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.

Lipids in Viruses

HERBERT A. BLOUGH AND JOHN M. TIFFANY

Division of Biochemical Virology and Membrane Research, Scheie Eye Institute of the Presbyterian—University of Pennsylvania Medical Center, Philadelphia, Pennsylvania

I. Introduction

Lipid forms an integral part of many viruses and exists either in the form of a continuous envelope or in lipoprotein complexes which surround a nucleoprotein core or helix. In general, the envelope can be described as a molecular container for the genetic material of the virus. The identification of lipid as a component of viruses was based initially on the loss or decrease of infectivity observed following exposure to organic solvents

(Andrewes and Horstmann, 1949; Hoyle, 1952). Although a review of lipid-containing viruses has appeared elsewhere (Franklin, 1962), lack of analytical data prevented this from being much more than a catalogue of solvent- or bile salt-sensitive viruses. The relationship of cellular membranes to the biogenesis of enveloped viruses was first pointed out for influenza virus more than 20 years ago by Hoyle (1950); at the time his results were received with some skepticism, primarily because his conclusions were based on dark-field microscopy. With the development of appropriate thin-sectioning techniques, electron microscopy provided the morphological evidence for the assembly of many viruses at cellular membranes (Morgan *et al.*, 1956, 1959, 1961; Hotz and Schäfer, 1955). There are, of course, exceptions: some DNA viruses appear to be synthesized *de novo*, i.e., without utilizing a preexisting "membrane template" (see Section VI). The application of negative contrast techniques not only demonstrated the symmetry of isometric viruses, but allowed visualization of the fine structure of the envelope; thickness, number and spacing of surface projections, and, in some cases, infrastructure, have been investigated by this means (e.g., Horne *et al.*, 1960; Apostolov and Flewett, 1969). Recently, freeze-etching techniques have been used as a visual probe of the apolar regions of the viral envelope (Bächi *et al.*, 1969; Nermut and Frank, 1971).

The first major compositional analysis of the lipids of influenza virus was performed by Frommhagen *et al.* (1959), and these studies were extended by Kates *et al.* (1961) to include the relationship between the lipids of influenza virus and of subcellular fractions of cultured host cells. These and similar studies initiated the present phase of virus research, in which the complex interactions of virus and host are studied as well as simply the lipid composition of the virus. Lipids are found to make up 20–35% by weight of many viruses (Armbruster and Beiss, 1958; Frommhagen *et al.*, 1959; Kates *et al.*, 1961; Pfefferkorn and Hunter, 1963a; Ahmed *et al.*, 1964; Rao *et al.*, 1966; Blough and Lawson, 1968) although there are notable exceptions such as the lipovirus PM2, 15% (Espejo and Canelo, 1968a,b); vaccinia, 5% (Zwartouw, 1964); and insect nuclear polyhedrosis virus, 1.6% (Bergold and Wellington, 1954).

Despite the large amounts of lipid found in some viruses, the status of lipids in the virologists' list of investigative priorities has remained low. This is probably due to a number of factors: the influence of classical bacteriophage genetics in virology, the emphasis in molecular biology on proteins and nucleic acids, and perhaps most importantly, the fact that didactic sessions on lipid biochemistry have not been presented to the young virologist in training. The low priority lipids have received is manifested by one's inability to find lipids as a part of the general taxon-

omy of viruses (see Wildy, 1971), and many of the current virology texts give only a cursory treatment of these important molecules in the discussion of assembly or in biochemical techniques (e.g., Fenner, 1968; Robinson and Duesberg, 1968). While it is undeniable that this emphasis on the study of the properties of nucleic acids and proteins is justified, since they are the source of virus-specific functions, lipids have an important role in maintaining structural integrity and infectivity of the virion, thereby permitting expression of these functions. Following recent advances in knowledge of the composition and structure of a number of viruses, and the application of more sophisticated techniques of analysis, lipids are now achieving a greater degree of attention than ever before, and it is to be hoped that this interest will lead to a more unified approach to the study and teaching of virology.

With the application of powerful tools such as thin-layer and gas-liquid chromatography and physical techniques such as X-ray diffraction and magnetic resonance spectroscopy, fresh light is being cast on the interactions of the constituent lipid and protein molecules in membranes. Viruses which are assembled at cell membranes, and contain a limited number of protein and lipid species, can serve as relatively simple biological models for study of these activities. The purpose of this review is to provide biochemists, cell biologists, and virologists with insight into the chemical composition of the envelopes of lipid-containing viruses and the effect of these viruses on host cell lipid metabolism. An attempt will be made to explain, by reference to models of membrane systems, how constituent molecules may be arranged and assembled to make up the molecular fabric of the viral envelope.

In many of the following sections, discussion will be found to center primarily on the myxoviruses, and particularly on influenza virus; this in part reflects the authors' own research interests, but also the fact that a large proportion of the work so far done on viral lipids has employed myxoviruses, since their propagation in either embryonated eggs or tissue culture is well established and samples can be obtained in good yield and high purity. The review of literature was substantially completed in March 1972, although a few references later than this have been included.

II. Classification

A. Types of Lipid-Containing Viruses

Viruses may be classified on the basis of their shape and size, type of nucleic acid, and the presence or absence of an envelope (Lwoff *et al.*,

1962). The presence of lipid within a virion does not, however, necessarily imply that the virus is enveloped, since the lipid may complex with protein to form a lipoprotein shell similar to that seen in low density lipoproteins (Pollard *et al.*, 1969), or lipid may associate with the nucleocapsid as in frog virus (E. Houts and M. Gravell, personal communication, 1972). An envelope is essentially a structural feature discernible by electron microscopy, where it may be recognized in one of two ways: (1) by appearing as a coherent shell in positively stained thin sections (when the lipid often stains in the well-known trilamellar pattern) and in negatively stained preparations where the stain has penetrated within the particle; (2) by its independent existence and retention of integrity even after loss of the internal component, or when produced as incomplete particles lacking a core (Almeida and Waterson, 1970).

Lipid-containing viruses are produced in mammalian, avian, bacterial, plant, and insect hosts. They may contain either RNA or DNA, and are characterized by lower densities than those of viruses containing no lipid, e.g., 1.16–1.18 gm cm^{-3} for influenza and murine leukemia viruses (Blough and Merlie, 1970; Johnson and Mora, 1967) up to 1.285 gm cm^{-3} for herpesvirus and iridoviruses (Spear and Roizman, 1967; Spring and Roizman, 1967; E. Houts and M. Gravell, personal communication, 1972); by contrast, the buoyant density of type 5 adenovirus, which contains no lipid, is 1.335 gm cm^{-3} (Wilcox and Ginsberg, 1963). The presence of unique lipids, which might aid in the classification of viruses, has not been reported. The proportions of certain classes of lipid may however differ considerably from those in the host cell (see Section IV).

For simplicity lipid-containing viruses will be divided into those containing RNA and those containing DNA (Table I). The majority of these viruses infect vertebrate and invertebrate hosts, although there are some plant viruses (rhabdoviruses, some iridoviruses, some baculoviruses, and tomato spotted wilt virus), but thus far only two lipid-containing viruses infecting microorganisms, i.e., lipovirus PM2 in *Pseudomonas* BAL-31 and *Mycoplasma laidlawii* type 2 virus (Gourley, 1971). The interested reader is referred to Wildy (1971) for a more comprehensive classification of viruses; even there, however, it will be found that the presence of lipid is in many cases only presumed from the ether sensitivity, without definite chemical proof. Because of this lack of knowledge about the composition of many viruses, we have restricted discussion in this review largely to those examples where electron microscopy, lipid and protein composition, and in some cases other studies such as X-ray diffraction, have all been done.

It will be seen in Table I that rubella virus has been assigned to a

Table I

CLASSES OF LIPID-CONTAINING VIRUSES AND SOME REPRESENTATIVE MEMBERS

Classes	Representative members
1. RNA viruses	
Orthomyxoviruses	Influenza (human, equine, avian, porcine), fowl plague viruses
Paramyxoviruses	Newcastle disease, parainfluenza, mumps viruses
Alphaviruses (Arbovirus group A)	Sindbis, Semliki Forest, equine encephalitis viruses
Arbovirus group B	Yellow fever, dengue, Japanese B encephalitis viruses
Rhabdoviruses	Vesicular stomatitis, rabies, *Drosophila* σ, potato yellow dwarf, Egtved viruses
Leukoviruses	Rous sarcoma, mouse leukemia (e.g., Rauscher, Moloney, Friend), mouse mammary tumor, visna, avian leukosis viruses
Tomato spotted wilt virus	
Coronaviruses	Avian infectious bronchitis, mouse hepatitis, human respiratory viruses
Arenaviruses	Lymphocytic choriomeningitis, Lassa, Parana viruses
Other unclassified enveloped RNA viruses	Rubella virus, measles virus; *Mycoplasma laidlawii* type 2 virus
2. DNA viruses	
Herpesvirus	Herpes simplex, varicella, infectious bovine rhinotracheitis, Epstein-Barr, Marek's disease, pseudorabies viruses
Baculoviruses	*Lymantria* (*Porthetria*) *dispar* nuclear polyhedrosis, *Choristoneura fumiferana* granulosis viruses
Iridoviruses	Amphibian polyhedral cytoplasmic (frog virus FV 3) and some invertebrate iridescent viruses
Lipoviruses	Marine bacteriophage PM2
Poxviruses	Vaccinia, orf, smallpox, fowlpox, Yaba monkey tumor, and entomopoxviruses

group of unclassified enveloped RNA viruses. There is still some controversy as to whether rubella virus belongs to the paramyxoviruses or to the arboviruses; comparison of its morphology and some of its properties with those of a number of arboviruses has led to the suggestion from two groups of workers that it should be reclassified (Holmes and Warburton, 1967; Carver and Marcus, 1968). At the time of writing, no group-specific antigen or arthropod vector has been reported. Measles virus has also

been assigned to this unclassified group rather than included in the paramyxoviruses since this virus has not officially been declared to be a paramyxovirus; the particle contains no neuraminidase, but otherwise shares many of the characteristics of the group.

B. Electron Microscopic Observations

This section is intended to provide only a brief review of the morphogenesis of virus particles as revealed by electron microscopy, rather than a detailed description of the morphology of the mature particles, as this has been given in many other texts. Based on the site and mode of assembly, three classes of lipid-containing viruses can be distinguished: (1) those where the virus buds from a preexisting cell membrane following insertion of virus-specific material into the membrane; the membrane is hence used essentially as a "scaffolding" or "template" to support viral material during maturation; (2) a *de novo* form of synthesis, where lipid and protein condense around the viral nucleic acid without utilizing a preexisting membrane; (3) a combination of the "scaffolding" and *de novo* methods for different parts of the virion.

Two terms used to describe structural features of viruses require definition: *capsomers* are morphological units of protein forming the capsid, and *capsid* is the protein structure of the virus which shields the nucleic acid (i.e., all the capsomers together make up the capsid). The capsid may be isometric (icosahedral) or helical (Caspar *et al.*, 1962).

1. *RNA Viruses*

a. Myxoviruses. These are "template" type viruses assembled at the cell surface membrane and released into the surrounding medium by a budding process (Fig. 1a); they are covered by an array of equidistant projections or spikes on the outer side of the envelope. In certain strains of influenza virus, particularly influenza C, a hexagonal reticular structure appears to exist within the envelope, connecting the bases of the spikes (Apostolov and Flewett, 1969). Extremely regular hexagonal arrays of spikes are also frequently observed (Almeida and Waterson, 1967, 1970). Many myxoviruses are roughly spherical in shape, although distortion may occur during preparation for microscopy, but filamentous strains are also seen, especially of influenza C (Archetti *et al.*, 1967); filamentous particles can also be produced by genotypic (Kilbourne, 1963) or phenotypic means (Blough, 1963) from normally spherical strains. Under conditions of high multiplicity of infection and continued passage, markedly pleomorphic particles of influenza virus are produced which show mor-

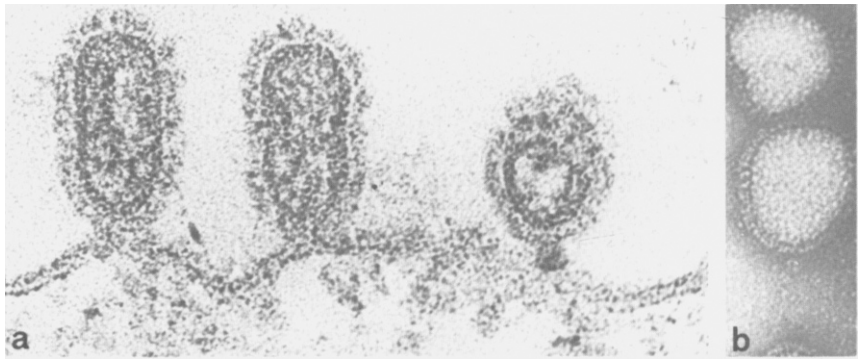

FIG. 1a. Assembly of influenza virus (A_0/PR8/34) at the cell surface membrane ("template" assembly). × 200,000. 1b. Negative contrast micrograph of influenza virus A_0/PR8/34. × 160,000. (H. A. Blough, unpublished micrographs.)

phological defects in the envelope and lack the larger of the pieces of RNA forming the viral genome (Pons and Hirst, 1969). Depending on the method of preparation and staining for electron microscopy, a variety of different structures may be detected within the envelope (Apostolov and Flewett, 1969; Compans and Dimmock, 1969; Nermut, 1970). The paramyxoviruses are larger, contain a continuous genome, and frequently appear pleomorphic; they are also assembled at cell surfaces, although some may be assembled also as single particles within intracytoplasmic vesicles (Blough, 1964).

b. Alphaviruses and Group B Arboviruses. In terms of structural components, these appear to be the simplest of the enveloped viruses. Thus Sindbis virus contains only two envelope glycoproteins and an inner nucleocapsid protein (Schlesinger *et al.*, 1972). They are assembled by budding at the cell surface (Fig. 2a), or by budding from the cytoplasm into intracytoplasmic vesicles by marsupialization of the vesicle wall to form the envelope (Fig. 2b) (Morgan *et al.*, 1961; Grimley and Friedman, 1970).

c. Rhabdoviruses. These viruses are found in plant, vertebrate, and invertebrate hosts, and are characterized by their bacilliform or "bullet shape" (Kitajima and Costa, 1966; Nakai and Howatson, 1968). In some cases, especially with rabies and the plant viruses, the infrastructure of the envelope is visible (Hummeler *et al.*, 1967). The vertebrate rhabdoviruses either bud off the surface membrane ("template" type) or through cytoplasmic membranes into vesicles, but large numbers of particles also appear to be formed *de novo* in the cytoplasm of infected cells (Fig. 3). Plant rhabdoviruses bud off the nuclear membrane or are found within

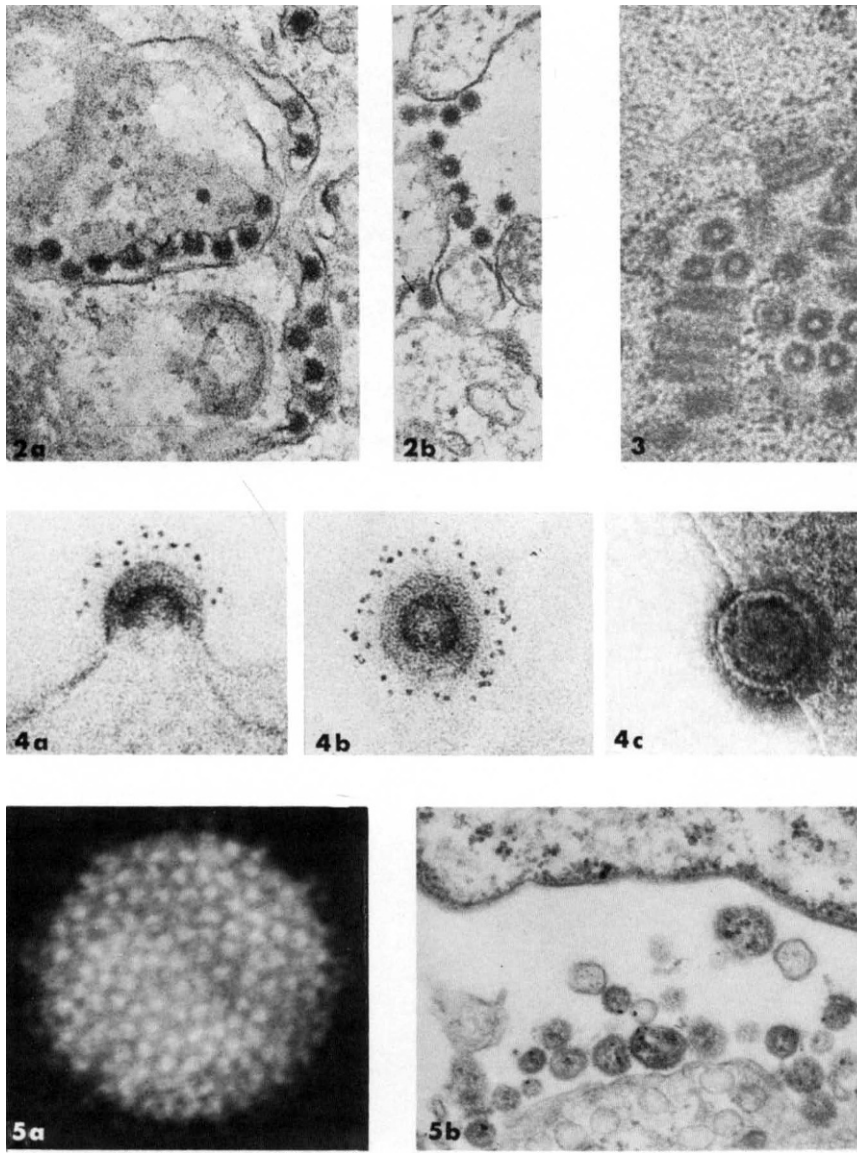

FIG. 2. Maturation of Semliki Forest virus in mouse neurons. 2a. Intracytoplasmic assembly. × 60,000. 2b. Assembly at the cell surface membrane. × 47,500. (Courtesy of Dr. P. Grimley.)

FIG. 3. Intracytoplasmic assembly of rabies virus. × 56,000. (Courtesy of Dr. K. Hummeler.)

FIG. 4. a and b. Maturation of avian myeloblastosis virus at the plasma mem-

intracytoplasmic vesicles derived from the endoplasmic reticulum (Kitajima and Costa, 1966). Extensive reviews have recently been published on rabies virus (Matsumoto, 1970) and on vesicular stomatitis virus and other rhabdoviruses (Howatson, 1970).

d. Leukoviruses. These are among the most complex of the RNA-containing viruses, and generally exhibit the lowest densities and highest lipid contents (Johnson and Mora, 1967). They appear to contain an inner membrane (possibly containing lipid in addition to protein) surrounded by an outer envelope acquired at the cell surface membrane (Fig. 4a–c). The outer envelope of Rous sarcoma virus appears to have a hexagonal subunit structure (Dourmashkin and Simons, 1961). The inner membrane fuses during budding to surround the nucleoid or RNA-containing central core (de Thé and O'Connor, 1966). Some particles of avian myeloblastosis virus have also been found to contain up to 100 molecules of transfer RNA and one or two ribosomes between the nucleoid and the envelope (Říman *et al.*, 1972). Virions are also seen within intracytoplasmic vesicles with certain cell types.

e. Tomato Spotted Wilt Virus. Particles of this virus appear isometric (van Kammen *et al.*, 1966) and are bounded by an envelope with surface projections. Assembly occurs in clusters in intracytoplasmic vacuoles, which may be formed from endoplasmic reticulum (Ie, 1964; Milne, 1970), as well as within the dilated lumen of the nuclear membrane (Kitajima, 1965).

f. Coronaviruses. These are enveloped viruses formed by budding, usually by marsupialization of the bounding membranes of large intracytoplasmic vacuoles (Becker *et al.*, 1967).

g. Arenaviruses. These are pleomorphic enveloped viruses, variable in size and with conspicuous surface projections. They are assembled at cell surface membranes (Fig. 5) and in intracytoplasmic vacuoles (Murphy *et al.*, 1970).

h. Other Viruses. Rubella virus has in the past proved difficult to obtain pure, and preparations examined in the electron microscope have frequently contained large amounts of nonviral material which made it impossible to obtain an accurate picture of the size and construction of the particles (Norrby, 1969). It has been found from purer preparations

---

brane, labeled with group-specific antigen coupled to ferritin. × 94,000. (Taken from Gelderblom *et al.*, 1972, by courtesy of Dr. H. Bauer and the Editors of *Virology.*) 4c. Negative contrast micrograph of mouse mammary tumor virus budding off a microvillus. × 186,000. (Courtesy of Dr. D. H. Moore.)

FIG. 5. Arenaviruses. 5a. Negative contrast micrograph of Tacaribe virus. × 413,600. 5b. Pleomorphic particles of Parana virus released at the cell surface membrane. × 42,000. (Courtesy of Dr. F. Murphy.)

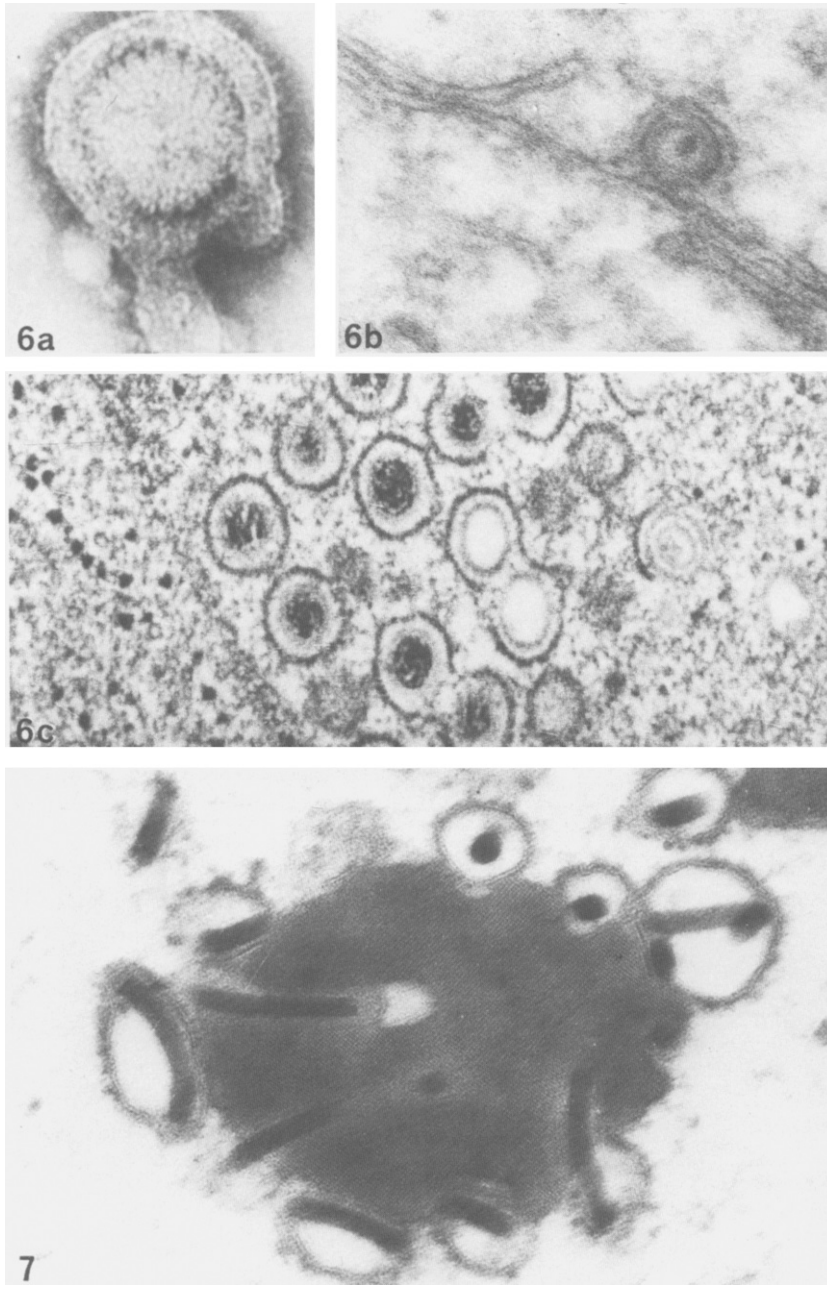

FIG. 6a. Negative contrast micrograph of herpes simplex virus. Note the envelope surrounding the isometric capsid. × 200,000. (Courtesy of Dr. D. H. Watson.)

that the virus buds largely into intracytoplasmic vesicles (McCombs *et al.*, 1968), specifically at Golgi membranes (Bonissol and Sisman, 1968), although Murphy *et al.* (1968) found that appreciable amounts of virus were produced by budding at the cell surface membrane. Particles generally appear to have a single envelope, although McCombs *et al.* (1968) report particles surrounded by a double membrane as occasionally seen with herpesvirus.

2. *DNA Viruses*

a. Herpesviruses. In general envelopment proceeds by budding through a membrane; here the inner nuclear membrane is preferred (Siegert and Falke, 1966; Darlington and Moss, 1968). However, any membrane may be used, including the Golgi apparatus, endoplasmic reticulum, and occasionally the plasma membrane; in some cases double membranes are formed. The viral core is icosahedral with a triangulation number of 16, containing 162 capsomers, and is surrounded by a lipoprotein envelope (Fig. 6a) containing 4–5 glycopeptides (Spear and Roizman, 1972). Treatment with phospholipase C suggests that lipid is not only in the envelope but may also be associated with the capsid of "naked" particles (Spring and Roizman, 1968; Asher *et al.*, 1969). Recent electron micrographs of herpesvirus of saimiri (squirrel monkey) (Fig. 6c) suggest that *de novo* synthesis of enveloped particles occurs within the nucleus of owl monkey kidney cells (Heine *et al.*, 1971). The structure of the virion and the usual site of assembly are presented in Figs. 6a and 6b.

b. Baculoviruses. Insect nuclear polyhedrosis viruses develop from a dense "virogenic stroma" to form large crystalline inclusions in the cell nucleus (Smith, 1967); considerable compression of the envelope occurs within the nuclear polyhedral crystals (Fig. 7). *De novo* synthesis of the viral envelope has been suggested in *Lymantria dispar* (= *Porthetria dispar*) and *Aglais urticae* (Harrap, 1969). Multiple genomes may be enclosed within a single envelope, and envelopes possessing different numbers of genomes can be separated by zonal centrifugation (Harrap, 1969). The complex structure of these viruses is indicated in Fig. 27).

c. Iridoviruses. Amphibian viruses such as the cytoplasmic frog virus FV 3 (Fig. 8) appear to be synthesized *de novo* in the cytoplasm of in-

---

6b. Herpes simplex virus acquiring its envelope at the nuclear membrane. × 87,000. (Taken from Morgan *et al.*, 1959, by courtesy of Dr. C. Morgan and the Rockefeller University Press.) 6c. Evidence of *de novo* synthesis of the viral envelope of herpesvirus of saimiri (squirrel monkey) within the nucleus of an owl monkey kidney cell. × 105,000. Taken from Heine *et al.* (1971).

FIG. 7. Development of a nuclear polyhedrosis virus of *Lymantria dispar* within the nucleus of a larval fat cell. × 60,000. (Courtesy of Dr. K. A. Harrap.)

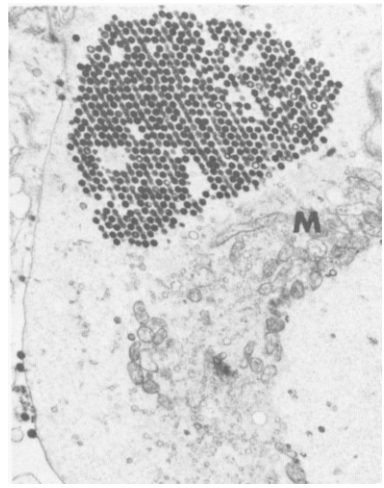

FIG. 8. An intracellular paracrystalline array of frog virus, FV 3 (iridovirus). × 6,000. (Courtesy of Dr. A. Granoff.)

fected cells and frequently appear in paracrystalline arrays (Darlington *et al.*, 1966; Granoff, 1969). Envelopment by budding at cytoplasmic membranes is rare. The lipid of the virion appears to be associated with the nucleocapsid (E. Houts and M. Gravell, personal communication,

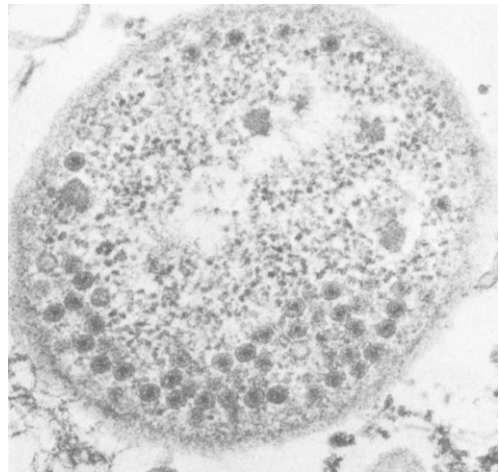

FIG. 9. Assembly of lipovirus PM2 in the marine bacterium *Pseudomonas* BAL-31. Virus particles are found beneath the cell envelope. × 63,000. (Courtesy of Dr. R. M. Franklin.)

1972). Members of this group infecting invertebrate hosts appear to be synthesized in a similar fashion (J. S. Robertson, personal communication, 1972).

d. Lipoviruses. The marine bacteriophage PM2, although assembled in close contact with the plasma membrane of the bacterial host (Fig. 9), appears to develop *de novo* by encapsidation of viral DNA by protein and lipid (Cota-Robles *et al.*, 1968); budding has not been observed (Dahlberg and Franklin, 1970). The virion is found not to contain enough lipid to form a complete bilayer shell (Harrison *et al.*, 1971a). Negative-contrast electron microscopy indicates that the virion is icosahedral.

e. Poxviruses. These are complex particles which are assembled in cytoplasmic "factories," probably *de novo* (Dales and Siminovitch, 1961). These large viruses ($3500 \times 2800 \times 2000$ Å) are covered by an outer layer of tubules or "spicules" giving the particle a mulberry-like appearance (Fig. 10a). The DNA is closely associated with protein to form a biconcave structure, and two lateral bodies can also be seen (Peters and Müller, 1963). Immature forms of the virus appear as membranous structures which probably contain DNA-protein complexes (Fig. 10b). Immature particles accumulate when protein synthesis is inhibited by 5-fluorodeoxyuridine or isatin β-thiosemicarbazone (Woodson and Joklik, 1965); on removal of the block, synthesis of mature particles proceeds normally (Dales and Mosbach, 1968). The viral envelope is assembled *de novo* (Dales and Mosbach, 1968; White *et al.*, 1968) in mammalian and avian

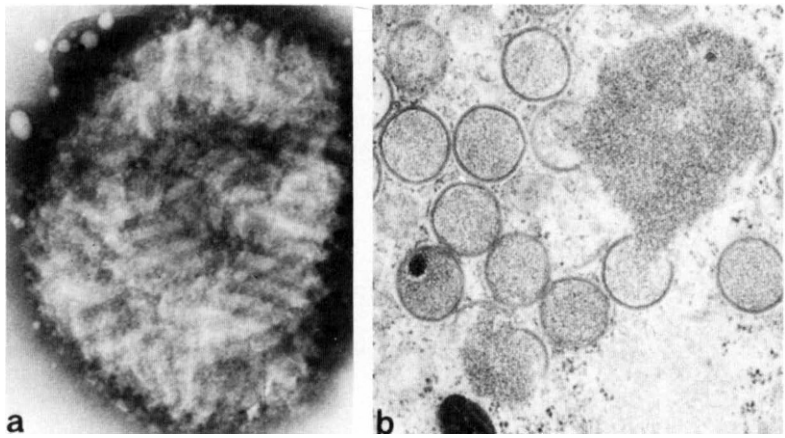

FIG. 10a. "Mulberry" form of vaccinia virus (poxvirus), by negative contrast. × 150,000. 10b. Immature forms of vaccinia virus in chick chorioallantoic cells. × 30,000. (Unpublished micrographs, courtesy of Prof. D. Peters.)

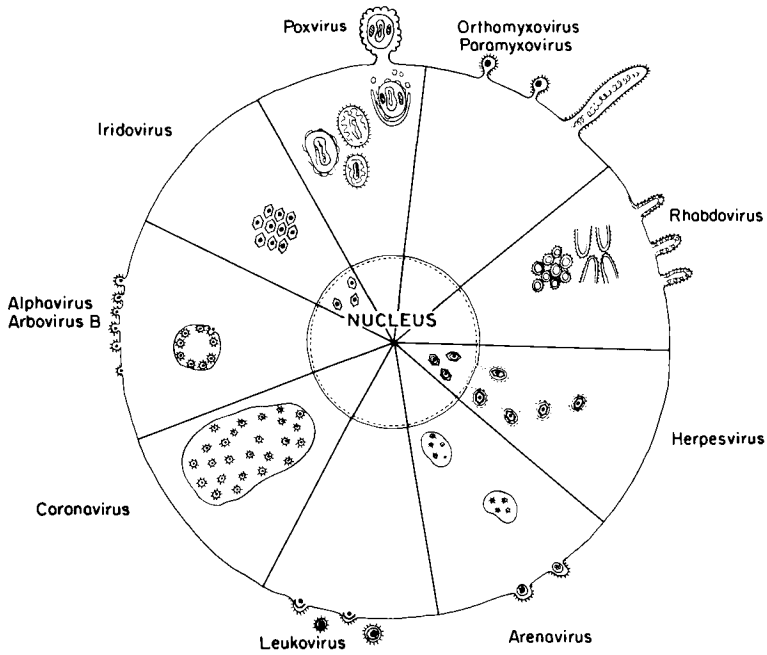

FIG. 11. Diagrammatic relationship between lipid-containing viruses and host cell membranes.

poxviruses; entomopoxviruses acquire an additional envelope by budding at the cell surface membrane (Devauchelle *et al.*, 1970, 1971).

The order of assembly of animal viruses and their relationship to preexisting cell membranes is shown in Fig. 11. It must be pointed out that, even though a virus may appear to derive the bulk of the lipids in its envelope from a preexisting membrane, evidence is accumulating that *de novo* synthesis of lipids or rearrangement of lipids occurs during virus infection, and these lipids may be incorporated into the virion. The relationship between viral infection and lipid metabolism is considered in greater detail in Section V.

III. Methods of Analysis of Viral Lipids

A. Purification of Virus

Until the development of tissue culture techniques, it was difficult to obtain any viruses in sufficiently large quantities to permit detailed study and lipid analysis. More recently, a wide variety of hosts has been used

for enveloped viruses, including calf kidney (Kates *et al.*, 1961), bovine and canine kidney (Klenk and Choppin, 1970a), primary rabbit kidney (Kaplan, 1957), baby hamster kidney (Vaheri *et al.*, 1965), HeLa (David, 1971), chick embryo fibroblast (Weinstein and Blough, 1973), and chick scalp epithelium (White *et al.*, 1968), but many other systems may be used, some of which are listed by Hoyle (1968) and Kaplan (1969). An older established method is growth in the allantoic membrane of embryonated eggs (Hoyle, 1968), especially for the myxoviruses where suitable egg-adapted strains are available. It has been suggested that the results of lipid analysis of virus produced in tissue culture under single-step growth conditions may be more consistent than in virus grown under multiple-cycle conditions in eggs (Klenk and Choppin, 1970a).

Methods of harvesting the progeny virus vary according to whether the virus is released into the medium or retained intracellularly. Following propagation in eggs, the virus is released into the allantoic fluid, and this can be harvested free from cells, although contaminated by cellular debris and "normal cell particles" pinched off from the surface membrane of chorioallantoic cells (Hoyle, 1950). Intracellular virus is released by conventional disruptive techniques such as Dounce homogenizing following harvest of cells, and virus released from cultured cells is obtained by collection of the medium, involving centrifugation to remove cells if suspension cultures are used. Purification of virus from cellular debris can then follow comparable techniques irrespective of the manner of growth of the virus.

In studying the lipids of viruses, it is of critical importance to obtain specimens uncontaminated by cellular material, particularly in cases where the composition of viral lipids is to be compared with that of the host cell or parent membrane. Generally, a first step involves removal of the bulk of cellular contamination: this may involve (1) one or more cycles of differential centrifugation; (2) liquid chromatography, e.g., on columns of aluminum phosphate (Miller and Schlesinger, 1955) or DEAE cellulose (Fuscaldo *et al.*, 1971) and elution with a salt gradient or buffered saline; (3) by employing some specific but reversible adsorption property of the virus. Using the latter technique, for viruses which hemagglutinate, washed erythrocytes are added to the viral suspension, causing attachment of virus but not of cellular debris. After centrifugation to remove debris, the virus is dissociated from the cells by addition of fresh saline medium or by lowering the divalent cation concentration (Furukawa *et al.*, 1967), and further centrifugation separates virus and cells. In the case of influenza virus, the release is through the action of viral neuraminidase (Hoyle *et al.*, 1954). In some cases an inert adsorbent such as barium sulfate for influenza virus (Mizutani, 1963), may be used

instead of erythrocytes. Additional techniques such as isoelectric focusing and density gradient electrophoresis have been used (e.g., Polson and Russell, 1967). Further purification usually involves banding of virus on a density gradient of sucrose, cesium or rubidium chloride or sulfate, or potassium tartrate (Blough *et al.*, 1967). Depending on the length of centrifugation and the concentrations necessary to achieve the desired densities, this procedure may prove disruptive of the virus (Norrby, 1969). For large quantities of virus, a considerable reduction of effort may be achieved by use of the zonal rotor; this has been found useful in separating influenza virus into spherical and filamentous populations (Reimer *et al.*, 1966). Isotope dilution techniques are frequently used to follow the extent of removal of contaminating host materials (Blough *et al.*, 1967; McSharry and Wagner, 1971); polyacrylamide gel electrophoresis may also be used with isotopically labeled host cell polypeptides as markers (Spear and Roizman, 1968; Holland and Kiehn, 1970). The above purification methods are necessarily brief and devoid of experimental detail; the reader is referred to standard virological texts for more detailed treatment (e.g., Brakke, 1967).

The adsorption methods of purification may be criticized on the grounds that no distinction is made between infectious and noninfectious particles, since only surface properties of the envelope are involved; similarly the use of erythrocytes may possibly lead to exchange of lipids with enveloped viruses, giving misleading results on lipid analysis.

B. Lipid Chemistry Techniques

In the past, compositional analyses of lipids in viruses were hindered not only by the unavailability of stocks of adequately purified virus, but also by inefficient biochemical techniques including methods of solvent extraction and separation and identification of individual species of lipid. The work of Folch *et al.* (1957) on extraction of lipids has led to a more systematic method of approach designed to free lipid classes from association with each other and with proteins. Workers differ in their preference for one solvent system or another, but most now use chloroform and methanol in various ratios. Most phospholipids and neutral lipids are removed by this means. An additional extraction with ethanol has been found to release an appreciable further amount of triglyceride, thought to be associated with envelope structural proteins of influenza virus (Tiffany and Blough, 1969a). Protocols of other workers differ largely in the proportions of solvents, temperatures and times of extraction, and the means of separating the lipid classes once extracted. The reader is referred to Kritchevsky and Shapiro (1967) for specific details on the

isolation of viral lipids, and to Weinstein *et al.* (1969, 1970) for techniques of isolation and analysis of cell membrane lipids.

Once they are extracted, it is particularly important to guard against breakdown of sensitive lipids by storage at low temperatures under an inert atmosphere such as nitrogen or argon, and by the addition of anti-oxidants such as *t*-butyl hydroxyanisole (BHA) or di-*t*-butyl hydroxy-toluene (BHT); thin-layer chromatographic separations of lipids may be carried out in the presence of anti-oxidant without interference (Blough and Lawson, 1968). Similarly, precautions should be taken to prevent breakdown of sialoglycolipids in aqueous solution by maintaining a neutral or alkaline pH.

Analysis of the lipids is carried out by well-established methods, largely detailed in the literature, e.g., Marinetti (1967), Renkonen (1967), or Dittmer and Wells (1969). Special techniques for certain classes of lipid may also be found elsewhere: e.g., Weinstein *et al.* (1970) for glycolipids; Baumann and Mangold (1966) and Baumann *et al.* (1970) for glyceryl ethers; White *et al.* (1968) for precursors of cholesterol biosynthesis; Renkonen (1967) for plasmalogens (phospholipids in which one acyl group is replaced by a long-chain-substituted vinyl ether group); Clamp *et al.* (1971) for carbohydrates. With the development of gas-liquid chromatography, many analyses formerly requiring considerable time and relatively large quantities of lipid may now be performed with microgram or even nanogram quantities.

Glycolipids remain among the most difficult classes of lipid to analyze, partly because they make up only a small proportion of the total lipid, but also because, in addition to acyl groups and a sphingosine base (together forming the ceramide portion of the molecule) they have a chain of sugars of variable length, which may be branched and which may also contain one, two, or three sialic acid units (Ledeen, 1966; Stoffel, 1971). Although glycolipids form only a small part of the total lipids of either viruses or membranes (Section IV, Table 2; Weinstein *et al.*, 1969), knowledge of their detailed composition is of importance since a possible role in cell fusion has been suggested for glycolipids (Blough and Lawson, 1968; Klenk and Choppin, 1970b).

Following initial separation of the glycolipid fraction from polar and neutral lipids, further fractionation into gangliosides, hematosides, and ceramide hexosides can be carried out by thin-layer chromatography (TLC) using known reference standards to give R_f values for each component (Klenk and Choppin, 1970b). Spots are scraped off the TLC plates and the glycolipids eluted from the gel with organic solvents, hydrolyzed, and converted to appropriate derivatives for analysis by gas-liquid chromatography (GLC): hexosamines and sialic acids as their

trimethyl silyl ether derivatives; hexoses as alditol acetates following reduction and acetylation, using internal standards (Weinstein *et al.*, 1970). The *N*-acetyl- and *N*-glycolyl- moieties of neuraminyl ceramide hexosides may be separated by TLC and quantitated by the thiobarbituric acid assay of Warren (1959), or by the much more sensitive GLC methods of Clamp *et al.* (1971) or Craven and Gehrke (1968), or the spectrofluorometric method of Hess and Rolde (1964). Sequencing of the sugar units can be done by periodate oxidation or following hydrolysis by specific glycosidases, but this technique has not yet been applied to viral lipids.

Equally as important as the determination of the lipid content of enveloped viruses is information on the amounts and classes of lipid available in the cell for incorporation into the virus particle. In the case of viruses assembled at membranes ("template" viruses, see Section VI), the lipids may be selected from those available at the site of assembly, and may be substantially identical to those of the parent membrane. However, the pattern of synthesis and turnover of cellular lipids, and hence the membrane lipid composition, is not necessarily the same in infected as in uninfected cells.

Studies of lipid metabolism in virus-infected and in control uninfected cells have just begun in the authors' laboratory and elsewhere, using isotopic precursors of various components of lipid molecules. Thus, in studying synthesis and turnover of phosphatidylcholine, a double label might be used of glycerol-2-^{3}H to label the glycerol skeleton and acetate-^{14}C to label the fatty acid chains, or alternatively choline-^{3}H to label the base and glycerol-^{14}C for the glycerol backbone. The acetate label will also appear in glycerides, sterols, and free fatty acids, but these are readily separated from other neutral lipids by a unidimensional TLC system such as that of Freeman and West (1966). To determine specific activities, individual spots are scraped off the plate and divided into two portions; one is used for radioactive assay by scintillation counting, and the other for mass measurement by a method such as the charring technique of Marsh and Weinstein (1966). Alternative precursor molecules include inositol-^{3}H for phosphatidylinositol and glucosamine-^{3}H for glycolipids. It should be stressed that the use of specific activities rather than counting rates is mandatory if turnover methods are to have any meaning.

It is hoped that by appropriate pulse-chase techniques it will be possible to trace the biosynthesis and turnover of lipids in a variety of cell types, and, more importantly, to determine how the effects of infection by lipid-containing viruses on host cell metabolism are related to envelope biogenesis and cell-surface-mediated phenomena. The results of some of these experiments are given in Section V. It should be borne in mind

that relatively little work of this type has as yet been done, and on relatively few different viruses. Conclusions on the overall effect of viral infection must therefore be considered tentative, until confirmation by other workers, and for a large number of viruses, is forthcoming.

The lipid compositions found experimentally for a number of viruses are considered in the next section.

IV. Composition of Viral Lipids

Viruses are obligate intracellular parasites and are not known to carry genetic coding for enzymes involved in lipid synthesis. Hence they generally contain the same classes of lipid as are found in the host cell or their membrane of assembly (Table II). Only one major lipid class has been found to be an exception: gangliosides are not detectable in the simian paramyxovirus SV5 but are present in the plasma membrane of the host cell (Klenk and Choppin, 1970b). The significance of this finding will be discussed later (Section VI).

Lipids are found to make up 20–35% by weight of most viruses; however, as mentioned earlier, there are exceptions such as vaccinia virus, which has only 5% lipid (Zwartouw, 1964) despite having a complex multimembrane envelope structure (Dales, 1963). Avian tumor viruses contain the most lipid, 31–35% (Rao *et al.*, 1966; Quigley *et al.*, 1971), and insect nuclear polyhedrosis virus the least, 1.6% (Bergold and Wellington, 1954). With animal viruses, the molar ratio of cholesterol to phospholipid is high ($\sim$1) if the virus is assembled at the cell surface membrane, but less in the case of cytoplasmic assembly (Blough, 1968). The lowest value for cholesterol is 4% for rabies virus grown in BHK-21 cells in chemically defined medium (Blough *et al.*, 1973), giving a cholesterol:phospholipid molar ratio of 0.5; this may reflect the site of assembly in BHK-21 cells, but some other mechanism may also be involved since some particles seen in thin sections appear to bud from the plasma membrane. Glycolipids make up 1–2% of the lipids of animal viruses, and these values (at least for viruses released by budding) are similar to those reported for plasma membranes (Weinstein *et al.*, 1970). Data on the lipids of other insect and plant viruses are largely lacking, but many of these are rhabdoviruses (e.g., potato yellow dwarf virus and wheat striate mosaic virus) and may resemble rabies or vesicular stomatitis virus in lipid content if not in detailed composition. Molecular models of some of the lipids found in viruses are depicted in Figs. 12a–c.

Compositional analyses of the lipids of viruses and of their parent membranes may be reported in two ways: (1) by comparing the lipids of

Table II

COMPOSITIONAL ANALYSIS OF VIRAL LIPIDS[a]

Virus	Host	Lipid (% of virion)	Phospholipid (% of virion)	Total cholesterol (% of virion)	Major phospholipids[b] (% of total phospholipid)	Neutral lipids (% of total neutral lipid)	Sphingo-glycolipids (% of total lipid)	Reference
Sindbis	CEF	28	20.3	6.8	PC 34; Sph 8.5; PE 32; PS 21	ND	ND	Pfefferkorn and Hunter (1963b)
Sindbis	BHK-21	28	ND	ND	PC 26; Sph 18; PE 35; PS 20	ND	ND	David (1971)
Semliki Forest	BHK-21	30.7	19	8.7	PC 34; Sph 21; PE 26; PS 12	TG 3; DG 2; FA 2	2.6	Renkonen *et al.* (1971)
Influenza A_0/PR8/34	EE	20.2	10.3	7.7	PC 38; Sph 23; PE 12	TG 3; DG 3; MG 1; FA 9	0.4	Blough and Merlie (1970)
Influenza (incomplete) A_0/PR8/34	EE	24.2	14.1	4.6	PC 16; Sph 16; PE 31; PS 16	TG 3; DG 10; MG 6; FA 25	1.2	Blough and Merlie (1970)
Influenza A_6/PR8/34	EE	18.5	11.5	6.5	PC 28; Sph 35	ND	ND	Frommhagen *et al.* (1959)
Influenza Mel	CaK	16	11.5	6.5	PC 11; PA 64; PS + PE 13	ND	ND	Kates *et al.* (1961)
Newcastle disease B1	EE	24	13	6.5	PC 12; Sph 18; PE 35; PS 12	Total glycerides 9	ND	Blough and Lawson (1968)
Sendai	EE	28	16	7.2	Sph 12; PE 37; PS 15; PA 10	Total glycerides 11	ND	Blough and Lawson (1968)
SV5	HaK	19	12.4	4.2	PC 44; Sph 26; PE 17	TG 4.6[c]	ND	Klenk and Choppin (1969b)
SV5	MDBK	18.6	9.4	4.3	PC 24; Sph 27; PE 40	TG 6.3[c]	ND	Klenk and Choppin (1970a)

Rous sarcoma	CEF	31	21	9	PC 28; Sph 29; PE 30	ND	ND	Quigley *et al.* (1971)
Avian myeloblastosis	Myelo	35	21.3	11.7	PC 18; Sph 26; PE 34; PS 12	1.8	ND	Rao *et al.* (1966)
Vesicular stomatitis (Ind)	L	20	12	5.8	PC 16; Sph 21; PE 33; PS 17	TG 9; DG 11; MG 5; FA 4	ND	McSharry and Wagner (1971)
Rabies	BHK-21	24	15.8	4	PC 23; Sph 31; PE 34	TG 9; DG 12; MG 2; FA 14	1.5	Blough *et al.* (1973)
Potato yellow dwarf	*Nicotiana rustica*	19	ND	ND	ND	ND	ND	Ahmed *et al.* (1964)
Tomato spotted wilt	*N. glutinosa*	19	ND	ND	ND	ND	ND	Best (1968)
Lipovirus PM2	*Pseudomonas* BAL-31	15	14	—	PG 65–68; PE 28	ND	ND	Braunstein and Franklin (1971)
Herpesvirus	BHK-21	ND	22	ND	PC + PI 69; Sph 17; PE 14	ND	ND	Asher *et al.* (1969)
Fowlpox	CS	34	5.3	7.5[d]	ND	TG 28; DG 0.3; MG 0.2; FA 19	ND	White *et al.* (1968)
Vaccinia	Rabbit	5	2.2	1.4	ND	ND	ND	Zwartouw (1964)
Iridovirus FV 3	FHM	ND	ND	ND	PC 58; LPC 11; PS + PE 16	ND	ND	G. Houts and M. Gravell (personal communication, 1972)

[a] Abbreviations used: Cell type: CEF, chick embryo fibroblasts; EE, embryonated eggs; CaK, calf kidney; HaK, hamster kidney; MDBK, Madin Darby bovine kidney; Myelo, avian myeloblasts; L, mouse fibroblasts; BHK-21, baby hamster kidney; CS, chick scalp epithelium; FHM, fat head minnow. Lipids: PC, phosphatidylcholine; Sph, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidyl inositol; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; TG, triglyceride; DG, diglyceride; MG, monoglyceride; FA, free fatty acid. ND = not determined.

[b] "Major phospholipids" indicates those present in amounts of 10% or more.

[c] Percentage of total lipid.

[d] Includes 5.9% cholesterol esters.

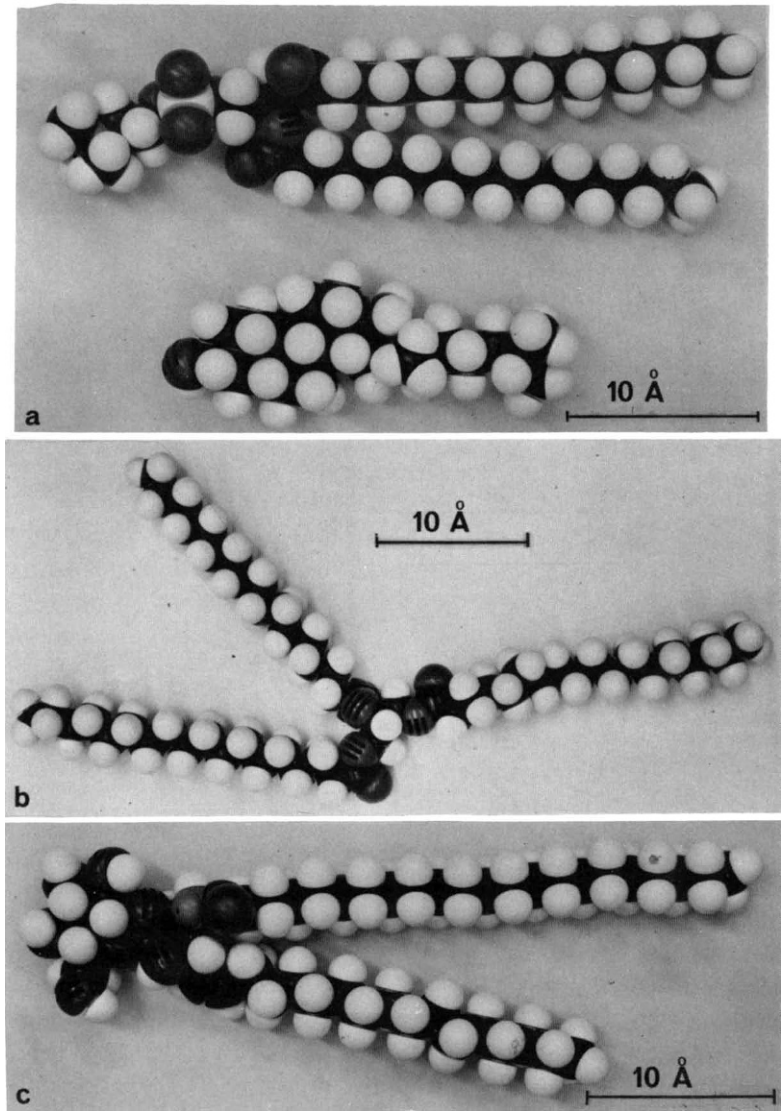

FIG. 12a. Molecular models of phosphatidylcholine and cholesterol. 12b,c. Molecular models of a triglyceride and a ceramide monohexoside.

different strains of the same virus propagated in homolog membranes, e.g., different strains of influenza virus in embryonated eggs (Tiffany and Blough, 1969a,b); (2) by comparing the lipids of purified virus with those of the isolated host membrane at which they are assembled. With several notable exceptions, phospholipid compositions (based on *polar*

group analysis) are essentially the same as those of the membrane from which the assembled virus originated (Klenk and Choppin, 1969b). Exceptions include Newcastle disease virus (strain Italy/Milano/1945), Sendai and influenza viruses in embryonated eggs (Blough and Lawson, 1968; Blough *et al.*, 1967); simian paramyxovirus SV5 in MDBK cells (Klenk and Choppin, 1970a); Sindbis virus in BHK-21 cells and chick fibroblasts (David, 1971); and avian myeloblastosis virus in myeloblasts (Rao *et al.*, 1966). The result with avian myeloblastosis virus is at variance with a recent report on the phospholipid composition of another leukovirus, Rous sarcoma virus (Quigley *et al.*, 1971). These authors employed orthophosphate-^{32}P labeling of chick embryo fibroblast host cells, and estimated the proportions of the different phospholipid classes from their radioactive content; essentially the same pattern of lipid classes as in the isolated host cell plasma membrane was reported for the Schmidt-Ruppin strain of Rous sarcoma virus, and also for Newcastle disease virus, Sendai virus, Sindbis virus, avian sarcoma virus B77, and another avian leukosis virus, RAV-2. However, under the conditions of labeling used by these workers, isotopic equilibrium might not be achieved, and further work is headed to resolve this point.

Paramyxoviruses (Blough and Lawson, 1968; Klenk and Choppin, 1969b), vesicular stomatitis virus (McSharry and Wagner, 1971), and rabies virus (Blough *et al.*, 1973) have all been found to contain more phosphatidylethanolamine than their parent membranes. Klenk and Choppin (1970a) observed that yields of paramyxovirus SV5 were higher in rhesus monkey kidney cells than in baby hamster kidney cells (BHK-21), and correlated this with a higher phosphatidylethanolamine:phosphatidylcholine ratio in plasma membranes of monkey kidney than in BHK-21 cells. The observed high values of phosphatidylethanolamine found in the other viruses mentioned above may be related in some way to this. Under conditions of high multiplicity of infection and repeated passage, alterations were noted in phospholipids, fatty acyl chains, and neutral lipids of incomplete influenza virus when compared to standard virus (Blough and Merlie, 1970).

The high level of phosphatidic acid found in influenza virus grown in calf kidney cells (Kates *et al.*, 1961) is most probably an artifact due to hydrolysis of other phospholipids during the extraction procedure, since no appreciable amount of phosphatidic acid has been reported by any other workers on ortho- or paramyxoviruses, using essentially similar techniques.

Fowlpox virus lipids include large amounts of plasmalogens (phospholipids having one acyl group replaced by a long-chain-substituted vinyl ether), which on hydrolysis yield both fatty acids and long-chain fatty

aldehydes. The proportion of saturated to unsaturated aldehydes was found to be higher in fowlpox virus grown in chick scalp epithelium than in the host cells (White *et al.*, 1968). Plasmalogens were also detected in Venezuelan equine encephalitis (an alphavirus), but no separation into molecular species was performed (Heydrick *et al.*, 1970).

Neutral lipids consist mainly of cholesterol and cholesterol esters, glycerides, and free fatty acids. Bacterial virus PM2 and plant viruses are exceptions to this in containing no cholesterol or cholesterol esters (Table II). The ratio of free to esterified cholesterol is generally high, but the reverse is found to be true for fowlpox virus (White *et al.*, 1968). A higher proportion of cholesterol was found in Semliki Forest virus than in its parent membrane (Renkonen *et al.*, 1971), and a lower proportion in incomplete influenza virus (Blough and Merlie, 1970). Squalene, a hydrocarbon precursor of cholesterol biosynthesis, accounted for 16% of the lipids of the fowlpox virion, but only 0.6% of lipids of the uninfected cell (White *et al.*, 1968). These authors suggest that squalene may serve to solubilize the lipids of the virion.

Lipid analyses of viruses considered here are given in Table II, as well as an indication of the host cells in each case. These figures should not be used as a basis for comparison of viruses, since they depend in some cases on the choice of host (e.g., paramyxovirus SV5), and in others on the site of assembly or the number of membranes incorporated into the virion (e.g., herpesvirus; Kaplan, 1969). We have attempted to keep this table as simple as possible, and have therefore not expressed the results in terms of deviations from the parent membrane composition, although this is one of the most interesting ways of looking at the results.

Table III shows the fatty acid composition of the polar lipids (PL: largely phospholipids) and neutral lipids (NL: glycerides, free fatty acids, and cholesterol esters) for three strains of influenza virus, and also for the "normal cell particles" (NCP) or fragments of chorioallantoic cell surface membrane released into the allantoic cavity of embryonated hen's eggs (Tiffany and Blough, 1969b). For convenience, acyl chains have been grouped at the foot of the table into saturated, monoenoic, and polyenoic categories. This permits a rapid comparison of the different virus strains. It is clear that the saturated fatty acid content of NCP is much lower than in virus, especially in the NL fraction, where NCP have a greatly increased oleic:stearic (18:1/18:0) ratio. The overall composition of the viruses shows differences between all the strains; this is particularly marked in the individual long-chain polyenoic components such as 22:polyene, where A_0 has 8.4% in PL and none in NL, but A_2 has only 1% in PL and 5.6% in NL, and B has 3% in PL only. If lipid were passively incorporated from the host membrane during budding, we should expect

Table III

FATTY ACID COMPOSITION OF THREE STRAINS OF INFLUENZA VIRUS AND NORMAL UNINFECTED CELL PARTICLES[a,b]

	Strains of influenza virus						Normal cell particles	
	A_0/PR8/34		A_2/Jap/305/57		B/Lee/40			
Acyl chains[c]	PL	NL	PL	NL	PL	NL	PL	NL
12:0	Tr	ND	1.0	5.8	ND	14.2	1.5	ND
14:0	1.6	4.1	3.1	16.8	Tr	14.8	3.5	2.8
16:0	15.1	28.5	17.5	23.7	20.9	25.0	23.2	22.3
16:1	5.2	6.6	1.1	3.2	0.8	1.1	3.1	5.9
18:0	14.8	18.8	9.7	6.2	16.9	16.9	17.3	8.5
18:1	15.8	22.0	20.5	10.3	17.0	9.6	17.1	35.5
18:2	4.3	4.6	7.0	0.9	4.2	6.5	3.9	12.3
18:3	0.9	Tr	Tr	0.8	2.8	4.8	1.1	0.6
20:0	8.4	6.1	5.0	3.1	3.9	1.9	0.9	0.9
20:1	Tr	ND	ND	ND	ND	ND	ND	ND
20:4	4.2	4.9	14.4	14.1	13.4	1.6	12.9	4.0
22:0	13.7	4.4	9.6	4.7	7.3	2.0	1.2	Tr
22:1	Tr	ND	ND	ND	ND	ND	ND	ND
22:polyene	8.4	ND	1.1	5.6	3.0	ND	6.5	3.7
24:0	6.8	Tr	8.1	1.0	9.0	0.5	1.6	2.8
24:polyene	Tr	ND	1.8	ND	ND	ND	ND	ND
Uncharacterized	0.6	—	—	3.9	0.8	1.1	6.3	0.5
Saturated	60	62	54	61	58	75	49	37
Monoenoic	21	29	21	14	18	11	20	41
Polyenoic	18	9	24	21	20	13	24	21

[a] Reproduced from Tiffany and Blough (1969b) by kind permission of the American Association for the Advancement of Science.

[b] Abbreviations used: PL = polar lipids; NL = neutral lipids; ND = not detected; Tr = Trace ($\leq 0.5\%$).

[c] Acyl chains are indicated by number of carbon atoms: number of double bonds.

these compositions to be substantially the same; if the composition of the lipid were determined in some way by the nucleocapsid of the virion, A_0 and A_2 viruses might have a similar composition since these strains have a common internal antigen. The fact that none of these correspondences was found led to the hypothesis that lipid composition of the virion is determined by some envelope component, probably a structural protein (see Section VI).

Table IV shows the result of acyl chain analyses on individual phospholipid fractions from two strains of influenza virus belonging to different subtypes (A and B). Many considerable differences can be seen, not only between A and B viruses, but between the phospholipids of each strain.

Table IV

FATTY ACID COMPOSITION OF INDIVIDUAL PHOSPHOLIPIDS OF TWO STRAINS OF INFLUENZA VIRUS DERIVED FROM HOMOLOG MEMBRANES[a,b]

Acyl chains[c]	PC		Sph		PE		PS	
	A	B	A	B	A	B	A	B
14:0	4.0	Tr	1.7	ND	Tr	2.3	2.9	ND
16:0	30.1	38.7	43.3	44.4	16.7	14.7	15.4	13.0
16:1	2.4	Tr	0.7	0.6	1.6	0.8	1.2	0.9
18:0	13.9	15.4	9.3	11.1	19.2	11.2	34.2	32.7
18:1	26.8	28.7	9.3	5.5	45.3	35.0	20.9	26.1
18:2	10.7	6.5	Tr	2.1	10.2	9.8	9.2	1.0
18:3	ND	0.7	Tr	Tr	Tr	1.8	1.8	ND
20:0	0.8	2.8	3.1	7.6	1.9	ND	2.3	Tr
20:4	5.3	7.0	2.7	3.1	4.1	24.4	3.6	10.3
22:0	0.9	ND	15.1	12.1	ND	ND	1.6	Tr
22:1	ND	ND	0.6	ND	ND	ND	ND	ND
22:polyene	4.9	ND	0.8	ND	1.1	ND	5.8	12.8
24:0	Tr	Tr	13.0	11.4	ND	ND	1.1	1.8
24:1	ND	ND	0.9	2.1	ND	ND	ND	ND

[a] Reproduced from Blough (1971a) by kind permission of Cambridge University Press.

[b] Abbreviations used: PC = phosphatidylcholine; Sph = sphingomyelin; PE = phosphatidylethanolamine; PS = phosphatidylserine; A = A_0/PR8/34 strain of influenza virus; B = B/Lee/40 strain of influenza virus; ND = not detected; Tr = Trace ($\leq 0.5\%$).

[c] Acyl chains are indicated by number of carbon atoms: number of double bonds.

For example, 16:0 content is roughly the same for A and B within any phospholipid class, but varies widely from class to class; the same is true for 18:1, varying between 5.5% in sphingomyelin and 35% in phosphatidylethanolamine of B virus. Almost all long-chain saturated acids (22:0 and 24:0) are in sphingomyelin. There is, however, no consistent pattern of acyl chain incorporation such as might be ascribed to selection of host lipids during budding only on the basis of their polar groups.

The neutral glycolipid content of paramyxovirus SV5, and of both the whole host cell and its plasma membrane, is given in Table V. It is not possible to compare the amounts of each of these lipids in membranes and virions in terms of micrograms of lipid per milligram of protein, since different proteins are inserted into the plasma membrane during budding; it can be seen, however, that the proportion of the ceramide tetrahexoside is much greater in the virion than in the parent membrane, and this may reflect a stimulation of glycosyltransferases during infection. Such differences have also been observed in the glycolipids of Semliki Forest virus

Table V

NEUTRAL GLYCOLIPID CONTENT OF MDBK CELLS, PLASMA MEMBRANES, AND SV5 VIRUS GROWN IN THESE CELLS[a]

Glycolipid[b]	Whole cells	Plasma membranes	SV5 virions
	(μg per 100 mg protein)		
Glc-Cer	160	1300	1550
GalNAc-Gal-Gal-Glc-Cer	510	2200	3000

[a] Reproduced from Klenk and Choppin (1970b) by kind permission of the authors and the Editors of *Proceedings of the National Academy of Sciences*, Washington.

[b] Cer = ceramide; Glc = glucose; Gal = galactose; GalNAc = *N*-acetyl galactosamine.

and BHK-21 cells (Renkonen *et al.*, 1971), and in incomplete versus normal influenza virus (Weinstein and Blough, quoted in Blough and Merlie, 1970).

A summary of differences between viral and host cell lipids is given in Table VI.

V. The Effects of Viral Infection on Host Cell Lipid Metabolism

The earliest studies on the effect of infection by fowl plague virus on host cell lipids were based on the incorporation of orthophosphate-^{32}P into chick cells and comparison of the specific activity of phospholipids of the virus with those of the host cell (Wecker, 1957). This was followed by the first definitive study on the origin of influenza virus lipids by Kates *et al.* (1961). Primary calf kidney cells were prelabeled with orthophosphate-^{32}P for 72 hours prior to infection, and the specific activities of the phospholipids of subcellular fractions of the host cells were compared to those of purified influenza virus grown in the same cell system. It was concluded that, with the exception of phosphatidic acid, newly synthesized lipids were not incorporated into the virion. Similar studies were made on Sindbis virus lipids by Pfefferkorn and Hunter (1963a,b), who demonstrated that the specific activities of viral sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were the same in the virus as in the host chick embryo fibroblast cell. They concluded that the lipids of arboviruses were preformed and that *de novo* synthesis of lipids did not play a role in the assembly of these viruses. The use of orthophosphate-^{32}P to study phospholipid turnover is open to

Table VI

SUMMARY OF DIFFERENCES IN LIPIDS BETWEEN HOST CELL AND ITS MEMBRANES AND VIRUS

Differences noted	Viruses	References
1. Acyl chain composition	Influenza, Newcastle disease	Tiffany and Blough (1969a,b)
	Incomplete influenza virus	Blough *et al.* (1969)
	Sendai	Blough and Lawson (1968)
	Paramyxovirus SV5	Klenk and Choppin (1970a)
	Vesicular stomatitis	McSharry and Wagner (1971)
	Sindbis	David (1971)
2. Absence of sialoglycolipids (gangliosides) in virus	Paramyxovirus SV5	Klenk and Choppin (1970b)
3. High cholesterol ester: free cholesterol ratio	Fowlpox	White *et al.* (1968)
4. Quantitative differences in polar groups of phospholipids	Newcastle disease, Sendai	Blough and Lawson (1968)
	Semliki Forest	Renkonen *et al.* (1971)
	Avian myeloblastosis	Rao *et al.* (1966)
	Iridovirus FV 3	E. Houts and M. Gravell (personal communication, 1972)
	Lipovirus PM2	Braunstein and Franklin (1971)
	Incomplete influenza virus	Blough and Merlie (1970)
	Vesicular stomatitis	McSharry and Wagner (1971)
	Paramyxovirus SV5	Klenk and Choppin (1970a)
5. Ratio of saturated to unsaturated plasmalogens	Fowlpox	White *et al.* (1968)
6. High concentration of unusual lipid in virion	Fowlpox	White *et al.* (1968)
7. Quantitative differences in neutral glycolipids	Paramyxovirus SV5	Klenk and Choppin (1970b)
	Sindbis	Renkonen *et al.* (1971)
8. Quantitative differences in cholesterol	Semliki Forest	Renkonen *et al.* (1971)
	Incomplete influenza virus	Blough and Merlie (1970)
9. Quantitative differences in glycerides	Incomplete influenza virus	Blough and Merlie (1970)
	Vesicular stomatitis	McSharry and Wagner (1971)
	Fowlpox	White *et al.* (1968)

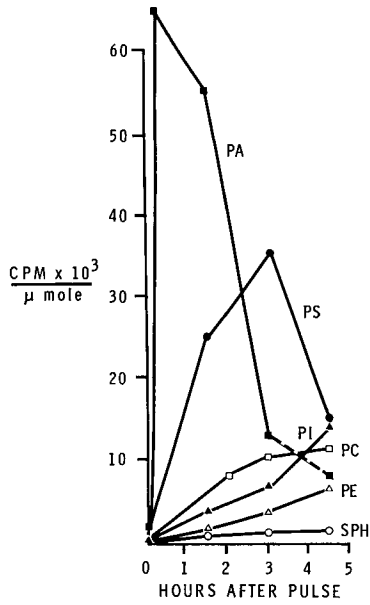

FIG. 13. Pulse-chase studies on uninfected chick embryo fibroblasts. Cells grown in a chemically defined medium, pulsed for 7½ minutes with orthophosphate-^{32}P and chased in carrier-free medium containing 10 m*M* α-glycerol phosphate (Weinstein and Blough, 1973).

question because it appears compartmentalized in an organic pool turning over slowly (Fig. 13).

Before embarking on any extensive description of measurements of lipid metabolism, we should define some of the terms used and give an indication of the ways in which they can be measured. *Synthesis* is the amount of a lipid produced in the cell, and may include newly formed material *de novo* as well as that formed by reutilization, or only that newly formed from small precursor subunits. It is measured by the rise in specific activity of a lipid class following uptake of a small isotopically labeled precursor molecule such as glycerol, choline, or acetate. *Turnover* is the balance between complete *de novo* synthesis of a class of lipid from its fundamental parts (choline, glycerol, phosphate, etc.) and its catabolism into simpler subunits. *Interconversion* or *exchange* is the transfer of individual moieties from one class of lipids to another without *de novo* synthesis or catabolism. Some authors will refer to this as *incomplete turnover*. Equilibrium labeling involves growth of the cell in medium containing an isotopically labeled precursor until the precursor pools within the cell have reached isotopic equilibrium and specific activity of a particular lipid class is maximal. To measure turnover, the cell is *pulsed* by supply-

ing a labeled precursor for a short period, and then *chased* in medium containing unlabeled precursor. Using short pulses, as much as 85% of acetate label appears in acyl chains of glycerides and phosphoglycerides as well as sterols and free fatty acids: however, the source of label in a lipid class may be complex if multiple pools are operative. Choline gives preferential labeling of zwitterionic phospholipids (phosphatidylcholine and sphingomyelin) and inositol is a useful label for phosphatidylinositol as it is not synthesized *de novo* in most mammalian cells. Glycerol is incorporated largely into glycerides and phosphoglycerides; in short pulses 95% of the label may enter these classes. In general, ^{32}P is the most widely used isotope, but is not specific for phospholipids since other molecules are also labeled (e.g., nucleotides). Compartmentalization and recycling into phospholipid synthetic pathways from these pools is possible, suggesting that ^{32}P is not a good precursor for the study of phospholipid synthesis and turnover. Similarly, if glycerol-1-^{3}H is used, tritium is lost during oxidation and decarboxylation of the molecule and may feed back into lipid via multiple pathways: the tritium label in glycerol-2-^{3}H is more stable and there is little or no randomization of label into other lipid precursor moieties. Studies in the authors' laboratory confirm the work of Bailey *et al.* (1964) that as little as 0.5% serum in the medium inhibits the synthesis of both phospholipids and neutral lipids by as much as 50%. Such large shifts in lipids may entirely mask changes in the level of a minor component if serum is present. Therefore all studies in the author's laboratory were done in chemically defined medium using fatty acid-poor bovine serum albumin in place of serum.

Recent studies by Pasternak and Bergeron (1970) have shown that in cultured mammalian cells in the presence of serum there are at least two classes of phospholipid, an "unstable" one consisting largely of phosphatidylcholine, and a "stable" one containing most of the sphingomyelin. Turnover rates for phospholipid using long pulses suggest a half-life of 18–24 hours for phosphatidylcholine in Novikoff rat hepatoma cells (Plagemann, 1971) and for mastocytoma cells in culture (Pasternak and Bergeron, 1970). Recently, however, Gallaher *et al.* (1972) found by using short pulses of 30 minutes and a double label of glycerol-2-^{3}H and acetate-^{14}C that the half-life of some phospholipids in BHK-21 cells and in chick embryo fibroblasts in confluent or nonconfluent monolayers is as low as 2–2½ hours. The half-lives of phospholipids are not all the same; using very short pulses of 7½ minutes on chick embryo fibroblasts, Weinstein and Blough (1973) have found that the turnover rate for phospholipids is multiple not only in separate classes (e.g., phosphatidylserine vs. phosphatidylethanolamine) but even within individual species.

A. RNA Viruses

RNA synthesis has been shown to be necessary to maintain phospholipid synthesis. In Sindbis virus-infected cells, synthesis of total proteins and host cell RNA appeared to decline more rapidly than phospholipid synthesis. Waite and Pfefferkorn (1970) used temperature-sensitive (*ts*) mutants of Sindbis virus which do not make RNA at 42.5°. Chick fibroblasts infected with the *ts* at the nonpermissive temperature failed to shut off phospholipid synthesis whereas wild-type virus did so. Using temperature-shift experiments and an RNA$^-$ mutant, it was possible to show that the depression in phospholipid synthesis occurred at 2–3 hours after infection, and paralleled the inhibition of host cell macromolecular synthesis (Fig. 14). At 28°, the small amount of viral RNA synthesized was enough to account for this inhibition. In addition, variability of response in different cell types was evident: in BHK-21 cells, phospholipid synthesis was not inhibited until 8–10 hours after wild-type infection.

Studies on phospholipid synthesis and renewal in influenza virus-infected cells were undertaken in the authors' laboratory using glycerol-2-^{3}H to label the glycerol skeleton and acetate-^{14}C to label the fatty acid chains, sterols, and free fatty acids (Weinstein and Blough, 1973). It was found that the level of *de novo* synthesis of phospholipids continued unchanged up to 7 hours after infection of chick fibroblasts with A_0/WSN influenza virus (Fig. 15) at low input multiplicities (3–5 pfu/cell), and up to 4 hours with higher multiplicities (10–30 pfu/cell) (Blough and Weinstein, 1972).

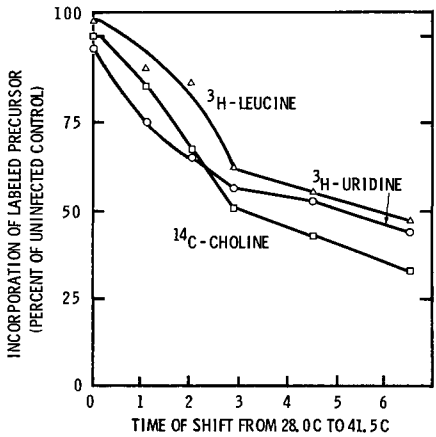

FIG. 14. Shutoff of phospholipid synthesis in chick embryo fibroblasts infected with a temperature-sensitive mutant of Sindbis virus, showing its dependence on RNA synthesis. From Waite and Pfefferkorn (1970).

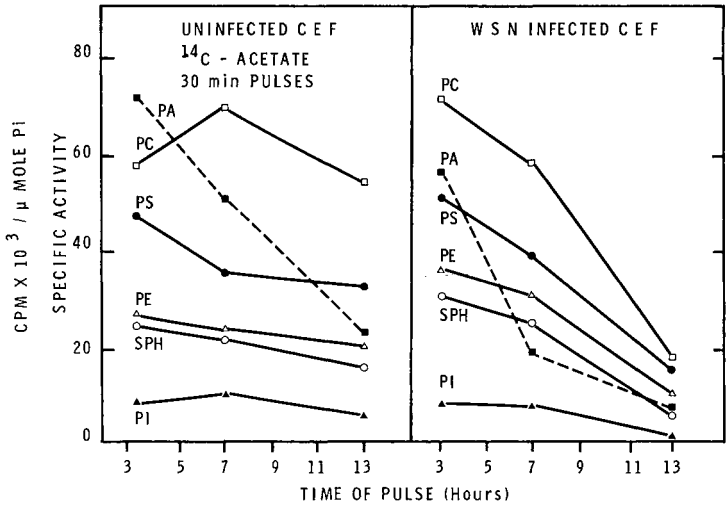

FIG. 15. Effect of influenza virus (A_0/WSN) on phospholipid synthesis in chick fibroblasts using a double label of glycerol-2-^{3}H and acetate-^{14}C. Cells were pulsed for 30 minutes at various time intervals after infection and sampled immediately. Depression of synthesis of various phospholipid species is seen to occur 7 hours after infection.

With influenza virus, all lipid synthesis was depressed between 8 and 12 hours following infection (Weinstein and Blough, 1972). This disproved the conclusion that *de novo* synthesis of lipid did not occur in virus-infected cells and stresses the importance of using multiple techniques of isotopic labeling.

Turnover studies were less conclusive. Short pulses with glycerol-^{3}H and acetate-^{14}C were done at various time intervals after infection. In the chick embryo fibroblast system, there appears to be an overall depression of all lipid synthesis 8 hours after infection, but turnover of the glycerol backbone (during a 4-hour chase) appears enhanced in infected cells in the case of phosphatidylcholine and phosphatidylserine. The fact that turnover rates appear remarkably similar in controls and infected cells despite an 80–90% decrease in phospholipid synthesis at these times is a paradox. A possible explanation is that preformed phospholipid molecules are transported for assembly in viral envelopes (see Section VI,A). Alternatively, the portion of lipid which is labeled may be a labile fraction whose turnover is necessary for viral envelope biogenesis.

It has been shown by Lands (1965) that enzymes of the monoacyl-diacyl phosphoglyceride cycle are responsible for the renewal of fatty acyl chains in a variety of tissues. These enzymes are located primarily in the microsomal and mitochondrial fractions of cells (Stoffel and

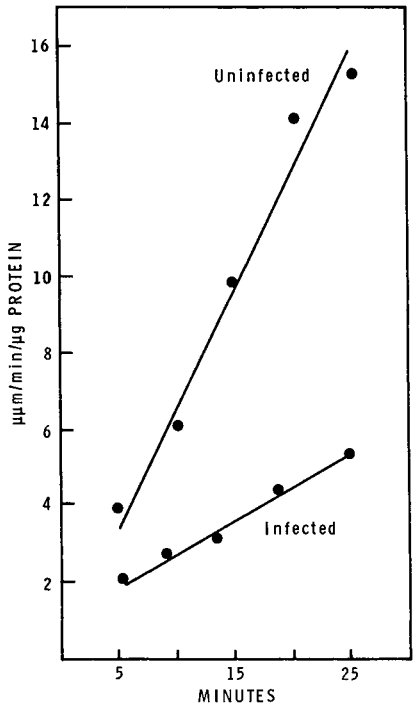

FIG. 16. Inhibition of host cell acyl-CoA : phosphoglyceride acyl transferase activity following infection with influenza virus. The reaction mixture contained 20 mμmoles of lysophosphatidylcholine as the acceptor, 200 μg of microsomal protein from chick chorioallantoic cells, and 5,5′-dithio-*bis*-nitrobenzoic acid (DTNB); the molar adsorptivity was used to calculate the release of free mercaptan. Microsomes were isolated 30 minutes after infection. From Blough and Smith (1973).

Schiefer, 1968; Turkki and Glenn, 1968; Eibl *et al.*, 1970). Due to the varying acyl chain composition observed in different strains of myxoviruses grown in embryonated eggs (Tiffany and Blough, 1969a,b) the myxovirus/chick cell system was investigated to see if influenza or Newcastle disease viruses could alter host cell enzymes responsible for phospholipid renewal. Microsomal fractions of chorioallantoic cells were isolated at various times after infection, and compared with uninfected controls; it was seen that the acyl-CoA:phosphoglyceride acyl transferase level was reduced to approximately 70% of that of uninfected controls (Fig. 16); this effect was noted within 30 minutes following infection. Acyl-CoA hydrolase was enhanced threefold with incomplete influenza virus of the von Magnus type at 40 hours postinfection (Fig. 17). These enzymes were *not* detected within the virion. The conclusion of these studies was that, *in ovo,* the enzymes of the monoacyl-diacyl phospho-

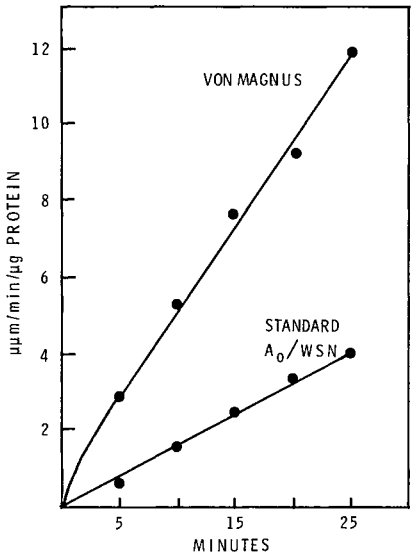

FIG. 17. Acyl-CoA hydrolase activity of the microsomal fraction of chick chorioallantoic cells infected with von Magnus influenza virus, compared to cells infected with standard virus. The reaction mixture is the same as for the experiment of Fig. 16, except that the acceptor molecule (lysophosphatidylcholine) has been omitted. Microsomal fractions were obtained 40 hours after infection or from 13-day-old uninfected chorioallantoic membranes. From Blough and Smith (1973).

glyceride cycle did not play a major role in determining the molecular species of phospholipid incorporated into the viral envelope (Blough and Smith, 1973).

Experiments were also designed to try to test the hypothesis that altered lipid metabolism in certain viruses (e.g., in cells infected with incomplete influenza virus) or certain nonpermissive cell types (e.g., HeLa cells) are responsible, in part, for failure of viral assembly. Influenza virus which has been passed at high multiplicity pleomorphic and loses a portion of its genome (Pons and Hirst, 1969), behaving in fact like a deletion mutation—the so-called incomplete or von Magnus virus. When compared to standard virus this virus has an altered lipid composition and shows obvious defects in the viral envelope (Blough and Merlie, 1970). When incorporation studies with glycerol-^{3}H and acetate-^{14}C were done, there was an immediate 50% decrease in diglyceride synthesis and a 75% increase in free fatty acids when compared to cells mock-infected with sterile allantoic fluid (Table VII). This correlated well with the studies on compositional analysis of von Magnus virus

Table VII

EFFECT OF INCOMPLETE INFLUENZA VIRUS ON INCORPORATION OF GLYCEROL-^{3}H AND ACETATE-^{14}C INTO NEUTRAL LIPIDS OF CHICK FIBROBLASTS[a,b]

Lipid class	Glycerol-^{3}H			Acetate-^{14}C		
	Control	Infected	Change (%)	Control	Infected	Change (%)
Monoglyceride	40,029	51,821	+29	44,000	46,607	+6
Diglyceride	613,760	521,503	−15	272,552	135,134	−50
Triglyceride	167,193	186,184	+11	14,570	15,225	+5
Fatty acids	—	—	—	10,175	17,809	+75
Cholesterol	—	—	—	79,351	64,210	−19
Cholesterol esters	—	—	—	766	762	No change

[a] Specific activities in cpm/μmole of neutral lipid.
[b] Samples taken immediately after a 30-minute pulse.

(Blough and Merlie, 1970), in which free fatty acids were increased in comparison to standard virus. Furthermore, preliminary studies (Blough and Weinstein, 1973) suggest that phosphatidylcholine and phosphatidylethanolamine synthesis is enhanced in chick embryo cells infected with von Magnus virus.

In HeLa cells infected with A_0/WSN influenza virus, viral structural polypeptides are synthesized, as are all the species of RNA, yet viral assembly does not occur (Lerner and Hodge, 1969). In HeLa cells, influenza virus (3–5 pfu/cell) affects both the synthesis and turnover of lipids immediately; diglyceride synthesis is inhibited by 15% and all phospholipids are inhibited by 27–80% (using glycerol-^{3}H label). Phosphatidylethanolamine and sphingomyelin turnover are decreased, whereas phosphatidylserine turnover was increased by 50% over controls. These studies suggest that host cell lipid metabolism plays an important role in determining whether or not some cell systems are permissive or not for certain viruses. In addition an uncoupling of synthesis and turnover of neutral lipids and phospholipids was evident (Blough, 1971b; Weinstein and Blough, 1973). Such systems provide a unique opportunity to study the interrelationship of host cell regulatory mechanisms and viral envelope biogenesis at the molecular level and are worthy of further exploration.

Fusion, a process whereby the viral envelope joins with the cellular membrane to form a coherent membrane is the means of entry of viral nucleocapsid into the cell under certain circumstances (Morgan and Rose, 1968). The molecular events responsible for fusion are unknown (Poste, 1970). Using BHK-21 cells infected with Newcastle disease virus (NDV),

and the appropriate conditions for fusion to occur, a twofold to fourfold depression of the synthesis of all lipid classes was noted immediately, which is quite different from results with orthomyxoviruses. This inhibition could be duplicated during a cytolytic infection with NDV in calcium-free medium or late in a productive infection (Gallaher and Blough, 1972); again, the phospholipid composition of the cells remained remarkably constant.

As mentioned earlier, SV5, a paramyxovirus, contains neutral glycolipids rather than the hematosides or gangliosides present in the host cell membrane; BHK-21 cells contain *N*-acetylneuraminyl lactose ceramide, and virtually none was detected in the virion produced in these cells (Klenk and Choppin, 1970b). Recent studies in sphingolipid synthesis have been done using choline-^{3}H and glucosamine-^{3}H as precursors (A. A. Scheid and P. W. Choppin, personal communication, 1972); in MDBK (Madin Darby bovine kidney) cells following SV5 infection, sphingomyelin synthesis decreases whereas globoside doubles. It had previously been shown by Hakomori (1970) that confluent cells had higher levels of sphingoglycolipids than had sparsely plated cells. Thus SV5 infection seems to produce the same effect as the attainment of confluence by a cell monolayer (A. A. Scheid and P. W. Choppin, personal communication, 1972). Hakomori *et al.* (1971) have studied the relationship of glycolipid synthesis to transformation of chick embryo fibroblasts by Rous sarcoma virus. Viral transformation led to a decrease in glycolipids with nonreducing terminals (e.g., disialosyl and monosialosyl hematosides), and increased amounts of precursor molecules (ceramides and glucosyl ceramides) within cells. At 20 hours after infection, when 30% of the cells were transformed, disialosylhematoside began to drop and reached barely detectable levels at 48 hours, when 70% of the cells were transformed. The decrease of hematoside was followed by an increase in neutral sphingoglycolipids. On the other hand, incorporation studies with glucosamine-^{14}C revealed a 50% increase in glycolipid synthesis in cells transformed by Rous virus (Hakomori *et al.*, 1971), suggesting a rapid turnover of the sugar moieties in virally transformed cells. In contrast Warren *et al.* (1972), using palmitate-1-^{14}C as a precursor, failed to show any major differences in the incorporation of this precursor into glycolipids of normal chick fibroblasts, those transformed by Rous virus (Schmidt-Ruppin strain), or cells transposed with a *ts* mutant (TF) which reverted to normal following growth at 41.5°. These differences may result from variability in "pool equilibria" with different isotopes (Hakomori *et al.*, 1971), or from palmitate exchange. Obviously, more studies along these lines are needed to map out the molecular events responsible for cell fusion, transformation, lysis, and death.

B. DNA Viruses

Studies of lipid metabolism in cells infected with DNA-containing viruses have been limited to three viral classes: herpesviruses, poxviruses, and lipovirus. It is the paucity of work on DNA viruses which accounts for the shortness of this section, rather than the authors' interests in the RNA-containing viruses.

1. *Herpesviruses*

Lipid synthesis in primary rabbit kidney cells infected with pseudorabies virus was studied using different radioactive precursors (Ben-Porat and Kaplan, 1971). These studies were based on the experimental design of Kates *et al.* (1961) for influenza virus except that 16- and 90-hour pulses of orthophosphate-^{32}P and choline-^{3}H were used instead of a single pulse of 72 hours. Several important findings were noted. First, the level of radioactivity of the inner nuclear membrane and that of purified virus closely resembled each other, confirming the morphological studies of Siegert and Falke (1966), which indicated that the virus acquired its envelope from this membrane. Second, the specific activities of the nuclear membrane and virion were quite different, suggesting to Ben-Porat and Kaplan (1972) that the viral envelope was derived from more metabolically active sites of the inner nuclear membrane than the remainder of the nuclear envelope. Ben-Porat and Kaplan (1972) prelabeled rabbit

Table VIII

Transfer of Phospholipids from Cytoplasmic to Nuclear Membranes after Infection with Pseudorabies Virus[a]

Experiment	Time after infection[b] (hours)		Type of membrane		
				Nuclear	
			Cytoplasmic	Outer	Inner
1	4.5	Infected	25,600[c]	3560[c]	5820[c]
		Uninfected	26,100	3120	3430
2	7	Infected	29,900	6780	5980
		Uninfected	33,500	3890	3020

[a] Reprinted from Table 2 of Ben-Porat and Kaplan (1972) by courtesy of Macmillan Press.

[b] Primary rabbit kidney cell monolayers were incubated in medium containing 3% bovine serum and 0.2 μCi/ml choline-^{3}H for 72 hours prior to mock infection or infection with 20 pfu/cell of pseudorabies virus, and chased with unlabeled medium.

[c] Values are in cpm/culture.

kidney cells with choline-^{3}H for 72 hours, and then isolated cellular organelles at various times following infection with pseudorabies virus. During the chase period, there was a 40% drop in radioactivity (cpm/culture dish) in the cytoplasm, and a doubling of radioactivity in the nuclear membrane at 7 hours postinfection (Table VIII), suggesting a flow of lipid from the cytoplasm to the nucleus for viral assembly. Since sphingomyelin and phosphatidylcholine turn over at different rates in P815Y cells (Pasternak and Bergeron, 1970) as well as in other cell types (Weinstein and Blough, 1973), it is surprising that they were found in the same ratio in infected and uninfected cells during the chase.

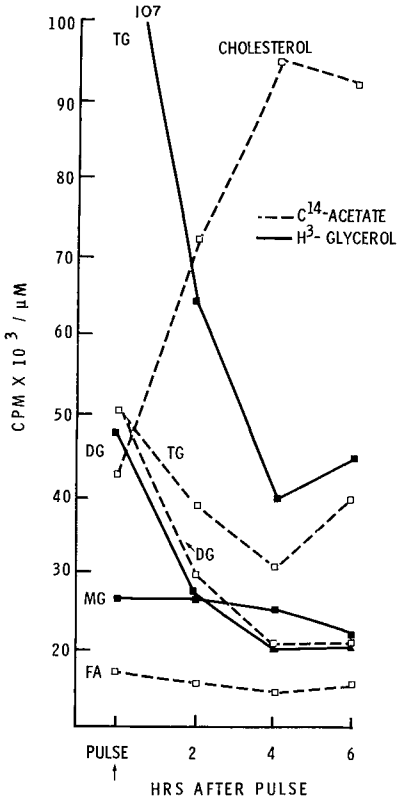

FIG. 18. a and b. Metabolism of glycerol-^{3}H (2 μCi/ml) and acetate-^{14}C (0.5 μCi/ml) by BHK-21 cells infected with herpes simplex virus. Cells were pulsed for 30 minutes at 2 hours after infection or mock-infection and samples taken after the pulse and during a 6-hour chase period. Fig. 18a. Control BHK cells. (MG = monoglycerides; DG = diglycerides; TG = triglycerides; FA = free fatty acids.) From Weinstein and Blough (1973).

Studies were done in the authors' laboratory on herpes simplex virus infection of BHK-21 cells using relatively short pulses of 30 minutes with glycerol-2-^{3}H and acetate-^{14}C, and various chase periods; both neutral lipids and phospholipids were analyzed. Very early in the infectious cycle (2 hours after infection), there was a preferential stimulation of phosphatidylserine and sphingomyelin synthesis over other phospholipids, whereas triglycerides were inhibited. At 3 to 5 hours, phosphatidylserine was inhibited, and all neutral lipids stimulated. At 8 hours, there was a stimulation of both isotopic precursors flowing into all lipids (Weinstein and Blough, 1973); this is the time of cell fusion and cytopathology. The different rates of uptake of acetate and glycerol into the same lipids suggest multiple pools are operative. Despite the cyclic changes in phospho-

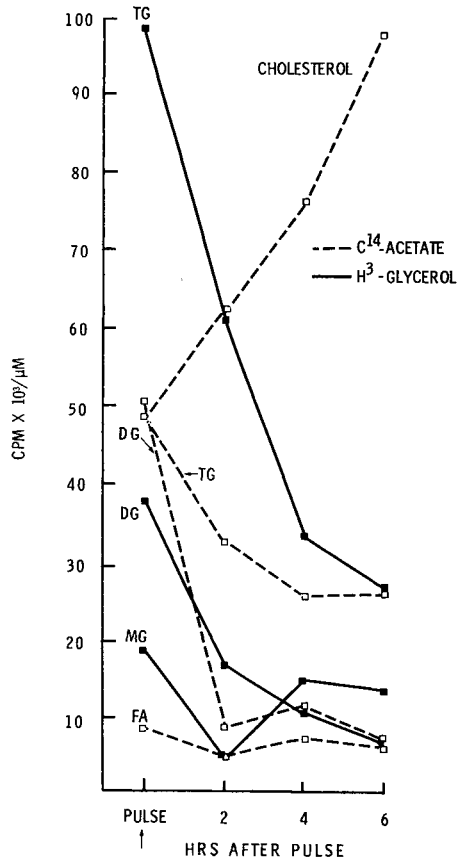

FIG. 18b. Herpesvirus-infected cells. See Fig. 18a legend.

lipids in herpesvirus-infected cells, the composition of host-cell lipid during the infectious cycle is stable (Gallaher and Blough, 1972), and increased levels of lysophosphatides are not detected. This suggests that monoacylated phospholipids do not play a part in cell fusion. Turnover of diglycerides and triglycerides is the same in infected and uninfected cells, that is, 2- to 2½-hour half-lives for both molecules. Fatty acids, sterols, and monoglycerides do not turn over under these experimental conditions (Figs. 18a,b). With phospholipids, the glycerol skeleton shows different patterns of turnover in phosphatidylserine and phosphatidylcholine, in comparing infected to uninfected cells. Turnover did not appear to follow first-order kinetics and the possibility of ^{3}H exchange in the case of glycerol-^{3}H could not be ruled out. It may well be that definitive measurements of turnover must await the isolation and quantitation of isotopic precursor and cellular product pool sizes. Obviously there may be a great deal of variation of lipid synthesis and turnover in cultured cells, which no doubt reflects cell types (multiple in the case of primary chick embryo fibroblasts), cell cycle conditions, contact inhibition, and other physiological and metabolic events responsible for cell growth and division.

2. *Poxviruses*

Vaccinia virus lipid biosynthesis was investigated in L cells using choline-^{3}H as a precursor (Dales and Mosbach, 1968); 92–98% of the radioactivity was found in phosphatidylcholine; the ratio of stearic acid ($C_{18:0}$) to oleic acid ($C_{18:1}$) was 1.0 in purified virions and 0.5 in microsomal fractions of the host cell, suggesting *de novo* synthesis of lipids. Inhibitor studies with streptovitacin A revealed that translation of messenger RNA was required before viral envelope biogenesis occurred at 3 hours postinfection.

3. *Lipovirus PM2*

Studies on the lipid composition of the bacteriophage PM2 and its host cell *Pseudomonas* BAL-31 suggest *de novo* synthesis or rearrangement of lipid for virus assembly. Within 60 minutes after infection there is a shift in the distribution of lysophosphatidylethanolamine and phosphatidic acid in the host cell (Braunstein and Franklin, 1971). Phosphatidic acid is a trace component only in uninfected cells, but makes up 2–4% of viral lipid. Phosphatidylethanolamine drops from 75% to 43% of cellular phospholipid and phosphatidylglycerol rises from 23% to 36%, following infection.

VI. Viral Assembly and Structure

A. Patterns of Assembly

Apart from electron microscopic observations on the site of synthesis or accumulation of viral material, and the relationship of this site to that of subsequent assembly of the virion, little is known about the assembly or transport of lipid molecules to cell membranes. The work of Kates *et al.* (1961) indicated no disturbance of lipid patterns of the host cell following viral infection; in contrast to this, *de novo* synthesis of lipid has been found to occur in chick embryo fibroblasts infected with influenza virus (see Section V). It is postulated that some of the differences in acyl chain composition seen in a variety of viruses may be due to the transport of newly synthesized (or preformed) lipid from the endoplasmic reticulum to the plasma membrane; in the case of viruses maturing at other membranes, such as herpesvirus, this transport would be toward the nuclear membrane. The most likely candidate to undertake this transport is an envelope structural polypeptide (Tiffany and Blough, 1970a). There is a precedent for this in the binding of lipid to enzymes of eukaryotic cells in the fatty acid synthetase system of yeast, where the apolar lipid binds to hydrophobic sites of the enzyme and allows transport of this multienzyme complex through the cell sap (Lynen *et al.*, 1968). Another transport system has been shown to operate in rat liver (Wirtz and Zilversmit, 1969), whereby phospholipids are transported from their site of synthesis in the endoplasmic reticulum to mitochondria.

The "inverted toadstool" model of Tiffany and Blough (1970a) for the structure of the envelope of influenza virus (considered in more detail below) suggests that the first step in the assembly process may be a phase transition of lipid within the cell membrane to a micellar state similar to that suggested by Lucy (1964). This would present an array of polar head groups of lipids with porelike spaces between them which would allow the hydrophilic parts of the external envelope polypeptides of the virus to be inserted through the cell membrane (Fig. 19). This is not unlike the "closed" to "open" pillar change in the membrane model of Kavanau (1963). There is either a lateral displacement of host cell membrane macromolecules or a dissolution of the preexisting membrane components to make room for the incoming polypeptides. The former mechanism has been shown to apply in the case of herpesvirus (Heine *et al.*, 1972).

The complexity of the problem of assembly is increased when one considers the need to glycosylate the glycolipids and the envelope polypep-

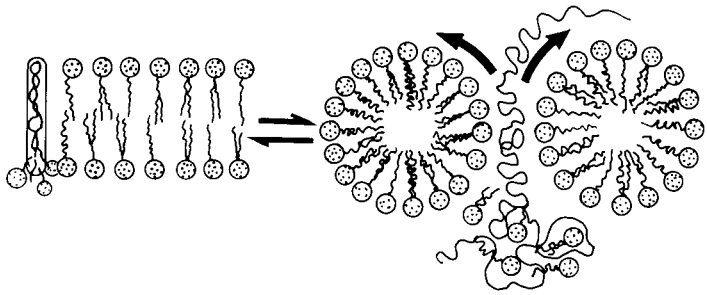

FIG. 19. Method of inserting viral polypeptide into a membrane. Polar groups of lipid in the micellar form feed the polypeptide through the membrane. The possible transition to a lamellar phase with an interdigitated polypeptide is shown on the left.

tides (which make up the surface projections of a large number of enveloped viruses). It seems that the majority of proteins exposed on the outer faces of cell surface membranes are also glycoproteins, and it has been proposed that the carbohydrate moiety promotes passage of glycoproteins across cell membranes (Eylar, 1966). This requires the presence in the cell of a battery of glycosylating enzymes. These are specific for each monosaccharide, and together build a host-specific carbohydrate structure, leading to observations such as that on Sindbis virus, where the carbohydrate moiety of envelope glycoproteins depends on the host cell (Grimes and Burge, 1971). Ortho- and paramyxoviruses grown in cells exhibiting blood group and Forssman antigen activities have been shown to possess the same surface activities, but not when grown in hosts without these groups (Isacson and Koch, 1965; Rott *et al.*, 1966). Burge (quoted by Scheele and Pfefferkorn, 1969) is reported to have been unable to find a pool of free glycosylated glycopeptide in the cytoplasm of cells infected with Sindbis virus. The enzymes responsible for glycosylation have been found in the microsomal fraction of cells (Grimes and Burge, 1971) and Golgi membranes (J. J. M. Bergeron, personal communication, 1972); thus far, no studies on the glycosylation of glycolipids in virus-infected cells have been reported. It is postulated that the glycosyltransferases responsible for addition and chain elongation of sugars are at the site of viral maturation. Klenk and Choppin (1970b) noted the absence of sialic acid in sphingoglycolipids synthesized in cells infected with the paramyxovirus SV5, and also showed the absence of detectable surface sialic acid in the budding virus envelope by electron microscopic staining (Klenk *et al.*, 1970b). It has yet to be determined whether this is a defect in glycolipid synthesis at the CMP-sialotransferase level, or the result of viral neuraminidase action. It seems unlikely that viral neuraminidase alone is responsible for total removal of

sialic acid, since nonterminal sialic acid is not readily removed from gangliosides by sialidases (Burton, 1963). However, sialic acid has been found in both glycoproteins and glycolipids of viruses which do not contain neuraminidase (Burge and Strauss, 1970; Burge and Huang, 1970; Klenk and Choppin, 1971). Budding is thought to occur when the envelope polypeptides reach a critical concentration or form a patch of critical area (Fig. 20). The ionic strength of the medium appears to play a part in the assembly process of enveloped viruses (Waite and Pfefferkorn, 1970). The separation of virus from the cell surface, endoplasmic reticulum, or nuclear membrane is probably mediated through electrostatic repulsive forces which shear the particle from its placental membrane.

Viruses such as PM2 (Harrison *et al.*, 1971a) and the envelope of more complex viruses such as poxviruses, baculoviruses, and possibly iridoviruses (Dales and Mosbach, 1968; Harrap, 1969; E. Houts and M. Gravell, personal communication, 1972), appear to be formed independently of any preexisting membrane, their proteins and lipids interacting directly to form a lipoprotein shell; the ordering of protein and lipid by hydroprobic interaction, or nonpolar protein-protein interactions, provide a net entropy decrease which is responsible for the self-assembly phenomenon. It is suggested that lipids bearing appropriate acyl chains are

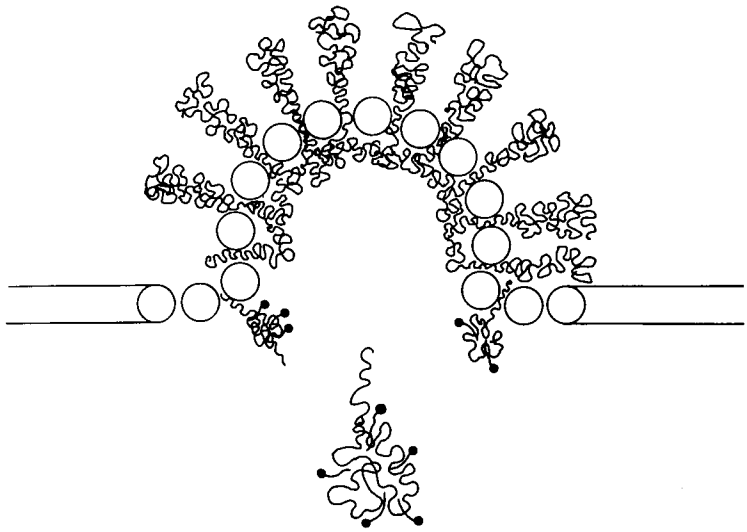

FIG. 20. Diagrammatic expression of the budding process of enveloped viruses at a cell membrane. Note molecules of lipid bound to structural polypeptide with polar groups on the exterior (●), facilitating transport through the cell cytoplasm. Spheres represent micelles of lipid.

necessary in order to provide or preserve configurations of some polypeptides which are needed for assembly. The incorporation of lipid permits a unique plasticity of structure not possible in the isometric viruses. It should be noted that lipid-protein structures are not necessarily devoid of an ordered structure; Pollard *et al.* (1969) found that low-density lipoproteins consisted of a core containing most of the neutral lipid, surrounded by a shell composed of the phospholipid and twenty protein subunits arranged in a dodecahedral pattern with icosahedral symmetry. It appears certain that union of protein and lipid occurs rapidly and probably leads to an almost instant encapsidation of viruses such as PM2. The functional need for lipids in this encapsidation process might be thought to resemble the lipid requirement in the model suggested by Green and Perdue (1966) for the assembly of lipid-depleted mitochondrial membrane building blocks. The hydrophobic protein regions of the virion may be considered as "lipid-depleted subunits"; the binding of lipid represents "information" at the molecular level, in which the stretches of hydrophobic amino acids specify the amount and type of lipid bound.

Some viruses such as entomopoxviruses (Devauchelle *et al.*, 1970, 1971), and some herpesviruses (Heine *et al.*, 1971), appear to be formed by a combination of "scaffolding template" and *de novo* synthesis. Why different viruses assemble at a specific site, such as poxviruses in cytoplasmic "factories" (Dales, 1963) or herpesvirus at the nuclear membrane (Morgan *et al.*, 1959), is at present unknown, although the availability of lipids bearing specific acyl chains is probably of paramount importance. Reference to Section II shows that many RNA viruses assembled in the cytoplasm will fall into the "scaffolding template" category, and some DNA viruses will fall into the *de novo* or mixed categories, but there are too many exceptions for this to be used as a basis for general classification of enveloped viruses.

Obviously these are extremely tentative hypotheses and a great deal more work is required to give them more formal substance. The isolation and characterization of unique hydrophobic polypeptides has not yet been achieved, and it is at present unknown whether the large DNA-containing viruses such as vaccinia, FV 3, etc., can induce or have the genetic capacity to code for enzymes which are necessary for lipid biosynthesis.

Figure 21 shows a general scheme for *de novo* synthesis of viruses.

B. Models of Structure

With the awakening of interest in the structure, composition, and function of membranes, and the realization that viral envelopes could be con-

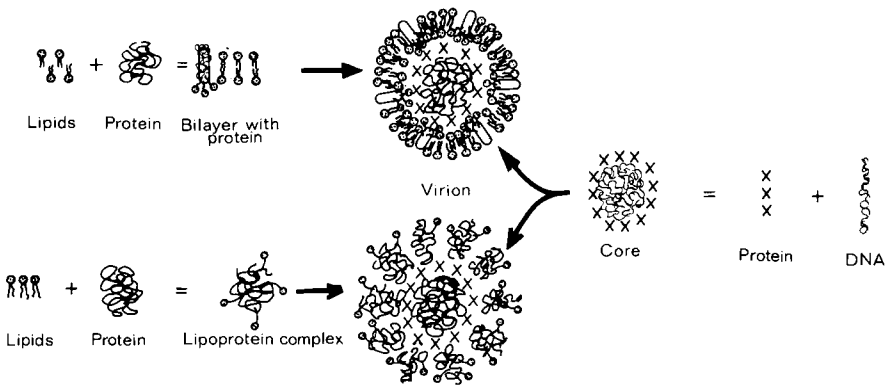

FIG. 21. Simplified diagram of self-assembly of lipid-containing viruses.

sidered as a special case in which a relatively small number of proteins was associated with lipid, much attention has been focused on the structure of viral envelopes, and it is probably safe to say that more papers have appeared on this subject in the last 3 years than in the 10 preceding years. The results up to the present cannot be considered complete for any single virus. It is the express purpose of this section to show the diversity of opinion that exists on envelope structure and to point out that not all lipid-containing viruses are constructed in the same fashion.

Prior to 1969 it was thought that the envelope of viruses was produced at cellular membranes by a simple budding process; the envelope lipids were therefore assumed in all cases to have the same composition as the host membrane, and to retain the presumed bimolecular leaflet structure of the cell membrane. The virus was pictured as a nucleocapsid core surrounded by a liposome-like structure in which were embedded repeating surface subunits. This posed a number of problems, chief among which was how the polypeptides forming the external projections of the virion were transferred from the cytoplasm to the outer side of the lipid bilayer prior to and during budding. This transport of charged materials through the hydrophobic interior of the lipid bilayer membrane would require considerable amounts of energy, and any involvement of a transmembrane pore system, such as might be used for macromolecular uptake or exclusion from the cell, would seem unlikely as host membrane proteins forming the pore might then be found in the viral envelope. This was shown not to be the case for influenza virus (Holland and Kiehn, 1970). With the application of high-resolution gas-chromatographic techniques, it became evident that the lipid composition of the virion did not necessarily mimic that of the uninfected host cell membrane from which it was derived (Tiffany and Blough, 1969a,b). The greatest differences were noted in the acyl groups of neutral lipids and phospholipids.

It has been suggested that viruses which contain lipid in an envelope do so in part because of the unique hydrophobicity of one or more of the envelope structural polypeptides. According to this hypothesis, viruses may have a predilection for maturing at certain membranes because of the phospholipid and glyceride (and hence acyl chain) composition at these points, or because of a favorable lipid transport process to this site; the virus may assemble incorrectly if the composition of the lipid available is altered, e.g., by growing virus in the presence of vitamin A (Blough, 1963) or an exogenous fatty acid (Blough and Tiffany, 1969), in incomplete influenza virus or influenza virus in a nonpermissive cell system (Blough, 1971b). As indicated in Section V, changes of lipid metabolism in the host cell following infection may be responsible for shifts in lipid composition at the site of assembly, or at the site of "charging" of transport proteins, thus accounting for at least some of the observed differences between virus and host lipids.

Of the viruses which possess classical cubic symmetry only those of the herpesvirus group appear to be enveloped, but the envelope itself has no such regularity and is capable of considerable variation in shape, such as during preparation for electron microscopy when ballooning may occur as negative stain penetrates within the envelope (Fig. 6a). Other regular viruses are known to contain lipid (e.g., lipovirus and frog virus FV 3), but in these the lipid appears to play a more integral part in the structure of the internal portion of the virion than in the true envelope. Some of the other enveloped viruses appear to possess an underlying network structure or scaffolding within the envelope which may be composed of hexamers and pentamers, in particular influenza C (Apostolov and Flewett, 1969), and the regular spacing of surface projections revealed by negative staining of some strains of influenza A has suggested a greater degree of regularity of envelope structure than had been previously considered (Almeida and Waterson, 1967, 1970; Tiffany and Blough, 1970b).

The external projections of the envelope of animal viruses have been found to be glycoproteins in all cases so far studied (Stollar, 1969; Compans *et al.*, 1970; Schulze, 1970; Burge and Strauss, 1970; Compans, 1971; Chen *et al.*, 1971), and recent studies confirm that an envelope structural protein of the type postulated by Blough (1969) and Tiffany and Blough (1970a) may be found within the lipid region of the envelope or just beneath it (Schulze, 1970, 1972; Compans *et al.*, 1970; Neurath *et al.*, 1972). In all cases the envelope must be regarded as an essential part of the virion since treatment with organic solvents or phospholipases, or the presence of "nonenveloped" particles occurring naturally, e.g., in herpesvirus-infected cells, are all associated with a lower infectivity (Watson *et al.*, 1964). Approaches to a plausible envelope structure can

be made by the construction of molecular models based on the compositional analysis of the virion, the possible type of molecular interaction between components of the virion, electron-microscopic appearance (using negative contrast, positively-stained thin sections, and freeze-etching), and the effect on virus structure of various enzymes such as proteases and phospholipases. We should stress, however, that the geometrical considerations (the packing of lipid, cross-sectional areas of molecules, etc.) are particularly important, since no matter what model of structure is chosen, it must be possible to pack all the components into the available space without incompatibility of hydrophobic and hydrophilic regions. Data from ultracentrifugal or polyacrylamide gel electrophoresis studies of individual polypeptides must be used with caution, since molecular weights so derived may be considerably in error, especially in the case of glycoproteins (Segrest *et al.*, 1971). An alternative approach is to use powerful tools such as X-ray diffraction and magnetic resonance spectroscopy in an attempt to discern the arrangement of lipid and protein within the virion (Harrison *et al.*, 1971a,b; Landsberger *et al.*, 1971). However, such methods are insufficient to solve the problem by themselves. At the present time there is enough information to construct tentative models for at least four groups of viruses: myxoviruses, rhabdoviruses, alphaviruses, and lipovirus. Models proposed for other viruses are also noted below.

1. *Myxoviruses*

We shall consider here principally influenza A virus as representative of the orthomyxoviruses, since almost all the work on structure has employed this type, although not always the same strain. There is also evidence that influenza C differs quite considerably in structure from influenza A and B (Apostolov and Flewett, 1969). These viruses are assembled at the cell surface membrane ("scaffolding template" viruses) and possess an envelope with regularly spaced projections 90–130 Å long. From measurements of spike spacing Tiffany and Blough (1970b) determined that a particle of overall diameter 1000 Å was covered by 500–600 spikes, in contrast to the frequently quoted figure of 2000; uncritical acceptance of this latter figure probably delayed attempts to establish a structure for the influenza virus envelope by leading to confusing estimates of numbers of functional molecules per virion (Kendal *et al.*, 1968). Protease digestion studies (Kendal *et al.*, 1969), negative-contrast electron microscopy (Berkaloff and Thiéry, 1963; Nermut and Frank, 1971), and freeze-etching studies (Bächi *et al.*, 1969) have all indicated the presence of "nanogranules" about 40 Å in size in the envelope; these appear to be embedded in the envelope toward the inner

side, and to correspond roughly to the positions of the spikes. Such a particle of protein would have a molecular weight of about 25,000.

The model proposed by Tiffany and Blough (1970a) was the first to attempt to rationalize the state of knowledge of the composition and structure of this virus by applying simple geometrical principles to the then available data on the nature and amounts of the constituent molecules of the envelope. At that time no detailed information was published on the proportions and molecular weights of the individual polypeptides, such as shortly afterwards became available from polyacrylamide gel electrophoresis studies (Compans *et al.*, 1970; Haslam *et al.*, 1969, 1970a,b; Schulze, 1970; Skehel and Schild, 1971). Nevertheless, this study led to some interesting conclusions and permitted some predictions to be made, particularly that there is an envelope structural protein underlying the lipid-containing region of the envelope; other parts of the model have been more seriously questioned, and in the light of more recent evidence require modification. The salient features of this model are given here, together with some indication of the data from other laboratories which reinforce or conflict with the model.

Typically a particule of influenza A (e.g., strain A_0/PR8/34) is spherical and 1000 Å in diameter with surface projections 100 Å long and 65 Å apart and an envelope 90–100 Å thick. The particle contains about 20% lipid (approximately 12% phospholipid and 8% cholesterol) and roughly 70% protein; protein has been estimated to weigh 4.2×10^{-16} gm per particle (Reimer *et al.*, 1966), and nucleocapsid to account for about 40% of this (Laver, 1964). We calculate that there are 550 spikes on this particle (Tiffany and Blough, 1970b), and by taking a typical value of 0.7 ml/gm for the partial specific volume of the proteins, and typical dimensions for the spikes (Laver and Valentine, 1969), we find that the total protein volume per particle is 2.94×10^8 Å^3, of which about 9.5×10^7 Å^3 is in the nonspike part of the envelope. A lipid bilayer structure similar to that proposed by Schulze (1972) was first considered, but comparison of the area occupied by the viral lipid in the form of a bilayer, and the area available in the envelope for such a bilayer, led to abandonment of this method of lipid arrangement. Taking De Bernard's (1958) figure of 50.3 Å^2 per molecule for the mean cross-sectional area of a lipid molecule at the observed cholesterol:phospholipid molar ratio of 1.5, the lipid is calculated to occupy 3.3×10^6 Å^2 as a bilayer. Other strains of influenza virus have been observed to have cholesterol:phospholipid molar ratios closer to unity, in which case the area per molecule is about 55 Å^2 but the bilayer area is still 3.4×10^6 Å^2. The largest area of bilayer which could be accommodated in the envelope (forming a shell of mean radius about 370 Å on the outer side of the envelope) is

only 1.54×10^6 Å^2. There hence appeared to be twice as much lipid present as would be required in the bilayer model, and the problem reduced to one of distributing this lipid in some uniform manner. Previous studies (Tiffany and Blough, 1969a) had suggested some hydrophobic interaction between lipids and an envelope protein; it was therefore postulated that the envelope protein might be arranged in the form of a shell of units with "stalks" directed outward and acting as supports for the external spikes. The spaces between the "stalks" would be filled by roughly spherical micelles of lipid, equal in number to the external spikes, and part of the lipid would also be bound to the units in the protein shell (Figs. 22 and 23). Because of the shape of the base and "stalk" this was referred to as the "inverted toadstool" model. The stalk is not necessarily to be thought of as an integral part of the toadstool; it is included with the base since their total volume was calculated as that of the nonspike envelope proteins, and it was in fact suggested that the stalk might form part of the spike. Assuming that the lipid-binding capacity of the toadstool base was similar to that of the lipid-binding chloroplast protein studied by Ji and Benson (1968), i.e., about 18 phos-

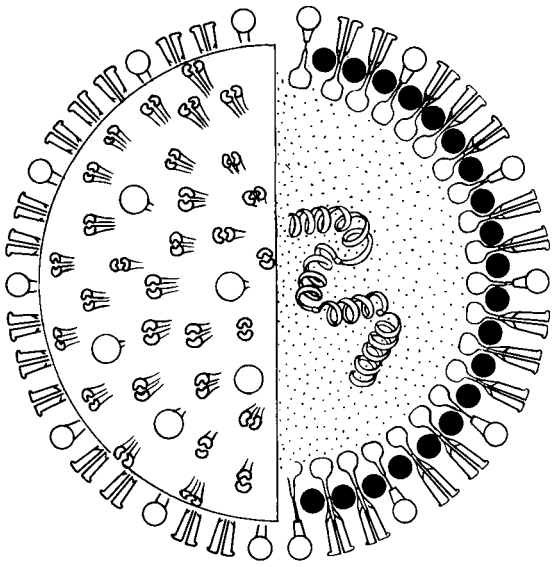

FIG. 22. Model of the influenza virus particle (Tiffany and Blough, 1970a). Right half shows internal fragmented nucleocapsid (coil) with "linker" molecules; the toadstool-shaped structural polypeptide goes through the membrane and interdigitates with envelope lipid in micellar form (black spheres). Surface projections are hemagglutinin and neuraminidase (sphere-topped spike). Left half shows an external view of the viral envelope with glycoprotein projections.

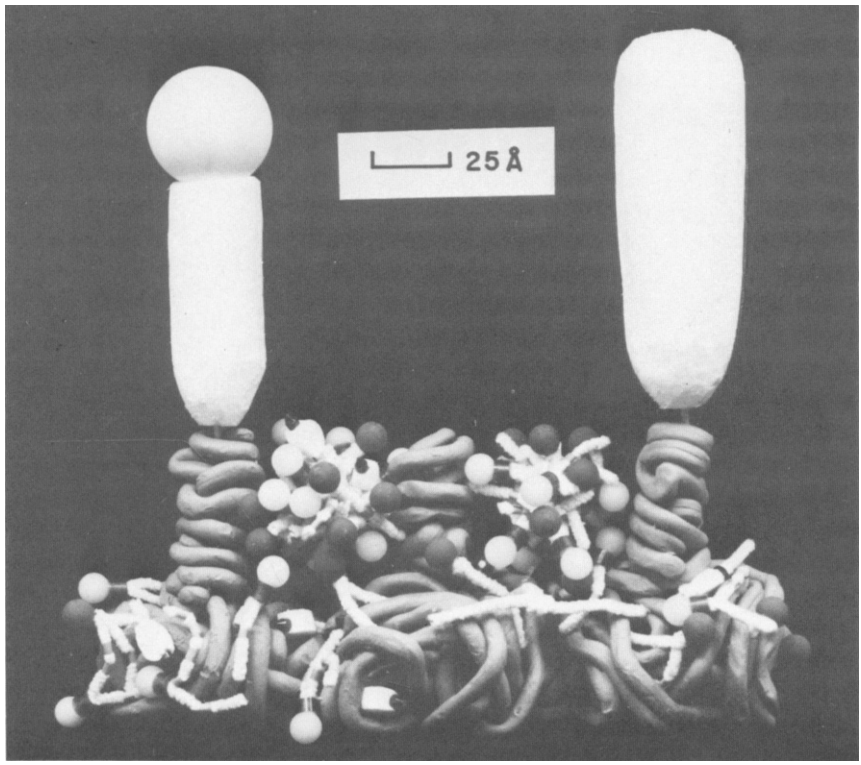

FIG. 23. Molecular model of micelles of lipid plus toadstool (viral envelope structural proteins). Acyl chains of lipid molecules are represented by pipe cleaners and their polar groups by small balls. Hemagglutinin (flat-topped projection) and neuraminidase (spherical-tipped projection) surmount the toadstools. The projection on the middle toadstool has been omitted. (From Blough, 1969, reprinted by courtesy of the Bulletin of the World Health Association.)

pholipid molecules bound per 23,000 molecular weight of protein, about 25% of the viral lipid would be hydrophobically bound, and the rest would form 550 spherical micelles 54 Å in diameter. This lipid, but not that hydrophobically bound, would be susceptible to lipase attack; Schulze (1970) found that 70% of the viral lipid was removed by this means. It seemed likely also that the bound lipid might confer greater resistance to proteolytic enzyme action on the base protein, and the toadstool bases would appear as "nanogranules" in partially digested particles (Kendal *et al.*, 1969). The appearance of the envelope in positively stained ultrathin sections would probably sufficiently resemble that of a conventional lipid bilayer structure to be interpreted as such. The molecular weight of the base would be about 120,000, and the stalk about

40,000; it was considered that the base might be composed of subunits of 20,000–25,000 molecular weight. This model also simplifies the assembly process at the cell membrane by permitting insertion of spike proteins through the membrane following a local phase change to the micellar form, induced by the presence of envelope proteins (Fig. 19).

Recently results have been published from several laboratories, indicating that there is in fact a shell of envelope structural protein underlying the lipid region (Compans *et al.*, 1970; Haslam *et al.*, 1969, 1970a,b; Schulze, 1970; Skehel and Schild, 1971), but as yet no studies showing lipid-binding properties have been reported for this protein. Schulze (1970, 1972) and Klenk *et al.* (1972) concluded, from the smooth appearance and resistance to proteolytic enzymes of influenza particles whose external spikes had been removed by bromelain treatment, that the lipid was probably in the form of a bilayer surrounding the outer part of the envelope, and that protein "stalks" supporting the spikes did not penetrate this lipid region. This view was reinforced by the spin-label studies of Landsberger *et al.* (1971). An unpaired-electron spin label was introduced into the virion by incorporating nitroxy-labeled analogs of stearic acid and androstane. A comparison was made of purified influenza virus (strain A_0/WSN) and human erythrocyte ghosts following labeling at 4° for 4–15 hours. Striking similarities between the spectra for human erythrocyte membranes and virus were noted (Fig. 24): the rotation of the nitroxy-label was more hindered when the label was close to the carboxyl end of the stearic acid molecule than when it was close to the terminal methyl group, and this was taken to be indicative of a bilayer type lipid structure. Taking values for the cross-sectional areas of the lipids which correspond more nearly to the packings achieved in crystals, 46.7 $Å^2$ for phospholipid and 35 $Å^2$ for cholesterol (Levine and Wilkins, 1971), Landsberger *et al.* (1971) calculated that the lipid of the virus could in fact all be contained in a bilayer shell.

Obviously the model of Tiffany and Blough (1970a) must be modified if it is to be brought into agreement with later results; it would, for instance, require little change to replace the lipid micelles by a bilayer through which the spikes protrude. De Bernard's (1958) values for cross-sectional areas of lipids have been criticized as being derived from monolayer studies at too low a value of surface pressure. However the figures chosen by Landsberger *et al.* (1971) may also be criticized since there is no evidence that the degree of lipid packing in the viral envelope approaches that in the solid phase. Engelman's (1969) calculations on the hydrophobic volume of lipids in a bilayer suggest a larger molecular area should be taken, especially as the virus is normally assembled at about 37°, whereas most area measurements on monolayers and packed

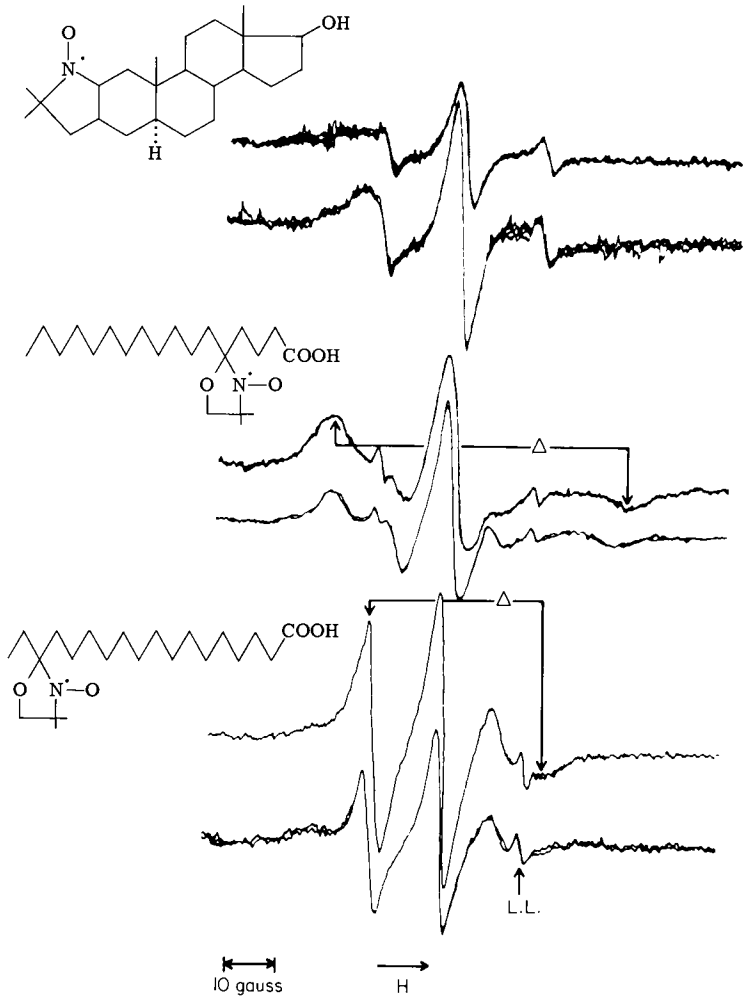

FIG. 24. Electron spin resonance traces for influenza virus particles (upper trace) and erythrocyte ghosts (lower trace). Nitroxy-derivatives of androstane and stearic acid were used. Δ = splitting between low- and high-magnetic-field peaks ("broad-line spectrum"). LL = high-field peak or "liquid lines." H = magnetic field. (Reprinted from Landsberger *et al.*, 1971, by courtesy of Dr. R. W. Compans and the Editors of the *Proceedings of the National Academy of Sciences.*)

lipids have been made at room temperature and the lipids can be expected to occupy significantly larger areas at the higher temperature. A control experiment which has not been performed is the determination of the spectrum of a micellar solution of lipids in which the micelles are *the*

same size as those postulated in the model of Tiffany and Blough; it is possible that this would not differ significantly from the erythrocyte membrane trace. Recent work by Lesslauer *et al.* (1972) indicates that the introduction of a bulky label into a lipid may cause a considerable change in the interactions of the membrane lipids. The above comments, rather than attempting to detract from the results of Landsberger *et al.*, indicate that the problem of the structure and function of the lipids of influenza virus is still not yet fully determined. The model of Tiffany and Blough (1970a) has served a useful purpose in postulating the presence of a structural protein whose existence was subsequently confirmed in several laboratories, and in promoting the use of a simple geometrical approach to the determination of envelope structure which is proving of use with other viruses (see below).

There appear to be two main reasons for the discrepancy in the amounts of lipid in the two models (bilayer and micellar). The first is the use by Tiffany and Blough of the value obtained by Reimer *et al.* (1966) of 4.2×10^{-16} gm of protein per virus particle, based on a Lowry protein determination and particle counting. This figure was also used by Skehel and Schild (1971) in calculating the numbers of molecules of each of the polypeptides per virion and may contribute to the differences found between the results of these authors and those of Compans *et al.* (1970). Alternatively, one may estimate the total molecular weight of viral lipid from the observed percentages of lipid and RNA and the molecular weight of the RNA, assuming one genome-equivalent per particle. Taking the RNA content to be 1% (Frisch-Niggemeyer and Hoyle, 1956), the lipid content as 20% (Blough *et al.*, 1967), and the molecular weight of one genome of RNA as 3.9×10^6 (Skehel, 1971), then the total molecular weight of lipid is 7.8×10^7; for a cholesterol:phospholipid molar ratio of unity this gives 6.75×10^4 molecules each of cholesterol and phospholipids, and using the cross-sectional area figures of Levine and Wilkins (1971) we find a lipid bilayer area of 2.8×10^6 Å^2. The available area from Landsberger *et al.* (1971) is only 1.47×10^6 Å^2, again indicating an excess of lipid. The lower limit found for the molecular weight of influenza virus RNA by Pons and Hirst (1969) is 2.4×10^6; use of this figure reduces the bilayer area to 1.7×10^6 Å^2, which is still greater than the figure of Landsberger *et al.*, and does not allow for the space occupied by the proximal ends of the spikes inserted into the outer leaflet of the bilayer. If we are to be able to bring the lipid areas calculated by different methods into agreement, it would seem necessary at least to reassess the figure for the proportion of RNA in the virion, and preferably the amount of protein per particle also. The second reason for the discrepancy between the two models is the value of 250×10^6 for the

total molecular weight of the virion used by Compans *et al.* (1970) in calculating the numbers of constituent polypeptides. This is much lower than the value calculated from the protein analysis of Reimer *et al.* (1966) of about 360×10^6.

The major difference still remaining between the two basic models is the question of whether the spikes are supported by "stalks" penetrating the lipid layer, or whether they float in the outer half of the lipid bilayer, stabilized partly by hydrophobic interactions between their bases and the acyl chains of the lipids surrounding them, and partly by electrostatic repulsive forces between the exposed parts of the spikes. Proteins traversing a membrane have been found in other systems (Bretscher, 1971), and such an interdigitated polypeptide would simplify the assembly problem.

Despite the considerable volume of knowledge which has been amassed on the paramyxoviruses, in particular on SV5 by Choppin and his coworkers (Compans *et al.*, 1966; Caliguiri *et al.*, 1969; Chen *et al.*, 1971; Klenk and Choppin, 1969a,b, 1970a,b, 1971; Klenk *et al.*, 1970a), no model comparable to that described above for orthomyxoviruses has yet been produced. It seems likely, however, that the role of lipids in the structure of the paramyxovirus envelope will closely parallel that in the orthomyxovirus envelope. Variation in the fatty acyl composition of lipids from different strains of Newcastle disease virus (Tiffany and Blough, 1969b) may indicate a lipid selection by envelope polypeptides such as was suggested for influenza virus (Tiffany and Blough, 1969a); differences of lipid composition between Newcastle disease virus and Sendai virus grown in the same host (Blough and Lawson, 1968) indicate that some process other than passive incorporation of normal cellular lipids takes place. If we perform the same kind of calculation on the extent of SV5 viral lipid in the form of a bilayer as given above for influenza virus, taking 0.9% RNA of molecular weight $5.7–6.8 \times 10^6$ and 20% lipid (Klenk and Choppin, 1969a) and a cholesterol:phospholipid molar ratio of 0.9 (Klenk and Choppin, 1969b), we find a bilayer area of $4.4–5.2 \times 10^6$ Å^2, corresponding to a spherical shell of mean diameter 1180–1290 Å. These figures are roughly the same as those given by Klenk and Choppin (1969a) for the diameter of the whole virion, including spikes. The diameter of a bilayer lipid shell in these particles would probably be 920–1000 Å, allowing 100 Å for the spike length and 40 Å for the bilayer thickness, so it appears that there is at least 60% too much lipid for a bilayer. It would seem reasonable here to reassess the diameter of the particle as seen in the electron microscope, making due allowance for shrinkage during specimen preparation. The particle size range of other paramyxoviruses is generally taken to be 1500–3000 Å.

2. *Rhabdoviruses*

Rabies virus is a rhabdovirus consisting of five or six polypeptides (Sokol *et al.*, 1971; Neurath *et al.*, 1972), 24% lipid (Blough *et al.*, 1973), and a single continuous nucleocapsid. The major part of the nucleocapsid is helically coiled into a hollow cylinder, with the remainder coiled in reducing turns to form a hemispherical end-cap (Matsumoto, 1970). Electron micrographs occasionally show released particles with the lipid-containing envelope ballooning at one end (Hummeler *et al.*, 1967); it is not known whether this is an error of assembly, or an artifact of preparation for electron microscopy. It is also not known whether the envelope is rigid or sufficiently elastic to distribute itself smoothly over the core when the particle is free from the parent membrane, in cases where ballooning is seen during budding. It seems possible that some of these ballooned regions of envelope are merely eversions of the invaginated portion lining the cylinder of nucleocapsid. Hummeler *et al.* (1967) refer to the flat or slightly reentrant base as "nocked" like the butt end of an arrow, and their results suggest that this is the normal form of the base. The surface area of the envelope has been calculated to be about 5.5×10^6 Å^2, taking the cap to be a hemisphere and the base flat. A hexagonal arrangement of surface projections can be seen in electron micrographs, with a row separation of about 75 Å; from this it can be calculated that there are about 790 surface projections per virion, covering the sides and cap. It is not known at present whether the base carries projections or not. Kuwert *et al.* (1972) confirm that the surface area of the envelope is about 5×10^6 Å^2. Calculations have been made which suggest that there may be rather less lipid than required to form a bilayer over the entire surface of the virion (Blough *et al.*, 1973); it has also been found that treatment of rabies virus with diethyl ether in the presence of Tween 80 detergent reduced infectivity but left the majority of the particles morphologically intact (Crick and Brown, 1970). These observations suggest that there may be some penetration of proteins through the lipid region of the envelope, rather in the manner proposed for the lipovirus PM2 (Harrison *et al.*, 1971a), and such an arrangement seems also to be suggested by Neurath *et al.* (1972) in drawing attention to the close correspondence between the numbers of the various polypeptide components of the envelope. These aspects of the structure of rabies virus obviously require further investigation. Kuwert *et al.* (1972) suggest the presence of hexagonal subunits over the surface of the particle and calculate that there are 580 of these on a particle 2000 Å long, but at present there seems to be no obvious connection between this and the other observations mentioned above.

Despite work on the structure of the nucleocapsid (Nakai and Howatson, 1968), and identification of the polypeptides of the virion (Wagner *et al.*, 1969), little seems to have been done to elucidate the structure of the envelope of another rhabdovirus, vesicular stomatitis virus. McSharry and Wagner (1971) have concluded from differences between viral and host lipids that a selection of lipids by polypeptides of vesicular stomatitis virus probably occurs, as in the case of myxoviruses (Tiffany and Blough, 1969a,b) and Sindbis virus (David, 1971), but as yet no specific interactions have been shown.

3. *Lipoviruses*

The marine bacteriophage PM2 contains 15% lipid, of which 90% is phospholipid consisting mainly of phosphatidylglycerol (67%) and phosphatidylethanolamine (27%) and a circular molecule of DNA of molecular weight 6×10^6 (Braunstein and Franklin, 1971). Harrison *et al.* (1971a) studied this virus intensively by combined electron microscopic, biochemical, and X-ray diffraction techniques. From the staining patterns using both positive and negative stains, it was possible to define "solvent excluding areas" occupied by nucleoprotein and lipid, and the electron density distribution in the solvent excluded areas was determined by X-ray diffraction. A deep minimum in electron density was found at a distance of 220 Å from the center of the particle and was considered to correspond to the hydrocarbon chain region of a lipid bilayer. Between 240 and 250 Å from the center was an area corresponding to the region of positive staining in thin section, which probably represented the polar head groups of the lipid interacted with protein. In the wide-angle X-ray scattering photograph shown in Fig. 25 the broad 4.6 Å band (middle) represents the lipid bilayer, while scatter in the 10 Å region is protein, and the inner 3.4 Å band is DNA. The lipid present in the virion (1.3×10^4 molecules, largely phospholipid) appears sufficient only to cover about 65% of the 220 Å shell as a bilayer. This suggests the presence of fenestrations or patches where protein might protrude through the lipid layer. The outer shell may be protein with a triangulation number of at least 12 or 13 (Caspar and Klug, 1962). Harrison *et al.* (1971a) suggest the virus is assembled *de novo* by condensation of protein and lipid around a core of DNA. A diagrammatic representation of the structure of PM2 is shown in Fig. 26.

4. *Alphaviruses*

Sindbis virus consists of only three proteins, two of which are envelope glycoproteins (of similar size or mobility in polyacrylamide gel electrophoresis), and the third a core protein associated with the single-

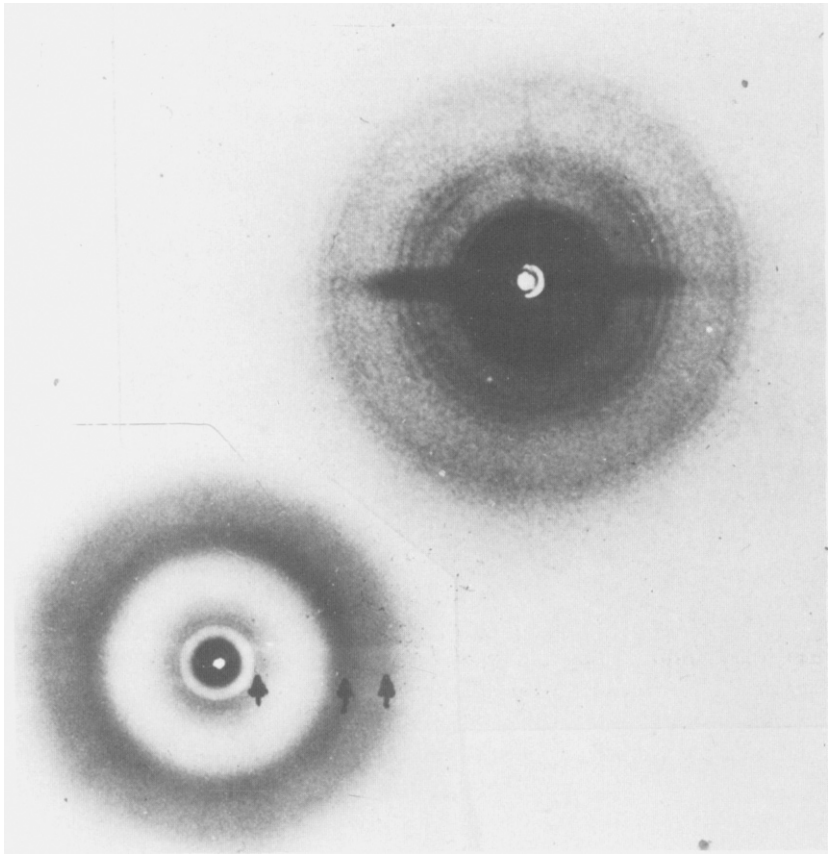

FIG. 25. X-ray diffraction pattern of marine bacteriophage PM2. Upper: small-angle pattern; the sharpness of the rings suggests the particles are isometric and identical. Lower: wide-angle pattern; arrows indicate sharp reflections at 12 Å, 4.6 Å, and 3.4 Å. (Reprinted from Harrison *et al.*, 1971a, by courtesy of Dr. R. M. Franklin and Macmillan Press.)

stranded RNA (Burge and Strauss, 1970; Schlesinger *et al.*, 1972); X-ray diffraction suggests that the envelope lipid is present in the form of a bilayer (Harrison *et al.*, 1971b).

Another member of this group, Semliki Forest virus, has been studied by Renkonen *et al.* (1971) and is found to have a cholesterol:phospholipid molar ratio near unity. These authors calculate, from data on the total molecular weight of the envelope, that there are about 10,000 cholesterol-phospholipid pairs in the viral envelope. Taking the electron-microscopic measurements of Acheson and Tamm (1967) and a cross-

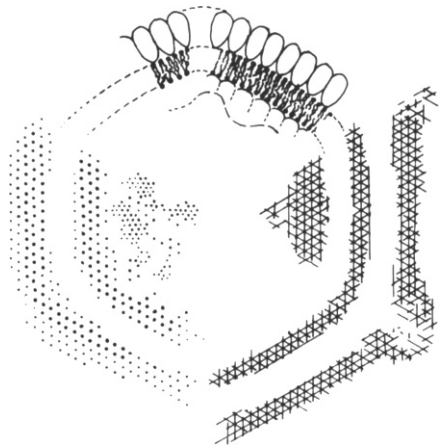

FIG. 26. Model of the structure of the lipid-containing bacteriophage PM2. (Reprinted from Harrison *et al.*, 1971a, by courtesy of Dr. R. M. Franklin and Macmillan Press.)

sectional area of about 100 Å^2 for each cholesterol-phospholipid pair, they calculate that 7000–8000 such pairs could be accommodated on the surface of the envelope; hence roughly twice this number could be incorporated in the form of a bilayer. These authors hence seem justified in concluding that a bilayer structure may be present. It may, however, be noted that according to the above calculation there is less lipid than required to form a complete bilayer, and there may therefore be penetration of a protein component through the lipid region, as was suggested above for rabies virus and influenza virus.

5. *Poxviruses*

Mitchiner (1969) suggested that the outer envelope of orf and vaccinia viruses is proteolipid in nature since these viruses are degraded by chloroform-methanol or 2-chloroethanol treatment, but not by carbon tetrachloride, acetone, ether, or lipolytic enzymes; however, treatment with ether produced a marked decrease in infectivity (Zwartouw, 1964). Mitchiner also proposed a model for the vaccinia envelope. This consists of (1) an internal envelope composed of a shell of laterally adherent blocks of envelope subunit protein, partially separated by deep clefts, and associated on both inner and outer faces and in the clefts with the polar groups of phospholipids and acyl chains of triglycerides; (2) an external membrane of closely associated protein and lipid. Spaces between the two membranes, and in the clefts, contain the cholesterol. However, although this model seems to agree with electron microscopic

observations and resistance to disruptive agents, a number of objections can be made. The amount of lipid present (2.1% phospholipid, 1.7% neutral lipid, and 1.2% cholesterol) seems inadequate to form the two membrane structures as envisaged in this model. There also seems no reason to suppose that the phospholipid associates with the envelope subunit protein only through its polar groups, since specific protein-acyl chain interactions have been shown to exist in some systems (DePury and Collins, 1966; Ji and Benson, 1968). There is also no evidence that triglycerides and phospholipids associate on a one-to-one basis as implied in the model. The indicated width of clefts between protein units (15–20 Å) seems insufficient to contain a double layer of phospholipid and triglyceride, as well as the cholesterol.

A model of this type seems even less acceptable in the case of fowlpox virus, where only 16% of the total lipid is phospholipid; the overall lipid composition is predominantly neutral, with 23% triglyceride, 16.8% squalene, and 17.6% cholesteryl esters (White *et al.*, 1968). The biological significance of this unusual pattern of lipid has not been elucidated.

6. *Leukoviruses*

Rao *et al.* (1966) analyzed the lipids of avain myeloblastosis virus, finding that total lipid made up 35% of the viral mass, comprising 21.3% phospholipid, 11.7% cholesterol, and only 1.8% "neutral fat." These authors also described the manner of assembly of the virion as seen in the electron microscope: barely discernible electron-dense material, probably either viral RNA or ribonucleoprotein, accumulates close to the plasma membrane and forms a segment of a sphere, enclosing material of even lower electron density; a second shell (referred to in the mature particle as the "inner membrane"), continuous with the cytoplasm, forms around the first, and during budding both these structures are enclosed in an external envelope continuous with the plasma membrane. Previous studies (Bonar and Beard, 1959) had established the dry weight of the virion as 7.5×10^{-16} gm, giving a lipid mass of 2.6×10^{-16} gm per virion; the mean overall diameter of the particle is 1000 Å. Using these figures, taking the average molecular weight of lipid to be 750 and the cross-sectional area per lipid molecule to be 88 Å^2, and assuming that the lipid is contained in a bimolecular lipid leaflet at the periphery of the virus particle, Rao *et al.* (1966) calculate that only 30% of the viral lipid can be accommodated in a shell of this size. It therefore appears that a large part of the lipid must be in the interior of the particle, possibly in the "inner membrane." This calculation is open to question since it takes much too high a value for the area per molecule, and seems to have con-

fused the average molecular weight of all lipids with that of phospholipid; however a recalculation using the lipid area figures of Levine and Wilkins (1971) shows there are 1.45×10^5 molecules of cholesterol and 1.34×10^5 molecules of phospholipid per virion and these occupy an area of 5.67×10^6 Å^2 in the form of a bilayer. The area of bilayer forming a spherical shell 1000 Å in external diameter is 2.8×10^6 Å^2, indicating that there is twice as much lipid in the virion as can be accommodated in this single spherical shell. The lipid shell of the outer membrane may in fact be even smaller, since this calculation does not take into account the size of the external projections of the envelope.

7. *Nuclear Polyhedrosis Virus of Lymantria dispar*

Chemical analysis of this insect virus indicated a lipid content of only 1.3%; however this low value may depend on the virus isolation technique or the lipid extraction procedure used. Alternatively, the apparent viral content may be low because of multigenomic particles or it may be low if not all capsids are enveloped.

Harrap (1969) suggests that the envelope of nuclear polyhedrosis virus is composed of an outer layer, presumably of protein, showing no regularity of structure, covering a layer of hexagonally packed subunits 200 Å in diameter beneath which is a bilayer of lipid 40 Å thick. The nucleic acid is complexed with protein to form a rodlike structure; this is encased by an inner protein shell composed of structural subunits 20–25 Å across packed in a rhomboidal lattice. A tentative model of this complex virus is presented in Fig. 27. Further confirmation of the envelope structure will have to await more detailed studies of purified virus from insect tissue culture.

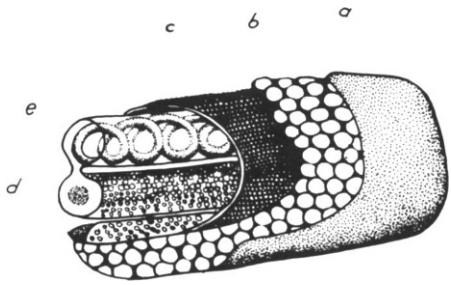

FIG. 27. Schematic representation of an enveloped bundle of *Lymantria dispar* nuclear polyhedrosis virus. a. surface layer; b. peplomers; c. virus membrane; d. virus capsid; e. coiled internal component. a, b, and c constitute the viral envelope; d and e represent the nucleocapsid. (Courtesy of Dr. K. A. Harrap.)

C. Factors Influencing the Composition of Viral Lipids

In concluding this section, we may list some of the factors most likely to influence the composition of viral lipids; some of these have already been covered in greater detail above.

1. *Envelope Structural Polypeptides*

Irrespective of the type of model favored for the structure of the envelope of a lipid-containing virus (i.e., basically a lipid-bilayer shell or a lipoprotein-complex structure), some lateral interaction of lipid and protein takes place. The closeness of packing of lipid acyl chains with nonpolar side chain regions of protein will be determined largely by the sequence and conformation of the amino acids in these regions, and this interaction will in turn influence the nature and degree of cohesion between other neighboring lipid molecules. The great importance of unsaturated chains in promoting or preventing close packing of lipid molecules in membranes has been stressed by Vandenheuvel (1963).

2. *Alterations in Host Cell Catabolic and Biosynthetic Pathways*

Host cell biosynthesis and catabolism of lipid during viral infection have not yet been extensively explored; however, compositional analyses of a variety of viruses and their host cell membranes show differences in lipid polar groups and precursors of cholesterol. Recent studies on virus-infected cells and uninfected controls reveal an uncoupling of phospholipid and neutral lipid biosynthesis which might account for some of the differences seen in viral lipids (see Section V,A).

3. *Environmental Factors*

Free fatty acids inoculated into embryonated eggs before infection with influenza virus (Blough and Tiffany, 1969) or added to tissue culture fluids (Klenk and Choppin, 1970a) were incorporated into the virion; differences in viral morphology and hemagglutinating ability were noted with branched-chain phytanic acid (Blough and Tiffany, 1969). Similarly, vitamin A alters the morphology of influenza virus from spherical to filamentous particles and this is accompanied by changes in polar lipids and acyl chains (Blough, 1963; Blough *et al.*, 1967). This points to the necessity, when comparing viruses and membranes, of using the same batches of serum and medium throughout a series of experiments.

4. *Virally-Coded Catabolic Enzymes*

The absence of detectable sialic acid in either glycolipids or glycoproteins of myxoviruses following release from the host cell, despite the demonstration of its presence in areas of the host surface membrane not involved in budding, has been discussed above. Klenk and Choppin (1970b) have suggested that viral neuraminidase is responsible for the removal of sialic acid, rather than a host enzyme, since vesicular stomatitis virus, grown in the same host but possessing no viral neuraminidase, has the same hematoside (neuraminosyl-galactosyl-glucosyl-ceramide) as its host cell plasma membrane.

5. *Physiological State of the Host Cell*

In cells where sterol metabolism has been altered by long passage in tissue culture, some functions such as the ability to desaturate desmosterol have been lost; this results in the replacement of cholesterol by desmosterol in vesicular stomatitis virus as well as in the mouse L cell in which it was grown (Bates and Rothblat, 1972). The biological activity and morphology of the virion were in this case unaffected. A virion may also mimic the acyl chain composition of the host cell in those cultured cells which have lost the ability to chain elongate or desaturate fatty acids. The flexibility of higher microorganisms, such as *Mycoplasma* strain Y, is striking in this respect; 97% of all its fatty acyl chains may be replaced with elaidic (*trans*-oleic) acid with little effect on growth (Rodwell, 1968).

6. *Multiplicity of Infection*

Infection of chick embryo fibroblasts with 10–30 plaque-forming units (pfu) per cell of influenza virus causes an inhibition of cell lipid metabolism at 4 hours after infection, whereas infection with 3–5 pfu per cell delays the onset of this inhibition until 7 hours after infection (Blough and Weinstein, 1972). In addition, infection at multiplicities sufficient to produce incomplete virus (von Magnus, 1951) causes an immediate alteration in host cell phospholipid and neutral lipid metabolism.

VII. Functional Role of Lipids

Despite the considerable accumulation of knowledge on the nature, amount, and composition of lipids of viruses and their hosts, it must be admitted that the role played by these components is still largely unknown. Several possible functions for lipids are suggested below; the

list is not considered to be exhaustive, and much more work obviously remains to be done before these suggestions can be conclusively proved or disproved.

A. Molecular Architecture of the Envelope

As mentioned earlier, viruses which possess lipid may do so in order to preserve or provide configurational changes in those polypeptides which are required for assembly of the envelope. Loss of viral activity on treatment with various agents, e.g., organic solvents (Andrewes and Horstmann, 1949), bile salts (Smith, 1939), or phospholipase C (Mizutani and Mizutani, 1964; Simpson and Hauser, 1966; Friedman and Pastan, 1969; Schulze, 1970), is brought about by removal of lipid and subsequent breakup of the envelope. The presence of a coherent envelope is in many cases necessary for normal levels of infectivity of the virus, presumably because the envelope carries the specific groups responsible for attachment to the host cell surface or for triggering the engulfment of the particle.

B. Transport

From experiments on the release of polypeptides by detergent, and the reaggregation of the envelope subunits on the removal of detergent, some at least of the viral envelope proteins have been shown to have extensive hydrophobic regions on their surfaces (Laver and Valentine, 1969). It seems unlikely that these regions will only be exposed by a configurational change after the proteins have reached the assembly site of the virus. Lipids could therefore fulfill a critical role in assisting the transport of these molecules from the site of synthesis to the site of assembly by shielding the hydrophobic portion. This would entail the formation of a partial micelle about these regions of the polypeptides; the lipids involved might not in all cases be those subsequently incorporated into the viral envelope, since the lipids conferring structural integrity on the envelope may requre properties other than solely that of solubilizing the polypeptide. This transport role could apply to both "template" type assembly and to *de novo* synthesis of the envelope.

C. Assembly

In Section VI a model of envelope assembly was discussed in which external envelope proteins were fed through the lipid region of the template membrane, probably mediated by a local phase transition of mem-

brane lipids to a micellar form. Once assembled, the viral lipids confer the unique plasticity seen in enveloped viruses, and the surface projections would be supported in a matrix which permitted sufficient lateral mobility for electrostatic repulsive forces between adjacent projections to maintain a uniform spacing (Hoyle, 1968; Schulze, 1972). This would be especially true if the lipid-bilayer model proves to be correct. In either a micellar or a lamellar configuration, polar groups of lipid would be on the exterior and interior of the lipid region of the envelope and could help to maintain the spacing of the projections as well as binding the nucleocapsid to the budding envelope (Schulze, 1972). Recent studies have shown that monovalent hemagglutinin solubilized from Newcastle disease virus, which had lost its ability to agglutinate erythrocytes, regained this biological activity when mixed with phosphatidylethanolamine, indicating reforming of a structure with functional groups directed outward (Iinuma *et al.*, 1971). Studies on the reconstitution of envelope subunits and surface projections have important biological implications, and this field merits further investigation. A report that lipid restores the infectivity of RNA from Newcastle disease virus (Dhar *et al.*, 1963) has never been verified.

D. Release

Naked herpesvirus capsids closely resemble non-lipid-containing viruses such as adenovirus or polyoma virus, which are also assembled in the nucleus but which show full infectivity without any envelope. Both naked and enveloped herpesvirus particles are found in infected cells, but only enveloped particles are found in extracellular fluids (Roizman *et al.*, 1968). It is not altogether clear whether only enveloped particles are infectious (Siegert and Falke, 1966; Watson *et al.*, 1964). The question may therefore be raised of why herpesvirus needs an envelope. During budding of the herpesvirus core through the inner nuclear membrane, virally coded polypeptides are incorporated into the membrane, which then forms the envelope. Morgan *et al.* (1959) have indicated that the enveloped virus is enclosed in vacuoles which are released from the cell by a process of "reverse phagocytosis." This is questioned by Schwartz and Roizman (1969), who found evidence for a network of branching tubules established in the cytoplasm of productively infected HEp-2 cells following infection with herpes simplex virus. The membranes lining these ducts were continuous at one end with the outer nuclear envelope, and at the other end with the cell surface membrane, thus offering a means of egress from the cell for the virus without being

exposed to the degradative or uncoating enzymes of the cytoplasm. The observations of Darlington and Moss (1968) are consistent with this hypothesis, but as yet no other confirmation of this mode of release has been reported. Schwartz and Roizman (1969) also suggest that the vacuoles containing virus seen by other workers are in fact cross sections of the tubules.

Two possible reasons for the acquisition of the envelope can be formulated. (1) The virus can only penetrate the inner nuclear membrane by this means, but having done so can escape from the cell by direct opening of the cytoplasmic tubules to the medium at the plasma membrane. (2) The envelope acts as a carrier for virally coded polypeptides which play some active part in the release process, such as by inducing vacuole formation in the cytoplasm, and expulsion of virus from the cell within vacuoles.

E. Fusion

Herpesvirus and some paramyxoviruses (e.g., Sendai and Newcastle disease virus) are capable of causing cell fusion, one form of which—"fusion from without" (Bratt and Gallaher, 1969)—arises from direct contact between the virus particles and the surface membranes of the cells. Monoacylated phospholipids such as lysophosphatidylcholine have been shown to be capable of producing membrane fusion (Howell and Lucy, 1969; Poole *et al.*, 1970), and it has been suggested that similar monoacylated phospholipids are responsible for cell fusion and hemolysis during viral infection (Rubin, 1967), and that the virus may carry a lipoid "fusion factor"; however, such lipids do not accumulate in cells infected with herpesvirus or Newcastle disease virus (Gallaher *et al.*, 1973) and do not make up significant amounts of the lipids of Newcastle disease or Sendai viruses (Blough and Lawson, 1968). Lysophosphatidylcholine has been shown not to be important in fusion of cells by Sendai (Falke *et al.*, 1967) or SV5 viruses (Elsbach *et al.*, 1969). In addition, exogenous monoacylated phospholipid inactivates the "fusion factor," suggesting that an intact viral envelope is necessary for fusion to occur (Kohn and Klibansky, 1967; Hosaka, 1970). While glycolipids have also been implicated as having a function in cell fusion (Blough and Lawson, 1968; Klenk and Choppin, 1970b), there is no supporting evidence for this at present. The possibility that the virus contains degradative enzymes, e.g., a sphingomyelinase (Moberly *et al.*, 1958; Hosaka, 1958), has not been verified by isolating such an enzyme from the virus and doing *in vitro* studies.

F. Penetration

In those viruses which fuse to cell membranes, lipids probably facilitate entry of the nucleocapsid (Morgan and Rose, 1968) by producing phase transitions at the site of entry (Bächi and Howe, 1972). This might involve the same type of mechanism as in virus-mediated cell fusion, but may involve a different "fusion factor" in the case of viruses which do not cause true fusion.

VIII. Conclusion

In the preceding pages we have attempted to indicate the large number of classes of lipid-containing viruses, their manner of assembly, and their lipid compositions, where this has been determined. It can be seen from Table II that the lipid analyses thus far available are few and incomplete, and do not in most cases cover all the common hosts for a given virus. It seems that we shall not be able to form a true picture of the function of lipids in the formation and structure of viruses until we have more consistent information on a number of fronts:

(1) More thorough compositional analyses are needed for many viruses; where extensive analysis has been carried out, confirmation is required from other workers in the field, including virus grown in different hosts.

(2) A thorough investigation is required on the vexing question of whether or not the lipid composition of template viruses is the same as that of their parent membranes; this involves more detailed knowledge of the changes in host cell lipid metabolism induced by viral infection, and hence of the varieties and relative amounts of different classes of lipids available during assembly. The major difficulty appears to be obtaining samples of the lipids of the parent membrane from the infected cell, without also including those regions of membrane at which the virus is budding. If there is appreciable lateral mobility of lipids in the membrane at this time, this may not prove possible.

(3) Further examination of virus particles by physical techniques such as X-ray diffraction and spin resonance spectroscopy is needed to establish the structure of the particle, and hence the degree of interaction of structural proteins and lipid. Isolation of structural proteins and investigation of their lipid-binding properties may also give valuable information.

In view of the wide variation in the proportion of lipid in different viruses, it seems unlikely that any unified structural pattern will emerge

which is capable of dealing with the virion as a whole. It is however possible that a common minimal structure will be discerned, upon which variations can be constructed. This may well be the lipid bilayer in association with an underlying layer of structural protein; where insufficient lipid is present in the virion to provide a complete bilayer shell, penetrating regions of protein can be expected, as suggested in structural models mentioned in Section VI. When too much lipid is present, the excess either may be accommodated in ill-defined association with the internal component of the virion or may form a concentric structure within the basic envelope.

ACKNOWLEDGMENTS

The senior author would like to thank Dr. Charles Pasternak, University of Oxford, and Dr. Ossi Renkonen, University of Helsinki, for the generous hospitality of their laboratories during the writing of this review. This review would also not have been possible without the enthusiastic and stimulating work of our colleagues Drs. W. R. Gallaher and D. B. Weinstein. This work was supported in part by the Commission on Influenza, Armed Forces Epidemiological Board, through the U. S. Army Medical Research and Development Command, Department of the Army (Research Contract No. DADA-17-67-C-7128), and by a Senior Post-Doctoral Fellowship of the National Multiple Sclerosis Society to H. A. Blough.

We should also like to thank Drs. P. W. Choppin, A. Granoff, K. A. Harrap, C. Howe and B. Roizman for permission to use unpublished data for inclusion in this review, Drs. M. S. Bretscher, W. R. Gallaher, K. A. Harrap, J. B. Marsh, C. A. Pasternak and H. Pollard for their stimulating discussions and criticism, and Dr. W. R. Gallaher in particular for his critical reading of the manuscript and many helpful suggestions. In addition, we are grateful to Ms. C. Court for the excellent technical illustrations.

References

Acheson, N. H., and Tamm, I. (1967). *Virology* **32**, 128.

Ahmed, M. E., Black, L. M., Perkins, E. G., Walker, B. L., and Kummerow, F. A. (1964). *Biochem. Biophys. Res. Commun.* **17**, 103.

Almeida, J. D., and Waterson, A. P. (1967). *J. Gen. Microbiol.* **46**, 107.

Almeida, J. D., and Waterson, A. P. (1970). *In* "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), p. 27. Academic Press, New York.

Andrewes, C. H., and Horstmann, D. M. (1949). *J. Gen. Microbiol.* **3**, 290.

Apostolov, K., and Flewett, T. H. (1969). *J. Gen. Virol.* **4**, 365.

Archetti, I., Jemolo, A., and Steve-Bocciarelli, D. (1967). *Arch. Gesamte Virusforsch.* **20**, 133.

Armbruster, O., and Beiss, U. (1958). *Z. Naturforsch. B* **13**, 75.

Asher, Y., Heller, M., and Becker, Y. (1969). *J. Gen. Virol.* **4**, 65.

Bächi, T., and Howe, C. (1972). Submitted for publication.

Bächi, T., Gerhard, W., Lindenmann, J., and Mühlethaler, K. (1969). *J. Virol.* **4**, 769.

Bailey, J. M., Gey, G. O., and Gey, M. K. (1964). *Proc. Soc. Exp. Biol. Med.* **113**, 747.
Bates, S. R., and Rothblat, G. H. (1972). *J. Virol.* **9**, 883.
Baumann, W. J., and Mangold, H. K. (1966). *Biochim. Biophys. Acta* **116**, 570.
Baumann, W. J., Takahashi, T., Mangold, H. K., and Schmid, H. H. O. (1970). *Biochim. Biophys. Acta* **202**, 468.
Becker, W. B., McIntosh, K., Dees, J. H., and Chanock, R. M. (1967). *J. Virol.* **1**, 1019.
Ben-Porat, T., and Kaplan, A. S. (1971). *Virology* **45**, 252.
Ben-Porat, T., and Kaplan, A. S. (1972). *Nature (London)* **235**, 165.
Bergold, G. H., and Wellington, E. F. (1954). *J. Bacteriol.* **67**, 210.
Berkaloff, A., and Thiéry, J. P. (1963). *J. Microsc. (Paris)* **2**, 583.
Best, R. J. (1968). *Advan. Virus Res.* **13**, 65.
Blough, H. A. (1963). *Virology* **19**, 349.
Blough, H. A. (1964). *Cell. Biol. Myxovirus Infect., Ciba Found. Symp., 1964* p. 120.
Blough, H. A. (1968). *Wistar Inst. Symp. Monogr.* **8**, 55.
Blough, H. A. (1969). *Bull. WHO* **41**, 487.
Blough, H. A. (1971a). *J. Gen. Virol.* **12**, 317.
Blough, H. A. (1971b). *Proc. Int. Congr. Virol., 2nd, 1971* p. 133.
Blough, H. A., and Lawson, D. E. M. (1968). *Virology* **36**, 286.
Blough, H. A., and Merlie, J. P. (1970). *Virology* **40**, 685.
Blough, H. A., and Smith, W. R. (1973). *J. Gen. Virol.* (in press).
Blough, H. A., and Tiffany, J. M. (1969). *Proc. Nat. Acad. Sci. U. S.* **62**, 242.
Blough, H. A., and Weinstein, D. B. (1973). *In* "Biology of the Fibroblast" Sigrid Jusélius Fd. Symp. (E. Kulonen and J. Pikkarainen, eds.). Academic Press, London. (in press).
Blough, H. A., Weinstein, D. B., Lawson, D. E. M., and Kodicek, E. (1967). *Virology* **33**, 459.
Blough, H. A., Merlie, J. P., and Tiffany, J. M. (1969). *Biochem. Biophys. Res. Commun.* **34**, 831.
Blough, H. A., Aaslestad, H. G., and Tiffany, J. M. (1973). In press.
Bonar, R. A., and Beard, J. W. (1959). *J. Nat. Cancer Inst.* **23**, 183.
Bonissol, C., and Sisman, J. (1968). *C. R. Acad. Sci., Ser. D* **267**, 1337.
Brakke, M. K. (1967). *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. 2, p. 119. Academic Press, New York.
Bratt, M. A., and Gallaher, W. R. (1969). *Proc. Nat. Acad. Sci. U. S.* **64**, 536.
Braunstein, S. N., and Franklin, R. M. (1971). *Virology* **43**, 685.
Bretscher, M. S. (1971). *J. Mol. Biol.* **58**, 775.
Burge, B. W., and Huang, A. S. (1970). *J. Virol.* **6**, 176.
Burge, B. W., and Strauss, J. H. (1970). *J. Mol. Biol.* **42**, 452.
Burton, R. M. (1963). *J. Neurochem.* **10**, 503.
Caliguiri, L. A., Klenk, H.-D., and Choppin, P. W. (1969). *Virology* **39**, 460.
Carver, D. H., and Marcus, P. I. (1968). *Abstr. 68th Annu. Meet. Amer. Soc. Microbiol.* V216.
Caspar, D. L. D., and Klug, A. (1962). *Cold Spring Harbor Symp. Quant. Biol.* **27**, 1.
Caspar, D. L. D., Dulbecco, R., Klug, A., Lwoff, A., Stoker, M. G. P., Tournier, P., and Wildy, P. (1962). *Cold Spring Harbor Symp. Quant. Biol.* **27**, 49.
Chen, C., Compans, R. W., and Choppin, P. W. (1971). *J. Gen. Virol.* **11**, 53.
Clamp, J. R., Bhatti, T., and Chambers, R. E. (1971). *Methods Biochem. Anal.* **19**, 229.
Compans, R. W. (1971). *Nature (London), New Biol.* **229**, 114.
Compans, R. W., and Dimmock, N. J. (1969). *Virology* **39**, 499.
Compans, R. W., Holmes, K. V., Dales, S., and Choppin, P. W. (1966). *Virology* **30**, 411.

Compans, R. W., Klenk, H.-D., Caliguiri, L. A., and Choppin, P. W. (1970). *Virology* **42**, 880.
Cota-Robles, E., Espejo, R. T., and Haywood, P. W. (1968). *J. Virol.* **2**, 56.
Craven, D. A., and Gehrke, C. W. (1968). *J. Chromatogr.* **37**, 414.
Crick, J., and Brown, F. (1970). *In* "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), p. 133. Academic Press, New York.
Dahlberg, J. E., and Franklin, R. M. (1970). *Virology* **42**, 1073.
Dales, S. (1963). *J. Cell Biol.* **18**, 51.
Dales, S., and Mosbach, E. H. (1968). *Virology* **35**, 564.
Dales, S., and Siminovitch, L. (1961). *J. Biophys. Biochem. Cytol.* **10**, 475.
Darlington, R. W., and Moss, L. H. (1968). *J. Virol.* **2**, 48.
Darlington, R. W., Granoff, A., and Breeze, D. C. (1966). *Virology* **29**, 149.
David, A. E. (1971). *Virology* **46**, 711.
De Bernard, L. (1958). *Bull. Soc. Chim. Biol.* **40**, 161.
DePury, G. G., and Collins, F. D. (1966). *Chem. Phys. Lipids* **1**, 1.
de Thé, G., and O'Connor, T. E. (1966). *Virology* **28**, 713.
Devauchelle, G., Bergoin, M., and Vago, C. (1970). *C. R. Acad. Sci., Ser. D* **271**, 1138.
Devauchelle, G., Bergoin, M., and Vago, C. (1971). *J. Ultrastruct. Res.* **37**, 301.
Dhar, M. M., Babbar, O. P., and Choud'hury, B. L. (1963). *Experientia* **12**, 100.
Dittmer, J. C., and Wells, M. A. (1969). *In* "Methods in Enzymology" (J. M. Lowenstein, ed.), Vol. 14, p. 483. Academic Press, New York.
Dourmashkin, R. R., and Simons, P. J. (1961). *J. Ultrastruct. Res.* **5**, 505.
Eibl, H., Hill, E. E., and Lands, W. E. M. (1970). *Eur. J. Biochem.* **9**, 250.
Elsbach, P., Holmes, K. V., and Choppin, P. W. (1969). *Proc. Soc. Exp. Biol. Med.* **130**, 903.
Engelman, D. M. (1969). *Nature (London)* **223**, 1279.
Espejo, R. T., and Canelo, E. S. (1968a). *Virology* **34**, 738.
Espejo, R. T., and Canelo, E. S. (1968b). *J. Virol.* **2**, 1235.
Eylar, E. H. (1966). *J. Theor. Biol.* **10**, 89.
Falke, D., Schiefer, H.-G., and Stoffel, W. (1967). *Z. Naturforsch. B* **22**, 1360.
Fenner, F. (1968). "Biology of Animal Viruses," Vol. 1, Chapters 1–3. Academic Press, New York.
Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). *J. Biol. Chem.* **226**, 497.
Franklin, R. M. (1962). *Progr. Med. Virol.* **4**, 1.
Freeman, C. P., and West, D. (1966). *J. Lipid Res.* **7**, 324.
Friedman, R. M., and Pastan, I. (1969). *J. Mol. Biol.* **40**, 107.
Frisch-Niggemeyer, W., and Hoyle, L. (1956). *J. Hyg.* **54**, 201.
Frommhagen, L. H., Knight, C. A., and Freeman, N. K. (1959). *Virology* **8**, 176.
Furukawa, T., Plotkin, S. A., Sedwick, W. D., and Profeta, M. L. (1967). *Proc. Soc. Exp. Biol. Med.* **126**, 745.
Fuscaldo, A. A., Aaslestad, H. G., and Hoffman, E. J. (1971). *J. Virol.* **7**, 233.
Gallaher, W. R., and Blough, H. A. (1972). *Abstr. 72nd Annu. Meet. Amer. Soc. Microbiol.* V179.
Gallaher, W. R., Weinstein, D. B., and Blough, H. A. (1973). Submitted for publication.
Gelderblom, H., Bauer, H., and Graf, T. (1972). *Virology* **47**, 416.
Gourley, R. N. (1971). *J. Gen. Virol.* **12**, 65.
Granoff, A. (1969). *Curr. Top. Microbiol. Immunol.* **50**, 107.
Green, D. E., and Perdue, J. F. (1966). *Proc. Nat. Acad. Sci. U. S.* **55**, 1295.
Grimes, W. J., and Burge, B. W. (1971). *J. Virol.* **7**, 309.
Grimley, P. M., and Friedman, R. M. (1970). *Exp. Mol. Pathol.* **12**, 1.

Hakomori, S.-I. (1970). *Proc. Nat. Acad. Sci. U. S.* **67**, 1741.
Hakomori, S.-I., Saito, T., and Vogt, P. K. (1971). *Virology* **44**, 609.
Harrap, K. A. (1969). Ph.D. Dissertation, Oxford University.
Harrison, S. C., Caspar, D. L. D., Camerini-Otero, R. D., and Franklin, R. M. (1971a). *Nature (London), New Biol.* **229**, 197.
Harrison, S. C., David, A. E., Jumblatt, J., and Darnell, J. E. (1971b). *J. Mol. Biol.* **60**, 523.
Haslam, E. A., Cheyne, I. A., and White, D. O. (1969). *Virology* **39**, 118.
Haslam, E. A., Hampson, A. W., Egan, J. A., and White, D. O. (1970a). *Virology* **42**, 555.
Haslam, E. A., Hampson, A. W., Radiskevics, I., and White, D. O. (1970b). *Virology* **42**, 566.
Heine, U., Ablashi, D. V., and Armstrong, G. R. (1971). *Cancer Res.* **31**, 1019.
Heine, J. W., Spear, P. J., and Roizman, B. (1972). *J. Virol.* **9**, 431.
Hess, H. H., and Rolde, E. (1964). *J. Biol. Chem.* **239**, 3215.
Heydrick, F. P., Comer, J. F., and Wachter, R. F. (1970). *J. Virol.* **7**, 642.
Holland, J. J., and Kiehn, E. D. (1970). *Science* **167**, 202.
Holmes, I. H., and Warburton, M. F. (1967). *Lancet* **2**, 1233.
Horne, R. W., Waterson, A. P., Wildy, P., and Farnham, A. E. (1960). *Virology* **11**, 79.
Hosaka, Y. (1958). *Biken J.* **1**, 90.
Hosaka, Y. (1970). *In* "Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), p. 684. Academic Press, New York.
Hotz, G., and Schäfer, W. (1955). *Z. Naturforsch. B* **10**, 1.
Howatson, A. F. (1970). *Advan. Virus Res.* **16**, 196.
Howell, J. I., and Lucy, J. A. (1969). *FEBS Lett.* **4**, 147.
Hoyle, L. (1950). *J. Hyg.* **48**, 277.
Hoyle, L. (1952). *J. Hyg.* **50**, 229.
Hoyle, L. (1968). *Virol. Monogr.* **4**, 1.
Hoyle, L., Jolles, B., and Mitchell, R. G. (1954). *J. Hyg.* **52**, 119.
Hummeler, K., Koprowski, H., and Wiktor, T. J. (1967). *J. Virol.* **1**, 152.
Ie, T. S. (1964). *Neth. J. Plant Pathol.* **70**, 114.
Iinuma, M., Yoshida, T., Nagai, Y., Maeno, K., Matsumoto, T., and Hoshino, M. (1971). *Virology* **46**, 663.
Isacson, P., and Koch, A. E. (1965). *Virology* **27**, 129.
Ji, T. H., and Benson, A. A. (1968). *Biochim. Biophys. Acta* **150**, 686.
Johnson, M., and Mora, P. T. (1967). *Virology* **31**, 230.
Kaplan, A. S. (1957). *Virology* **4**, 435.
Kaplan, A. S. (1969). *Virol. Monogr.* **5**, 1.
Kates, M., Allison, A. C., Tyrrell, D. A. J., and James, A. T. (1961). *Biochim. Biophys. Acta* **52**, 455.
Kavanau, J. L. (1963). *Nature (London)* **198**, 525.
Kendal, A. P., Biddle, F., and Belyavin, G. (1968). *Biochim. Biophys. Acta* **165**, 419.
Kendal, A. P., Apostolov, K., and Belyavin, G. (1969). *J. Gen. Virol.* **5**, 141.
Kilbourne, E. D. (1963). *Progr. Med. Virol.* **5**, 79.
Kitajima, E. W. (1965). *Virology* **26**, 89.
Kitajima, E. W., and Costa, A. S. (1966). *Virology* **29**, 523.
Klenk, H.-D., and Choppin, P. W. (1969a). *Virology* **37**, 155.
Klenk, H.-D., and Choppin, P. W. (1969b). *Virology* **38**, 255.
Klenk, H.-D., and Choppin, P. W. (1970a). *Virology* **40**, 939.

Klenk, H.-D., and Choppin, P. W. (1970b). *Proc. Nat. Acad. Sci. U. S.* **66**, 57.
Klenk, H.-D., and Choppin, P. W. (1971). *J. Virol.* **7**, 416.
Klenk, H.-D., Caliguiri, L. A., and Choppin, P. W. (1970a). *Virology* **42**, 473.
Klenk, H.-D., Compans, R. W., and Choppin, P. W. (1970b). *Virology* **42**, 1158.
Klenk, H.-D., Rott, R., and Becht, H. (1972). *Virology* **47**, 579.
Kohn, A., and Klibansky, C. (1967). *Virology* **31**, 385.
Kritchevsky, D., and Shapiro, I. L. (1967). *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. 3, p. 77. Academic Press, New York.
Kuwert, E., Böhme, U., Lichfeld, K. G., and Böhme, W. (1972). *Zentralbl. Bakteriol., Parasitenk., Infektionskr., Hyg., Abt. 1: Orig. A* **219**, 39.
Lands, W. E. M. (1965). *Annu. Rev. Biochem.* **34**, 313.
Landsberger, F. R., Lenard, J., Paxton, J., and Compans, R. W. (1971). *Proc. Nat. Acad. Sci. U. S.* **68**, 2579.
Laver, W. G. (1964). *J. Mol. Biol.* **9**, 109.
Laver, W. G., and Valentine, R. C. (1969). *Virology* **38**, 105.
Ledeen, R. (1966). *J. Amer. Oil Chem. Soc.* **43**, 57.
Lerner, R. A., and Hodge, L. D. (1969). *Proc. Nat. Acad. Sci. U. S.* **64**, 544.
Lesslauer, W., Cain, J. E., and Blasie, J. K. (1972). *Proc. Nat. Acad. Sci. U. S.* **69**, 1499.
Levine, Y. K., and Wilkins, M. H. F. (1971). *Nature (London) New Biol.* **230**, 69.
Lucy, J. A. (1964). *J. Theor. Biol.* **7**, 360.
Lwoff, A., Horne, R. W., and Tournier, P. (1962). *Cold Spring Harbor Symp. Quant. Biol.* **27**, 51.
Lynen, F., Oesterholt, D., Schweitzer, E., and Willeke, K. (1968). "Cellular Compartmentalization and Control of Fatty Acid Metabolism," Fed. Eur. Biochem. Soc. Symp., p. 1, Universitetsforlaget, Oslo.
McCombs, R. M., Brunschwig, J. P., and Rawls, W. E. (1968). *Exp. Mol. Pathol.* **9**, 27.
McSharry, J. J., and Wagner, R. R. (1971). *J. Virol.* **7**, 59.
Marinetti, G. V., ed. (1967). "Lipid Chromatographic Analysis," Vol. 1. Dekker, New York.
Marsh, J. B., and Weinstein, D. B. (1966). *J. Lipid Res.* **7**, 574.
Matsumoto, S. (1970). *Advan. Virus Res.* **16**, 257.
Miller, H. K., and Schlesinger, R. W. (1955). *J. Immunol.* **75**, 155.
Milne, R. G. (1970). *J. Gen. Virol.* **6**, 267.
Mitchiner, M. B. (1969). *J. Gen. Virol.* **5**, 211.
Mizutani, H. (1963). *Nature (London)* **198**, 109.
Mizutani, H., and Mizutani, H. (1964). *Nature (London)* **204**, 781.
Moberly, M. L., Marinetti, G. V., Witter, R. F., and Morgan, H. R. (1958). *J. Exp. Med.* **107**, 87.
Morgan, C., and Rose, H. M. (1968). *J. Virol.* **2**, 925.
Morgan, C., Rose, H. M., and Moore, D. H. (1956). *J. Exp. Med.* **104**, 171.
Morgan, C., Rose, H. M., Holden, M., and Jones, E. P. (1959). *J. Exp. Med.* **110**, 643.
Morgan, C., Howe, C., and Rose, H. M. (1961). *J. Exp. Med.* **113**, 219.
Murphy, F. A., Halonen, P. E., and Harrison, A. K. (1968). *J. Virol.* **2**, 1223.
Murphy, F. A., Webb, P. A., Johnson, K. M., Whitfield, S. G., and Chappell, W. A. (1970). *J. Virol.* **6**, 507.
Nakai, T., and Howatson, A. F. (1968). *Virology* **35**, 268.
Nermut, M. V., (1970). *Acta Virol. (Prague), Engl. Ed.* **14**, 185.
Nermut, M. V., and Frank, H. (1971). *J. Gen. Virol.* **10**, 37.

Neurath, A. R., Vernon, S. K., Dobkin, M. B., and Rubin, B. A. (1972). *J. Gen. Virol.* **14**, 33.
Norrby, E. (1969). *Virol. Monogr.* **7**, 115.
Pasternak, C. A., and Bergeron, J. J. M. (1970). *Biochem. J.* **119**, 473.
Peters, D., and Müller, G. (1963). *Virology* **21**, 266.
Peters, D., Müller, G., and Geister, R. (1963). *Arch. Gesamte Virusforsch.* **13**, 435.
Pfefferkorn, E. R., and Hunter, H. S. (1963a). *Virology* **20**, 433.
Pfefferkorn, E. R., and Hunter, H. S. (1963b). *Virology* **20**, 446.
Plagemann, P. G. W. (1971). *J. Lipid Res.* **12**, 715.
Pollard, H., Scanu, A. M., and Taylor, E. W. (1969). *Proc. Nat. Acad. Sci. U. S.* **64**, 304.
Polson, A., and Russell, B. (1967). *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. 2, p. 391. Academic Press, New York.
Pons, M., and Hirst, G. K. (1969). *Virology* **38**, 68.
Poole, A. R., Howell, J. I., and Lucy, J. A. (1970). *Nature (London)* **227**, 810.
Poste, G. (1970). *Advan. Virus Res.* **16**, 303.
Quigley, J. P., Rifkin, D. B., and Reich, E. (1971). *Virology* **46**, 106.
Rao, P. A., Bonar, R. A., and Beard, J. W. (1966). *Exp. Mol. Pathol.* **5**, 374.
Reimer, C. B., Baker, R. S., Newlin, T. E., and Havens, M. L. (1966). *Science* **152**, 1379.
Renkonen, O. (1967). *Advan. Lipid Res.* **5**, 329.
Renkonen, O., Kääriäinen, L., Simons, K., and Gahmberg, C. G. (1971). *Virology* **46**, 318.
Římen, J., Korb, J., and Michlová, A. (1972). "Virus-Cell Interactions and Viral Antimetabolites," *Fed. Eur. Biochem. Soc. Symp.* (D. Shugar, ed.). **22**, 99.
Robinson, W. S., and Duesberg, P. M. (1968). *In* "Molecular Basis of Virology" (H. Fraenkel-Conrat, ed.), p. 255. Van Nostrand-Reinhold, Princeton, New Jersey.
Rodwell, A. (1968). *Science* **160**, 1350.
Roizman, B., Spring, S. B., and Schwartz, J. (1968). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **28**, 1890.
Rott, R., Drzeniek, R., Saber, M. S., and Reichert, E. (1966). *Arch. Gesamte Virusforsch.* **19**, 273.
Rubin, H. (1967). *In* "The Specificity of Cell Surfaces" (B. D. Davis and L. Warren, eds.), p. 181. Prentice-Hall, Englewood Cliffs, New Jersey.
Scheele, C. M., and Pfefferkorn, E. R. (1969). *J. Virol.* **3**, 369.
Schlesinger, M. J., Schlesinger, S., and Burge, B. W. (1972). *Virology* **47**, 539.
Schulze, I. T. (1970). *Virology* **42**, 890.
Schulze, I. T. (1972). *Virology* **47**, 181.
Schwartz, J., and Roizman, B. (1969). *Virology* **38**, 42.
Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971). *Biochem. Biophys. Res. Commun.* **44**, 390.
Siegert, R. S., and Falke, D. (1966). *Arch. Gesamte Virusforsch.* **19**, 230.
Simpson, R. W., and Hauser, R. E. (1966). *Virology* **30**, 684.
Skehel, J. J. (1971). *J. Gen. Virol.* **11**, 103.
Skehel, J. J., and Schild, G. C. (1971). *Virology* **44**, 396.
Smith, K. M. (1967). "Insect Virology," Chapter 2. Academic Press, New York.
Smith, W. (1939). *J. Pathol. Bacteriol.* **48**, 557.
Sokol, F., Stanček, D., and Koprowski, H. (1971). *J. Virol.* **7**, 241.
Spear, P. G., and Roizman, B. (1967). *Nature (London)* **214**, 713.

Spear, P. G., and Roizman, B. (1968). *Virology* **36**, 545.
Spear, P. G., and Roizman, B. (1972). *J. Virol.* **9**, 143.
Spring, S. B., and Roizman, B. (1967). *J. Virol.* **1**, 294.
Spring, S. B., and Roizman, B. (1968). *J. Virol.* **2**, 979.
Stoffel, W. (1971). *Annu. Rev. Biochem.* **40**, 57.
Stoffel, W., and Schiefer, H.-G. (1968). *Z. Phys. Chem.* **349**, 1017.
Stollar, V. (1969). *Virology* **39**, 426.
Tiffany, J. M., and Blough, H. A. (1969a). *Science* **163**, 573.
Tiffany, J. M., and Blough, H. A. (1969b). *Virology* **37**, 492.
Tiffany, J. M., and Blough, H. A. (1970a). *Proc. Nat. Acad. Sci. U. S.* **65**, 1105.
Tiffany, J. M., and Blough, H. A. (1970b). *Virology* **41**, 392.
Turkki, P. R., and Glenn, J. L. (1968). *Biochim. Biophys. Acta* **152**, 104.
Vaheri, A., Sedwick, W. D., and Plotkin, S. A. (1965). *Proc. Soc. Exp. Biol. Med.* **125**, 1086.
Vandenheuvel, F. A. (1963). *J. Amer. Oil Chem. Soc.* **40**, 455.
van Kammen, A., Henstra, S., and Ie, T. S. (1966). *Virology* **30**, 574.
von Magnus, P. (1951). *Acta Pathol. Microbiol. Scand.* **28**, 278.
Wagner, R. R., Schaitman, T. C., and Snyder, R. M. (1969). *J. Virol.* **3**, 395.
Waite, M. R. F., and Pfefferkorn, E. R. (1970). *J. Virol.* **6**, 637.
Warren, L. (1959). *J. Biol. Chem.* **234**, 1971.
Warren, L., Critchley, D., and MacPherson, I. (1972). *Nature (London)* **235**, 275.
Watson, D. H., Wildy, P., and Russell, W. C. (1964). *Virology* **24**, 523.
Wecker, E. (1957). *Z. Naturforsch. B* **12**, 208.
Weinstein, D. B., and Blough, H. A. (1973). Submitted for publication.
Weinstein, D. B., Marsh, J. B., Glick, M. C., and Warren, L. (1969). *J. Biol. Chem.* **244**, 4103.
Weinstein, D. B., Marsh, J. B., Glick, M. C., and Warren, L. (1970). *J. Biol. Chem.* **245**, 3928.
White, H. B., Powell, S. S., Gafford, L. G., and Randall, C. C. (1968). *J. Biol. Chem.* **243**, 4517.
Wilcox, W. C., and Ginsberg, H. S. (1963). *J. Exp. Med.* **118**, 295.
Wildy, P. (1971). *In* "Monographs in Virology" (J. L. Melnick, ed.), Vol. 5, p. 28. Karger, Basel.
Wirtz, K. W. A., and Zilversmit, D. B. (1969). *Biochim. Biophys. Acta* **193**, 105.
Woodson, B., and Joklik, W. K. (1965). *Proc. Nat. Acad. Sci. U S.* **54**, 946.
Zwartouw, H. T. (1964). *J. Gen. Microbiol.* **34**, 115.