–850
150 Hosaka, Y. and Shimizu, Y. K. (1972) Virology 49, 627–639
151 Hosaka, Y. and Shimizu, Y. K. (1972) Virology 49, 640–646
152 Nermut, M. V. (1972) J. Gen. Virol. 17, 317–331
153 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661
154 Skehel. J. J. (1971) Virology 44, 409–417
155 Schulze, I. T. (1970) Virology 42, 890–904
156 Compans, R. W., Content, J. and Duesberg, P. H. (1972) J. Virol. 10, 795–800

157 Zee, Y. C., Hackett, A. J. and Talens, L. (1970) J. Gen. Virol. 7, 95–102
158 Acheson, N. H. and Tamm, I. (1967) Virology 32, 128–143
159 Matsumura, T., Stollar, V. and Schlesinger, R. W. (1971) Virology 46, 344–355
160 Howatson, A. F. and Whitmore, G. F. (1962) Virology 16, 466–478
161 Howe, C., Morgan, C., de Vaux St. Cyr, C., Hsu, K. C. and Rose, H. M. (1972) J. Virol. 1, 215–237
162 Gelderblom, H., Bauer, H. and Graf, T. (1972) Virology 27, 416–425
163 Darlington, R. W. and Moss, L. H. (1968) J. Virol. 2, 48–55
164 Nii, S., Morgan, C. and Rose, H. M. (1968) J. Virol. 2, 517–536
165 Hotchin, J. E., Cohen, S. M., Ruska, H. and Ruska, C. (1958) Virology 6, 689–701
166 Baker, R. F., Gordon, I. and Stevenson, D. (1965) Virology 27, 441–445
167 Duc-Nguyen, H. (1968) J. Virol. 2, 494–506
168 Compans, R. W. and Choppin, P. W. (1971) in Comparative Virology (Maramorosch, K. and Kurstak, E., eds), pp. 407–432, Academic Press, New York, N.Y.
169 Duc-Nguyen, H., Rose, H. M. and Morgan, C. (1966) Virology 28, 404–412
170 Klenk, H. D., Compans, R. W. and Choppin, P. W. (1970) Virology 42, 1158–1162
171 Spear, P. G., Keller, J. M. and Roiman, B. (1970) J. Virol. 5, 123–131
172 Spear, P. G. and Roizman, B. (1970) Proc. Natl Acad. Sci. U.S. 66, 730–737
173 Wagner, R. R., Kiley, M. P., Snyder, R. M. and Schnaitman, C. A. (1972) J. Virol. 9, 672–683
174 Compans, R. W. (1973) Virology 51, 56–70
175 Stanley, P., Gandhi, S. S. and White, D. O. (1973) Virology 53, 92–106
176 Klenk, H. D., Wollert, W., Rott, R. and Scholtissek, C., (1974) Virology, in the press
177 White, D. O., Taylor, J. M., Haslam, E. A. and Hampson, A. W. (1970) in The Biology of Large RNA Viruses (Barry, R. D. and Mahy, B. W. J., eds)., pp. 602–618, Academic Press, London,
178 Burge, B. W., personal communication
179 Cohen, G. H., Atkinson, P. H. and Summers, D. F. (1971) Nature New Biol. 231, 121–123
180 Heine, J. W., Spear, P. G. and Roizman, B. (1972) J. Virol. 9, 431–439
181 Roizman, B. (1971) Transplantation Proc. 3, 1179–1183
182 Heine, J. W. and Roizman, B. (1973) J. Virol. 11, 810–813
183 Kraemer, P. M. (1971) in Biomembranes (Manson, L. A., ed.), Vol. I, pp. 67–190, Plenum Press, New York
184 Molnar, J. and Sy, D. (1967) Biochemistry 6, 1941–1947
185 Zagury, D. Uhr, J. W. Jamieson, J. D. and Palade, G. E. (1970) J. Cell Biol. 46, 52–63
186 Bennett, G. and Leblond, C. P. (1970) J. Cell Biol. 46, 409–416
187 Compans, R. W. (1973) Virology 55, 541–545
188 Kaluza, G., Scholtissek, C. and Rott, R. (1972) J. Gen. Virol. 14, 251–259
189 Gandhi, S. S. Stanley, P., Taylor, J. M. and White, D. O. (1972) Microbios 5, 41–50
190 Klenk, H. D., Scholtissek, C. and Rott, R. (1972) Virology 49, 723–734
191 Bikel, I. and Knight, C. A. (1972) Virology 49, 326–332
192 Dales, S. and Mosbach, E. H. (1968) Virology 35, 564–583
193 Holowczak, J. A. (1970) Virology 42, 87–99
194 Garon, C. F. and Moss, B. (1971) Virology 46, 233–246
195 Moss, B., Rosenblum, E. N. and Garon, L. F. (1971) Virology 46, 221–232
196 Crick, F. H. C. and Watson, J. D. (1956) Nature 177, 473–475
197 Nermut, M. V. and Frank, H. (1971) J. Gen. Virol. 10, 37–51
198 Cartwright, B., Smale, C. J., Brown, F. and Hull, R. (1972) J. Virol. 10, 256–260
199 Almeida, J. D. and Waterson, A. P. (1967) J. Gen. Microbiol. 46, 107–110
200 Archetti, I., Jemolo, A. and Steve-Bocciarelli, D. (1967) Archiv. Gesamte Virusforsch. 20, 133–136
201 Blasie, J. K., Dewey, M. M., Blaurock, A. E. and Worthington, C. R. (1965) J. Mol. Biol. 14, 143–152
202 Blasie, J. K. and Worthington, C. R. (1969) J. Mol. Biol. 39, 417–439
203 Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731
204 Oda, T. and Sato, R. (1965) J. Electron Microscopy 14, 210–217
205 Johnson, C. F. (1967) Science 155, 1670–1672
206 Eichholz, A., personal communication
207 Goodenough, D. A. and Stoeckenius, W. (1972) J. Cell Biol. 54, 646–656
208 Marchesi, V. T., and Steers, E., Jr (1968) Science 159, 203

209 Frye, C. D. and Edidin, M. (1970) J. Cell Sci. 7, 319–335
210 Compans, R. W. and Caliguiri, L. A. (1974) in Negative Strand Viruses (Barry, R. D. and Mahy, B. W. J., eds), Academic Press, in press
211 Robinson, D. J. and Watson, D. H. (1971) J. Gen. Virol. 10, 163–171
212 Landsberger, F. R. and Compans, R. W., unpublished observations
213 Bretscher, M. (1973) Science 181, 622–629
214 Caliguiri, L. A., Klenk, H. D. and Choppin, P. W. (1969) Virology 39, 460–466
215 Scheid, A. and Choppin, P. W. (1971) Bacteriol. Proc. 205
216 Choppin, P. W., Compans, R. W., Scheid, A., McSharry, J. J. and Lazarowitz, S. G. (1972) in Membrane Research (Fox, C. F., ed.), pp. 163–185, Academic Press, New York, N.Y.
217 Helenius, A. and Soderlund, H. (1973) Biochim. Biophys. Acta 307, 287–300
218 Choppin, P. W. and Compans, R. W. (1970) J. Virol. 5, 609–616
219 McSharry, J. J., Compans, R. W. and Choppin, P. W. (1971) J. Virol. 8, 722–729