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CHAPTER III

FUNCTIONS AND ALTERATIONS OF CELL MEMBRANES DURING ACTIVE VIRUS INFECTION

P. M. Grimley and A. Demsey

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I. Introduction

Molecular and macromolecular alterations in host cytomembranes occur as direct or secondary consequences of virus assault in animal cells, and active interactions with celluar membranes characterize at least some portion in the life cycle of nearly all animal viruses. These may include steps of virus uptake, nucleic acid replication, protein synthesis, macromolecular assembly, virion maturation, and emergence or release. Indeed, the success of animal virus infections at a cellular level can be largely attributed to an efficient harnessing of the versatile molecular and biophysical properties of host membrane systems. Even the central process of membrane biogenesis by the host cell may become indentured to a general virus strategy of competitively dominating or subverting the preexisting regulatory and synthetic machinery (Mosser *et al.*, 1972b; Blough and Tiffany, 1975).

In addition to direct participation in virus developmental processes, the membrane surfaces in virus-infected cells may retain their specialized transport, protective, and metabolic functions. Membrane-limited host compartments also maintain spatial segregations favorable to regulation of positive-entropic biochemical processes, and confinement of DNA virus genomes within the nuclear envelope represents a relatively sophisticated deployment of this advantage (Gautschi *et al.*, 1976). Control of viral nucleic acid and protein synthesis within the intranuclear microenvironment can remarkably simulate host cell regulatory mechanisms (Chantler and Stevely, 1973; Seebeck and Weil, 1974; Su and DePamphilis, 1976). This may even involve selective molecular transport through the nuclear envelope (Kozak and Roizman, 1974).

In the cytoplasm, virus adaptation of host membranes facilitates the initiation of viral nucleic acid or protein synthesis (Caliguiri and Mosser, 1971; Mosser *et al.*, 1972a; Friedman *et al.*, 1972; Wagner *et al.*, 1972; Hay, 1974; Wirth *et al.*, 1977) and glycosylation of integral virus envelope proteins can depend entirely upon membrane-associated host enzymes involved in oligosaccharide synthesis (Sefton, 1976). These processes and the final maturation of enveloped viruses by budding through modified host membrane can be viewed as developmental mechanisms which minimize the requirement for direction of new macromolecular synthesis by viruses with a relatively limited genetic-coding capacity (see Portner and Pridgen, 1975). Indeed, an ample provision of host membrane material may be critical to the final steps of virion maturation (Choppin *et al.*, 1971; Blough and Tiffany, 1975). Infections by relatively well-adapted viruses need not grossly disrupt normal membrane biosynthesis (Quigley *et al.*, 1972; Luftig, *et al.*, 1974).

From a pathobiological viewpoint, host reactions to virus infection may be conditioned by cell membrane alterations. Insertion of viral proteins or

modification of host membrane proteins may modulate immune recognition antigens at the cell surface (Edelman, 1976; Zinkernagel and Oldstone, 1976), modify intercellular adhesive properties (Wallach, 1972), promote cell fusion or aggregation (Feldman *et al.*, 1968; Scheid and Choppin, 1974; Larke *et al.*, 1977), or influence differentiation (Aoki, 1974). The possible significance of such virus-related phenomena in neoplastic, neurologic, and other chronic diseases offers a fertile area for future investigation.

With the exponential growth of experimental virologic observations and data, it becomes necessary within the practical confines of a subject review to choose between thorough analysis of specialized topics or a more general survey. The burgeoning interest of pathologists, immunologists, and other "nonvirologists" in cell membrane phenomena during virus infections suggested that a broad scope might be timely. Since molecular and biochemical features of virus structural membranes and assembly processes have been amply treated in a number of recent and comprehensive reviews (e.g., Klenk, 1973; Rifkin and Quigley, 1974; Blough and Tiffany, 1975), this presentation aims toward providing a somewhat broader perspective on those membrane functions and alterations which ultimately relate to the pathobiology of virus infections. We attempt to compensate for some unavoidable superficiality by directing attention to comprehensive current articles and earlier reviews at appropriate points in the text.

II. Investigation of Viral Membrane Constituents

A. *Membrane Isolation and Composition*

With the exception of naturally pure membranes obtained from erythrocyte ghosts or nerve sheaths, accurate biochemical analysis of native cell membranes awaited refinement of cell fractionation techniques. A number of reproducible methods now available for isolation of plasma membrane or cell "ghosts" (Warren and Glick, 1969; Steck, 1972; Atkinson, 1973; Neville and Kahn, 1974), internal cytomembranes (Bosmann *et al.*, 1968), and nuclear membranes (Berezney, 1974; Aaronson and Blobel, 1975) can be applied to the study of membranes in virus-infected cells (e.g., Spear *et al.*, 1970; Caliguiri and Tamm, 1970a; Friedman *et al.*, 1972; Heine and Roizman, 1973; Buck *et al.*, 1974; LeBlanc and Singer, 1974; Hay, 1974; Atkinson *et al.*, 1976). In general, these methods rely on mechanical cell disruption in a hypotonic buffer or nitrogen cavitation (Blough *et al.*, 1977) followed by differential velocity sedimentation to remove nuclei and large cell fragments and to prepare crude membrane supernatants or pellets. Specific membrane fractions are then separated by isopycnic centrifugation in discontinuous and

continuous sucrose or ficoll gradients. The significance of biochemical analyses depends largely on the consistency of these preparations. Thin-section electron microscopy of pelleted cytomembrane fractions is an indispensible monitor (Bosmann *et al.*, 1968; Spear *et al.*, 1970; Caliguiri and Tamm, 1970a; Friedman *et al.*, 1972).

The predominant molecular species in animal cell biomembranes, regardless of source, are lipids and proteins in roughly balanced proportions. The lipids may be characterized as phospholipids, cholesterol, fatty acids, and glycolipids or glycosphingolipids (Law and Snyder, 1972; Klenk, 1973; Brady, 1975). Values of 30–40% lipid and 60–70% protein are typical for many clean mammalian cell membrane isolates (Stoeckenius and Engelman, 1969); however, considerable variation is recognized and differences in the organic composition of the naturally pure membranes of erythrocyte ghosts and in myelin dramatize this diversity: erythrocyte membranes contain lipid to protein ratio of about 1:1, whereas the ratio in myelin is approximately 4:1 (Singer, 1974). HeLa cell surface membranes contain about 40% lipid and 60% protein, and the proportion in most enveloped animal viruses is grossly similar (Klenk, 1973). The lipid pattern of highly purified virus particles in effect characterizes their envelope composition since the core (nucleocapsid) is a nucleic acid–protein structure (see reviews by Klenk, 1973; Rifkin and Quigley, 1974; Blough and Tiffany, 1975). Nevertheless, alertness to artifacts of preparation is necessary (Blough and Tiffany, 1975). Loosely-bound membrane proteins can solubilize, while cellular microproteins may contaminate the surface of virions (Pinter and Compans, 1975).

Small amounts of carbohydrate usually represent no more than 10% of the dry weight in cell membranes or virus particles (Rifkin and Quigley, 1974) and this is distributed in glycoslated proteins as well as in glycolipids. Since carbohydrate chains are completed stepwise through the action of specific glycosyltransferases present in smooth and rough cytomembranes, glycosylated proteins may show microheterogeneity depending on the degree to which oligosaccharides are completed (Heath, 1971). With the exception of some large viruses, the nature of sugar residues in viral envelope glycoproteins is determined by the function of host cell glycosyltransferases rather than viral-encoded enzymes (Keegstra *et al.*, 1975; Sefton, 1976).

The molar ratio of cholesterol to phospholipid in certain virus envelopes approximates unity, whereas the ratio in whole cells is closer to 1:5 (Klenk, 1973). This apparently reflects a predominance of virus budding through the cell surface which contains a larger proportion of cholesterol than the internal cytomembranes; however, preferential selection of host lipids during the process of virion assembly is also possible (Blough and Tiffany, 1975). Viral phospholipids may contain a significantly higher proportion of sphingomyelin and a lower proportion of phosphatidylcholine than the plas-

malemma (Quigley *et al.*, 1971, 1972). In general, the pattern of lipids in virus envelopes qualitatively resembles that of the host cell source (Klenk, 1973; Lenard and Compans, 1974; Rifkin and Quigley, 1974; Hirschberg and Robbins, 1974). A striking illustration is provided by togaviruses grown in baby hamster kidney cells and in mosquito cells: lipid composition of the progeny virions resembles that of the mammalian or insect host respectively, so that virions from the two sources contain only 36% of their lipids in common (Renkonen *et al.*, 1972). Microviscosity of togavirus membranes, measured by fluorescence depolarization, is also influenced by the host cell lipid (Moore *et al.*, 1976). In contrast, envelope proteins of viruses are largely specified by the virus genome and displace host proteins in the membrane plane (Hay, 1974; Birdwell and Strauss, 1974a; Dubois-Dalcq *et al.*, 1976b; Demsey *et al.*, 1977). This dichotomy in content of lipids and proteins is accounted for by a fluid-mosaic molecular construct of biological membranes (Section III,B).

B. *Topographic and Topological Localization of Membrane Proteins*

Dramatic progress in the elucidation of virus membrane structure since a review by Allison (1971) owes largely to a multidisciplinary convergence of techniques both for topographical and topological localization of membrane proteins. Conventional electron microscopy remains a basic tool in this armamentarium, and high resolution of negatively stained samples, or even thin sections, permits recognition of asymmetrical macromolecular structures such as glycoprotein spikes in a virion envelope (e.g., Cartwright *et al.*, 1969; Klenk *et al.*, 1970; Garoff and Simons, 1974). At the same time, new ultrastructural techniques such as surface replication (Birdwell *et al.*, 1973; Demsey *et al.*, 1978; Dubois-Dalcq *et al.*, 1976a), freeze-fracture (Bächi *et al.*, 1969; Brown *et al.*, 1972; Sheffield, 1974; Haines and Baerwald, 1976), and secondary electron scanning (Wong and MacLeod, 1975; Dubois-Dalcq *et al.*, 1976b) offer a more integrated view of virus membrane topography. Freeze-drying of intact cells is a simple, but elegant method, which allows direct visualization of virus macromolecular elements and has provided resolution of at least two distinct virus components at a host surface (Demsey *et al.*, 1976, 1977). Figure 1 illustrates results of these procedures.

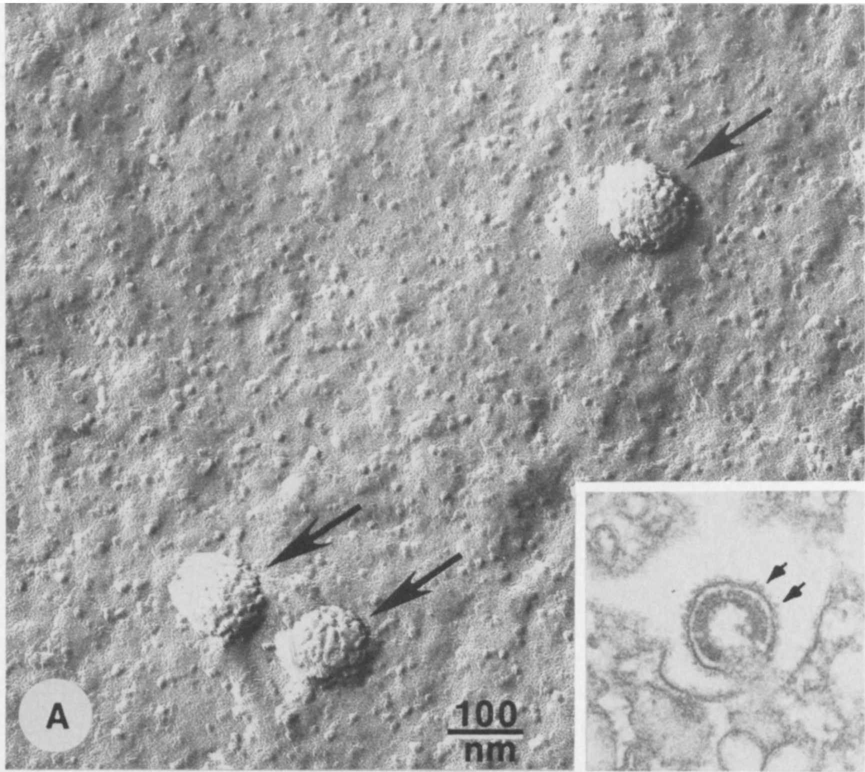
During the last decade, emphasis has also shifted toward ultrastructural localization of specific virus envelope constituents, sometimes combined with experimental manipulations to dissect individual steps in the complex chain of maturational events (e.g., Tiffany and Blough, 1971; Klein and Adams, 1972; Bächi *et al.*, 1973; Birdwell and Strauss, 1974a; Lampert *et al.*, 1975). Powerful tools for this work have been provided by biochemists

and immunologists who pioneered the development of techniques in which antibodies or macromolecular probes are coupled to markers appropriate for electron microscopy and immunofluorescence or autoradiography. In general, the application of particulate electron-dense markers (e.g., ferritin, small viruses) coupled to specific antibodies or macromolecular probes has proven most suitable for high-resolution localization of virus-specific constituents. For a full discussion of technical details the reader is referred to Wagner (1973). Enzyme-labeled antibody can serve as a very sensitive marker for intracellular or surface cites of virus protein attachment. Hemocyanin or small virus particles are useful in conjunction with surface replication (Birdwell and Strauss, 1974a) or secondary scanning electron microscopy of cells prepared by critical point drying (Hämmerling *et al.*, 1975).

Table I summarizes representative samples of several major approaches which have been successfully applied for high-resolution immunolabeling of virus-infected systems. Perhaps the most versatile procedures are based upon construction of hybrid antibody molecules from Fab′ fragments (Hämmerling *et al.*, 1973). Dual monovalent specificities are directed both against the IgG of a species in which a specific antibody probe is raised and against a protein particle such as ferritin or a virus nucleocapsid. An example of this technique is illustrated in Fig. 2.

Although much emphasis has been placed upon antibody localization of specific viral proteins, the uses of colloidal iron to identify neuraminic acid residues (Klenk *et al.*, 1970) or of lectins with selective oligosaccharide affinities (Klein and Adams, 1972; Birdwell and Strauss, 1973) have been successfully exploited in the study of virus infection. Some novel technical approaches remain on the horizon. These include use of antibody fragments (Mannik and Downey, 1973; Kraehenbuhl *et al.*, 1974) and haptenic conjugates (Lamm *et al.*, 1972). Refinement of methods for labeling ultrathin sections of frozen (Tokuyasu and Singer, 1976) or embedded tissues (e.g., Kraehenbuhl and Jamieson, 1972) would be most advantageous in studying intracellular cites of virus protein biosynthesis without gross disruption of the membrane systems by subcellular fractionation.

Fig. 1. (A) Replica of a freeze-dried mouse JLSV$_9$-RLV cell surface revealing three budding Rauscher leukemia viruses (arrows). Note viral knoblike components concentrated on the buds and distributed randomly over the rest of the cell surface. Inset is a thin section of a virus particle budding from the membrane of a JLSV$_9$-RLV cell. Densities probably representing knobs are evident (arrows), as is the forming crescent-shaped viral nucleocapsid underneath the membrane. (B) Mid, and (C) late stages of Friend leukemia virus budding from STU-Eveline cells as seen in freeze-fracture replicas. Intramembranous particles are excluded from those regions of the host membrane enveloping the viral nucleocapsid. As virus budding progresses, release occurs by a pinching off of the membrane (arrows). ×95,000 (from Demsey *et al.*, 1977).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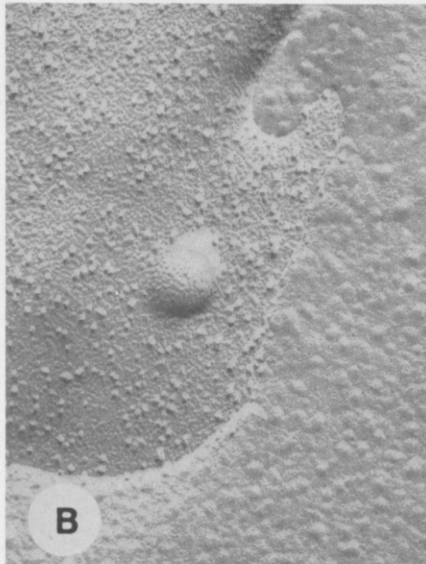
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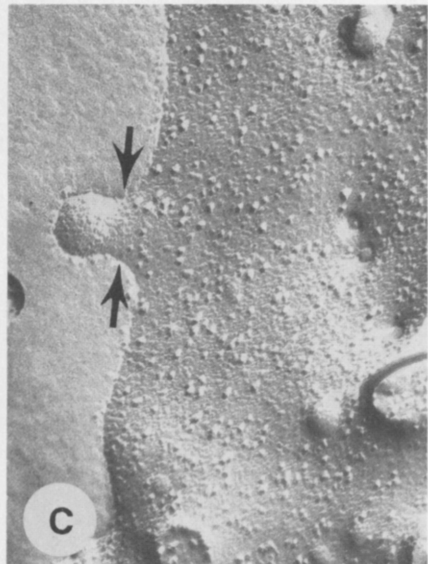
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TABLE I

Examples of High Resolution Immunolabeling Procedures for Localization of Membrane-Associated Viral Antigens[a]

Method	*First reagent*	*Second reagent*	*Third reagent*	*Selected references*
Direct ferritin	γ/Ferritin			Howe *et al.* (1969), Pedersen and Sagik (1973), Bächi and Howe (1973)
Direct enzyme	γ/HPO	DAB + H_2O_2		Hoshino *et al.* (1972), Suzuki (1972)
Indirect ferritin	γ^*	Anti-γ/ferritin		Levinthal *et al.* (1969), Shigematsu *et al.* (1971), Coward *et al.* (1972)
Indirect hemocyanin	γ^*	Anti-γ/hemocyanin		Birdwell and Strauss (1974a)
Indirect enzyme	γ^*	Anti-γ/HPO	DAB + H_2O_2	Ciampor *et al.* (1974), Lampert *et al.* (1975), Hiraki *et al.* (1974)
Hybrid antibody	IgG	$F(ab')_2$ (*anti*-γ/anti-ferritin)	Ferritin	Wagner *et al.* (1971), Gelderblom *et al.* (1972) Aoki and Takahashi (1972 Heine and Schnaitman (1971)

[a] γ, Total globulin fraction from specific antibody (first animal species); anti-γ, total globulin fraction from antibody raised in second animal species against globulin of first species; HPO, horseradish peroxidase; DAB, diaminobenzidine; SBMV, southern bean mosaic virus; and γ^*, use of IgG fraction of Fab′ fragments may improve localization.

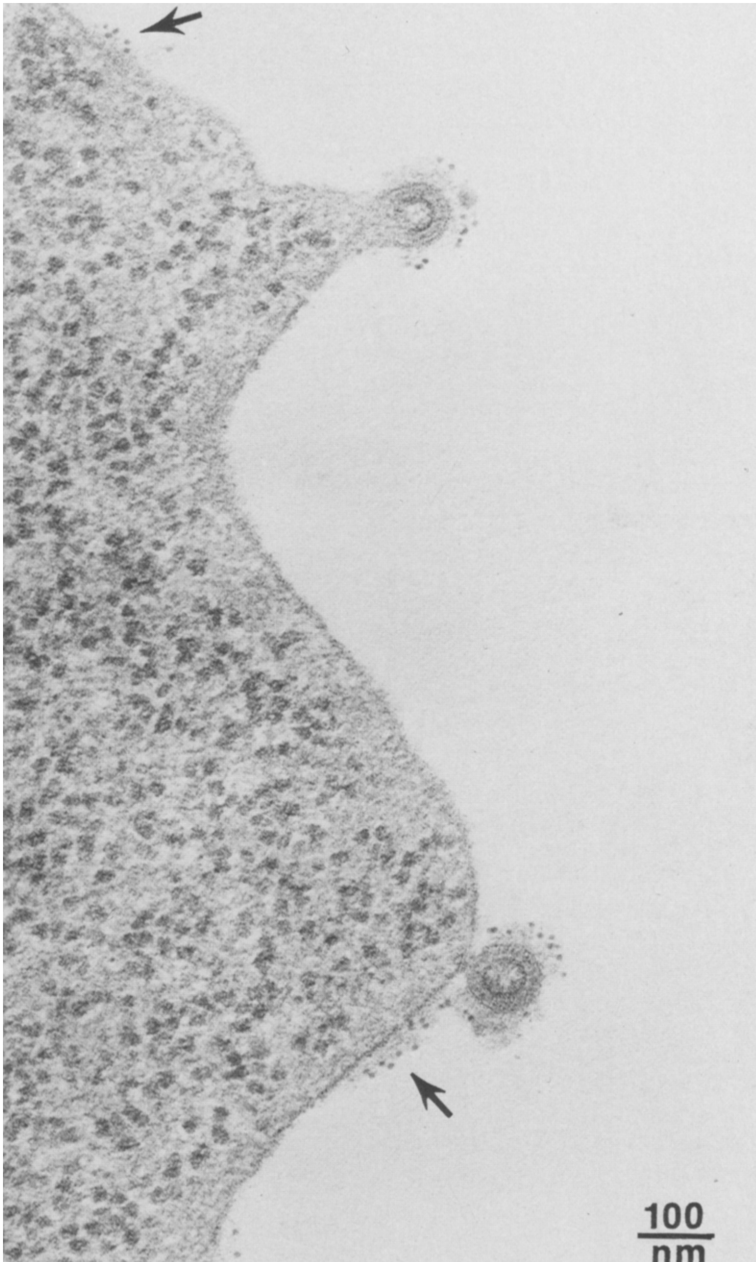

Fig. 2. Thin section of a FLC745 (Friend erythroleukemia) cell after labeling sequentially with rabbit anti-gp71 serum, hybrid sheep anti-rabbit IgG/anti-ferritin antibodies, and ferritin. Ferritin label is found on the surfaces of the Friend leukemia virus buds, as well as on other parts of the cell surface (arrows). ×95,000.

At a molecular level, the subcellular distribution and topological positioning of viral polypeptides within membranes may be elucidated by a number of relatively refined enzymatic techniques including gentle proteolytic cleavage on isolated membrane vesicles (Katz *et al.*, 1977; Wirth *et al.*, 1977; Witte *et al.*, 1977) or virus envelopes (Cartwright *et al.*, 1969; Schloemer and Wagner, 1974) and catalytic radiolabeling (see Juliano and Behar-Bannelier, 1975) followed by physical separation of radioiodinated molecules (Witte and Weissman, 1976; Knipe *et al.*, 1977c). More specific references to these approaches appear in appropriate sections below.

III. Membrane Dynamics Relating to Virus Infection

A. *Continuous Circulation and Biogenesis of Cell Membranes*

Cellular membranes exist in a state of dynamic equilibrium, both physical and metabolic, with continuous active synthesis and degradation (see Schimke and Dehlinger, 1972; Warren, 1972; Singer and Rothfield, 1973). Nascent membranes, synthesized in continuity with the endoplasmic reticulum (Higgins, 1974) eventually incorporate into the Golgi region through an "assembly-line" process (Morré *et al.*, 1971). Cell surface membrane is generated or replaced by assimilation of modified membrane vesicles which move peripherally from the Golgi region (Hicks, 1966; Grove *et al.*, 1968; Singer, 1974). The cell membranes utilized by many groups of enveloped viruses evidently arise in a similar fashion by continuous amplification of preexisting templates (Amako and Dales, 1967b; Mosser *et al.*, 1972b; Luftig *et al.*, 1974; Blough and Tiffany, 1975).

Exchanges of material between the cell surface and internal cytomembranes (see Whaley *et al.*, 1971) compensate for macromolecular migrations involved in the vectorial transport of fluids (phagocytosis) to the cell interior as well as the centrifugal export of secretions or waste products (exocytosis). Pinocytosis, for example, can result in internalization of cell surface at the rate of up to 20% per minute (Gosselin, 1967). The intimate linkage of virus uptake to membrane movements will be discussed below (Section IV,B). While this necessitates no gross interruption of the membrane circulatory dynamics, production and emergence of enveloped virus eventually taxes the process of membrane biogenesis. In the absence of compensatory membrane production, prolonged virus infection leads to a chronic depletion of surface membrane resources (Choppin *et al.*, 1971; Quigley *et al.*, 1972).

In bacterial models there is some degree of feedback control between protein and fatty acid synthesis (Fox, 1972), but coordination of protein and

lipid synthesis during vertebrate membrane biogenesis appears more complex. Production of lipid constituents depends upon the availability of appropriate enzymes which are subject to metabolic or genetic regulatory controls (Majerus and Kilburn, 1969; Brady, 1975), but the synthesis of membrane proteins is not necessarily concerted, either temporally or spatially. In liver microsomes, for example, phospholipid synthesis occurs *in situ* in smooth membranes, while some complementary proteins may be synthesized on the ribosome-bound endoplasmic reticulum and later transferred to cites of insertion (Higgins and Barrnett, 1972). Phospholipid to protein ratios in smooth and rough microsomal fractions can vary independently (Higgins, 1974) and the half-life of barbiturate-stimulated microsomal enzymes may be shorter than the membrane half-life (Orrenius and Ericsson, 1966). Schimke and Dehlinger (1972) reproted differential rates of membrane protein turnover, with larger molecules renewing more rapidly.

Changes in the patterns of protein and lipid biosynthesis during animal virus infections is providing some useful clues to normal controls regulating membrane biogenesis. Early in the course of virus development, for example, there can be direct and selective inhibition or stimulation of membrane biogenesis (Plagemann *et al.*, 1970; Ben-Porat and Kaplan, 1971; Mosser *et al.*, 1972b; Willis and Granoff, 1974; Makino and Kenkin, 1975; Vance and Lam, 1975). The poxviruses offer unique models for further studies of biomembrane assembly mechanisms (Dales and Mosbach, 1968; Grimley *et al.*, 1970; Moss *et al.*, 1971a; Stern and Dales, 1974), since they synthesize membrane *de novo* from degraded host lipids. Investigations of other enveloped viruses are illuminating the subcellular pathways of membrane specialization (see Section V,C).

B. Fluid-Mosaic Structure

It is now well established that viral and cellular proteins occupy contiguous and mobile domains in the cell surface membrane (Heine and Roizman, 1973; Birdwell and Strauss, 1973, 1974a; Hay, 1974; Blough and Tiffany, 1975; Edelman, 1976). Indeed, the capacity of host membranes to accommodate virus-specified proteins into a preexisting molecular framework is a biophysical property critical to the processes of virus maturation. A "recycling" of host cytomembranes by attachment or insertion of new viral gene products underlies the survival of virus groups which lack enzymatic resources to synthesize their own structural membranes.

Before 1960, hypotheses of biomembrane structure usually assumed a relatively uniform bilayer. The phospholipid molecules were considered to be relatively rigid while the protein was typically represented in an extended

configuration applied to the exterior surfaces. This model appeared to explain the "railroad-track" image of cell and virus membranes observed in ultrathin sections after osmium tetroxide fixation (with a profile thickness in the range of 60–100 Å). Critical analysis of the essentially static bilayer concept revealed many discrepancies with experimental observations, and in the late 1960s evidence rapidly mounted for the presence of macromolecular subunits within various types of membranes (see Branton and Deamer, 1972). The conceptual developments have been lucidly recapitulated by Stoeckenius and Engleman (1969).

Freeze-fracture studies of plasma membranes by Branton and others were most revealing when they disclosed the presence of 80-Å diameter particles within the membrane plane. Internal localization was established by marking the membrane exterior with ferritin (Pinto da Silva and Branton, 1970) or with F-actin (Tillack and Marchesi, 1970). Independent evidence for the globular conformation of proteins was obtained with physical methods (see Branton and Deamer, 1972). These also indicated extensive "bareness" of the extended phospholipids. In favor of hydrophobic-protein-phospholipid interactions Singer (1974) found that neutral salt solutions do not dissociate large amounts of protein from membranes, and that cytochrome *b*5 isolated from liver microsomes and an erythrocyte surface glycoprotein each appeared to possess hydrophobic regions which could be cleaved from polar regions by limited proteolytic digestion. Additional experimental evidence consistent with penetration of protein macromolecules into the membrane lipid (Singer and Nicolson, 1972; Steck and Fox, 1972), phospholipid fluidity (Scandella *et al.*, 1972; Lee *et al.*, 1973), and protein mobility within the membrane plane (Frye and Edidin, 1970; DePetris and Raff, 1973) culminated in the proposal of a "fluid lipid-globular protein mosaic" structure (Singer and Nicolson, 1972).

This "fluid-mosaic" concept currently offers the most attractive synthesis of biophysical, biochemical, immunological, and ultrastructural observations in virus-infected and virus-transformed cells. Principal features of the model have been concisely summarized by Nicolson (1975) and Fig. 3 is based on prevalent conceptions: the membrane is composed of lipid molecules (principally phospholipid) arranged in an extended bimolecular configuration. Hydrocarbon, nonpolar tails of the lipid molecules are directed inward, away from the bulk aqueous phase. Thus, they form a semifluid matrix for integral membrane proteins which are stabilized within the hydrophobic plane. Hydrophilic moieties of these integral proteins, such as the glycopeptide portions of glycoprotein macromolecules, thrust outward into the aqueous environment. The integral proteins may comprise either single macromolecules or macromolecular complexes formed by weak interaction with peripheral proteins. The latter do not penetrate through the lipid bilayer and

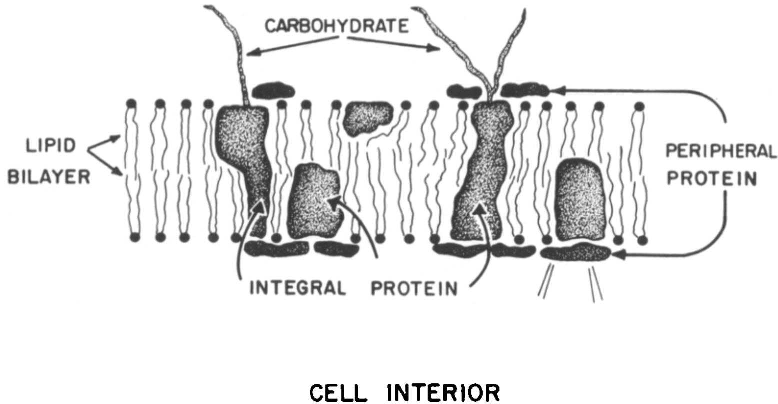

Fig. 3. Membrane structure based upon fluid-mosaic concept. Globular proteins are embedded in the plane of the lipid bilayer. "Integral" glycoproteins, penetrating into the hydrophobic region of the bilayer, could represent the G proteins in a virion envelope. The peripheral proteins, more loosely attached to the membrane surface, could be represented by the M proteins (see Section V).

are believed to account for up to 30% of all membrane-associated proteins. They often serve as membrane attachment cites to the cytoskeleton. Both the "integral" and "peripheral" membrane proteins have counterparts in virus envelopes (Blough and Tiffany, 1975; Knipe *et al.*, 1977b).

In recent studies, some of the dynamic techniques which led to devleopment of the fluid-mosaic concept have been applied to studies of virus-infected cells and virion envelopes. These techniques include visual or ultrastructural observation of virus antigen movements within the cell surface (Birdwell and Strauss, 1974a; Lampert *et al.*, 1975), incorporation of spin-labeled phospholipids for electron paramagnetic resonance spectroscopy (Sefton and Gaffney, 1974; Sharom *et al.*, 1976), or incorporation of a diphenylhexatriene probe for fluorescence depolarization analysis (Lenard *et al.*, 1974; Moore *et al.*, 1976; Levanon *et al.*, 1977). Detailed discussion of the biophysical techniques may be found in Branton and Deamer (1972) and Fox and Keith (1972).

C. *Freeze-Fracture Observations*

Freeze-fracture methods for ultrastructural observation of membranes were devised in order to avoid potential artifacts associated with extensive dehydration and plastic embedding prior to thin sectioning. The approach has been successfully applied to examine morphogenesis of enveloped viruses in several groups: herpesvirus (Haines and Baerwald, 1976),

myxoviruses (Bächi *et al.*, 1969; Bächi and Howe, 1973; Dubois-Dalcq *et al.*, 1976a), togaviruses (Brown *et al.*, 1972; Demsey *et al.*, 1974), visnavirus (Dubois-Dalcq *et al.*, 1976b), and oncornavirus (Sheffield, 1974; Demsey *et al.*, 1977). It can be profitably integrated with scanning electron microscopy (Dubois-Dalcq *et al.*, 1976b).

Biological material for freeze-fracture study is usually fixed briefly and infiltrated with a cryoprotectant solution to prevent ice crystal formation during the rapid freezing. The freeze-fracture process begins with a "snap freezing" at about −150°C, followed by cleavage (e.g., with a cold knife) and replication of the surface topography by deposition of fine molecular layers under high vacuum. Typically, platinum is deposited at an angle (about 45°), and "backed" perpendicularly by carbon. The carbon helps maintain integrity of the replica during digestion of the subjacent cellular material (e.g., with sodium hypochlorite or chromic acid). This is necessary to clean the replica for examination by transmitted electrons. Ice or glycerol–ice can be sublimed away from exposed biological components immediately after fracturing by allowing the cleaved specimen to remain in high vacuum for a brief time at an increased temperature (e.g., 2 min at −100°C). The controlled process is commonly known as freeze-etching.

With few exceptions, cell membranes are either (a) cross-fractured—the fracture plane cuts through the plasma membrane and enters the cell contents; or (b) split through the region of hydrophobic bonding of the lipid bilayer. The latter split is most informative and reveals either of two fracture faces: the PF (also A or +) face is characterized by numerous particles as seen from an exterior view; the EF (also B or −) face normally exhibits a sparser particulation as seen from an interior view (Branton and Deamer, 1972). These intramembranous particles (IMP) or membrane-associated particles (MAP) seen on freeze-fractured membrane faces are evidently large proteins (Tillack *et al.*, 1972; Marchesi *et al.*, 1976) and asymmetry of the inner- and outer-facing leaflets is anticipated in the fluid-mosaic model. Figure 4 illustrates planes in a freeze-fracture of cultured HeLa cell surface membranes. The PF face of an intramembranous fracture of one cell surface comprises most of the visible surface. A few patches of attached EF belong to a neighboring cell. The concept may be clarified by imagining the PF surface covers the remainder of the first cell which has lost only the outer half of its

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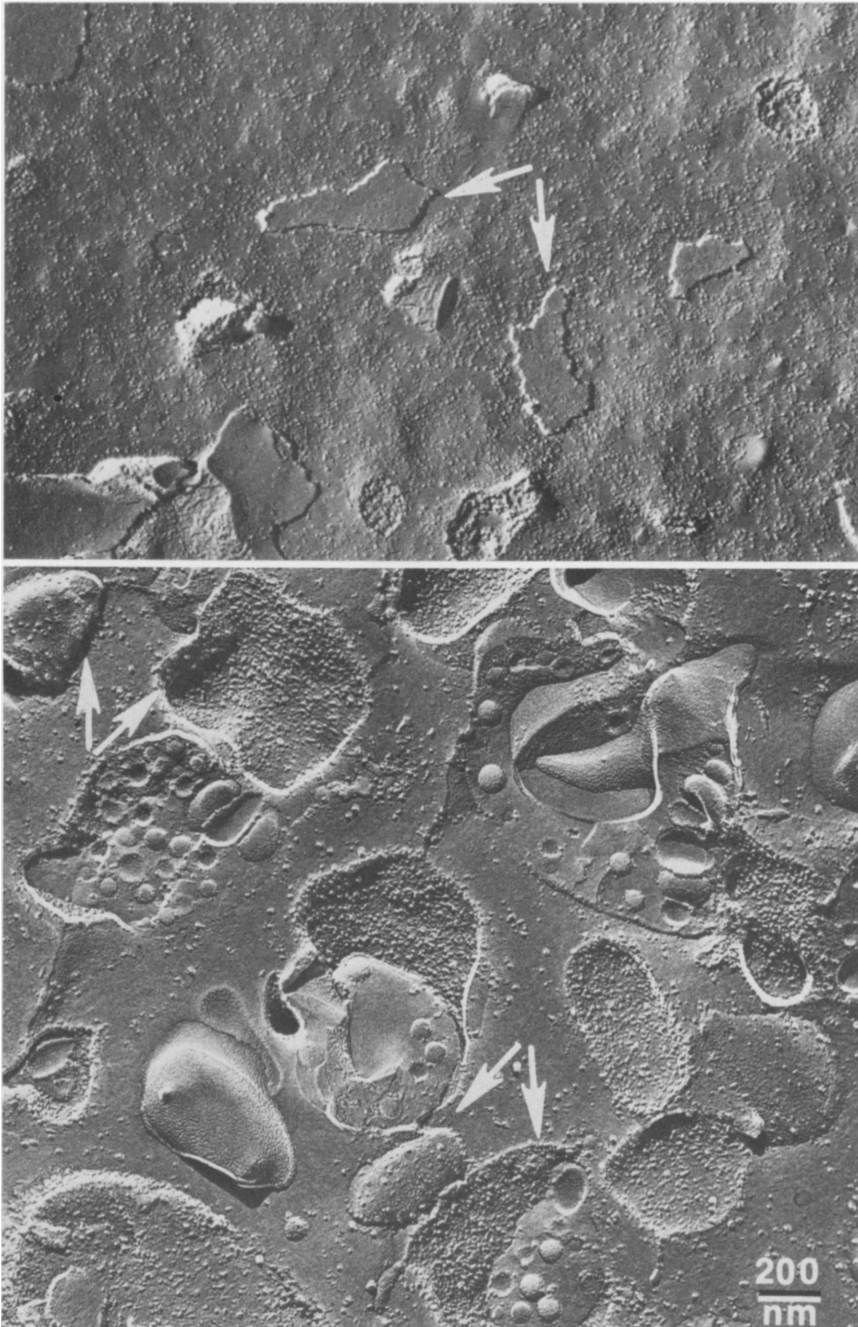
Fig. 4. (Top) Replica of freeze-fractured HeLa cells. Mostly PF surface is revealed, although some small patches of EF surface from an adjacent cell are seen (arrows). ×40,000.

Fig. 5. (Bottom) Replica of freeze-fractured LLC-MK_2 (monkey kidney) cell infected with Dengue virus. Arrows indicate "inside-out" membrane polarity of vacuoles, some of which contain virus. ×40,000.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membrane, whereas the EF surface has been left behind by tearing the remainder of the second cell away. Between the PF and EF faces would remain the outer half of the first cell's membranes, intercellular space, and the outer half of the second cell's membrane. In principle it is possible to replicate and retain the material that was fractured away, and such a complementary replica can be very useful for orientation purposes.

D. *Internal Cytomembranes*

The fluid-mosaic structure evidently extends to internal cytomembranes which continuously fuse to the cell surface (Scandella *et al.*, 1972). Exocytic cytoplasmic vacuoles, which discharge certain viruses, demonstrate a similar internal structure to the cell surface after freeze-fracturing and etching (Demsey *et al.*, 1974); however, the intramembranous polarity is oriented with respect to the vacuolar lumen rather than to the cell surface (Fig. 5).

The intramembranous structure of the endoplasmic reticulum (ER) is of special interest, since certain RNA viruses, including togaviruses (Filshie and Rehacek, 1968; Blinzinger, 1972), coronaviruses (Caul and Egglestone, 1977), and intracisternal A-type oncornaviruses (Perk and Dahlberg, 1974) mature predominantly by budding through the ER or derivative cytomembrane systems. Furthermore, ER membranes indirectly support the envelopment of viruses which bud from the cell surface, since they are the source of new membrane biogenesis (see Section V,C). Macromolecular dynamics of the ER membranes, however, have only lately been examined. Lateral movements of membrane-bound ribosomes resemble those of surface proteins and suggest comparable internal fluidity with temperature-dependent phase transitions (Ojakian *et al.*, 1977). Freeze-fracture of the nuclear envelope which is homologous to the ER also provides evidence for structural similarities (Wunderlich *et al.*, 1974).

Certain cytomembranes exhibit a more regular and stable internal structure than the fluid-mosaic cell surface. For example, mitochondrial inner membranes, retinal photoreceptor membranes, and chloroplast membranes are highly enriched in enzymes which promote energy conversion by means of electron transfer or ATP synthesis. Efficiency of these processes is promoted by a tightly packed organization of protein subunits, perhaps involving polypeptide cross-linkage (Hendler, 1974). Even under such conditions, however, a potential for intramembranous movement of protein "particles" appears to persist (Apel *et al.*, 1976; Staehelin, 1976). Although there has been at least one report of virus envelopment within mitochondrial membranes (Lunger and Clark, 1973), there is yet insufficient information for any specific discussion of mitochondrial membrane structure, function, or turnover in relation to virus infections.

IV. Virus Uptake and Penetration of Cytomembrane Barriers

A. *Techniques for Investigating Virus Uptake*

Development of plaque assay and hemagglutination techniques encouraged early experimental efforts to quantitate virus uptake by host cells (e.g., Joklik and Darnell, 1961). This was accomplished by measuring the adsorption of infectious particles from an inoculum of known biological potency. Since the ratio of physical particles to infectious units ranges up to 1000:1 in a virus inoculum, only a fraction of the total virions can be measured by biological titration. Production of virus with radiolabeled nucleic acid and determination of cell-associated radioactivity as a percentage of radioactivity applied provides a more precise tool for quantitation of virion binding (e.g., Philipson, 1967; Sussenbach, 1967; Schloemer and Wagner, 1975). Investigations at a molecular level became more feasible as procedures for purification and fractionation of virus particles were refined (see Fraenkel-Conrat, 1969). This encouraged analysis of the virus envelope by selective extraction of lipids (see Klenk, 1973; Rifkin and Quigley, 1974) or electrophoretic separation of proteins (Cords *et al.*, 1975). Adaptation of methods for subcellular fractionation and separation of host membrane systems (e.g., Chan and Black, 1970; Roesing *et al.*, 1975) further expanded opportunities for experimental analysis.

Analysis of virus–cell interactions has become increasingly sophisticated with use of proteolytic or lipolytic enzymes (Cartwright *et al.*, 1969; Friedman and Pastan, 1969; Tillack *et al.*, 1972; Scheid and Choppin, 1974; Schloemer and Wagner, 1974), plant lectins (Ito and Barron, 1974), individual glycoproteins (Schloemer and Wagner, 1975), or purified virion subunits (Philipson *et al.*, 1968) to explore the surface properies of virus envelopes and host cells. Reconstitution of virus envelopes (Shimizu *et al.*, 1972) or construction of artificial membrane models offer further means for probing the molecular roles of specific phospholipid or protein components in virus attachment and penetration (Tiffany and Blough, 1971; Haywood, 1974; Mooney *et al.*, 1975; Sharom *et al.*, 1976). Virus–cell receptor complexes (Philipson *et al.*, 1968) can be isolated by buoyant density, and analysis of "cytotrophic" subunits of viruses or "viroceptive" proteins removed during the elution of virions from cell surfaces has been accomplished by polyacrylamide gel electrophoresis (see Crowell and Philipson, 1971; Philipson *et al.*, 1976).

Transmission electron microscopy has proven to be a uniquely valuable tool for examining virus uptake despite limitations in sampling and an inherent inability to discriminate the fate of infective and noninfective virus

particles (Dales, 1973; Lonberg-Holm and Philipson, 1974; Smith and de Harven, 1974). High-resolution autoradiography has proven particularly rewarding since it can localize early events in virus replication (Amako and Dales, 1967b; Silverstein and Dales, 1968; Grimley *et al.*, 1968; Hummeler *et al.*, 1969, 1970; Willis and Granoff, 1974; Mackay and Consigli, 1976). Freeze-cleaving of membranes, described in Section III,C, provides a new and essential approach to the ultrastructural examination of virus receptors without treatment by organic solvents (Tillack *et al.*, 1972; Dubois-Dalcq *et al.*, 1976a). Improvement of resolution in secondary mode scanning electron microscopy (cf. deHarven, 1974; Panem and Kirsten, 1975; Wong and MacLeod, 1975; Dubois-Dalcq *et al.*, 1976b), should ultimately permit a more representative and topographical view of virus entry events, complementing conventional ultrastructural techniques. Other potentially valuable approaches are the use of high-voltage electron microscopy with stereoscopy (Grimley, 1971; Stokes, 1976) and a technique of freeze-drying intact cells (Demsey *et al.*, 1978). The latter method also avoids organic solvents and affords a relatively high resolution (10–15 Å). Most recently Levanon *et al.* (1977) used fluorescent polarization analysis with molecular probes to explore changes in intramembranous lipid fluidity of the cell surface during virus adsorption.

B. Functional Routes for Virus Invasion of Host Cells

The obligate dependence of viruses upon host machinery for protein synthesis requires that instructions encoded by the virus genome gain direct access to intracytoplasmic or intranuclear compartments. Thus, virus *penetration* as defined by Dulbecco (1965) is consummated only when nucleic acid of the inoculum virus has escaped from its protective wrappings (uncoating) and reached intracellular sites where expression and replication of the parental genes can ensue. These virus entry mechanisms have proven to be unexpectedly complex and specialized for each major group of animal viruses. From an evolutionary perspective, perhaps they can be viewed as a process in which gross and molecular membrane dynamics of potential host cells have been adopted to assist the virus invasion. Examples of membrane-associated phenomena, to be discussed more fully in appropriate sections, include processes of membrane fusion and repair, interiorization and digestion of particulates (see Jacques, 1975), and nuclear cytoplasmic exchange (see Goldstein, 1974). In two comprehensive reviews, Dales (1965, 1973) cogently summarized the extensive evidence that virus penetration is a resultant of interacting virus and host influences. More recently, Lonberg-Holm and Philipson (1974) provided another lucid analysis of these events.

Since there normally exist no direct openings between the cell sap and exterior environment, viral components must traverse a membrane barrier. Physical disruption occurs when some bacteriophage penetrate their microbial hosts, and this may be accompanied by transient leakage of cell contents (Luria and Darnell, 1967). Transient dissolution of plasma membrane continuity has also been suggested by thin section observations of mammalian cells during penetration of a small nonenveloped adenovirus (Brown and Burlingham, 1973), and a murine leukemia virus (Miyamoto and Gilden, 1971). While local breaches of animal cell surfaces produced by physical, chemical, or immunological means can be rapidly restored as shown by the entry and resealing of microparticulates (Seeman, 1974), cytosol leakage is not notable during animal virus infections, and transmembranous entry is generally considered to involve more subtle molecular mechanisms. These include the attachment of the virus nucleocapsid or virus envelope to host membrane by means of mutually specific receptor molecules, with molecular translocation through the fluid-mosaic membrane structure (nucleocapsids) or membrane fusion (virus envelopes). In addition, there is a potential for active virus uptake by engulfment. This phenomenon may either resemble nonselective inhibition of small particulates (Epstein *et al.*, 1964; Morgan *et al.*, 1969) or occur subsequent to specific virus binding. The latter process was termed *viropexis* by Fazekas de St. Groth (1948).

In the sense of virus engulfment combined with specific membrane-binding mechanims, viropexis is probably very common. The microvesicular-limiting membrane around internalized virions remains topologically and functionally homologous to the plasma membrane from which it originates (Abodeely *et al.*, 1970; Choppin, 1976), so that penetration of intact virions engulfed within microvesicles can proceed by specialized mechanisms including nucleocapsid translocation or membrane fusion as observed at the cell surface (e.g., Smith and de Harven, 1974; Crowell, 1976).

Operationally, it is important to distinguish between mere interiorization of an infectious virion and actual genomic penetration. In either case, the infective element is no longer accessible to neutralizing antibody and particles cannot be eluted from the membrane exterior (Mandel, 1967). Nevertheless, interiorized virus particles may be sequestered for several hours within cytoplasmic vesicles (phagosomes) (Smith and de Harven, 1974; Ogier *et al.*, 1977), functionally quarantined from the cytoplasmic compartment by a limiting membrane. In the special case of nonenveloped nucleotropic viruses (adenoviruses and papoviruses), electron microscopic studies show an almost immediate attachment and engulfment of inoculum virions which are swiftly transported to the periphery of the nuclear envelope. Intact nucleoids of papovavirus arrive in the nuclei of host cells within 30 min

(Hummeler *et al.*, 1970; Mackay and Consigli, 1976), and centripetal migration of adenovirus is equally rapid (Morgan *et al.*, 1969; Dales and Chardonnet, 1973). This movement appears to be guided by cytoplasmic microtubules (Dales and Chardonnet, 1973). Virion uncoating may occur at orifices of the nuclear envelope (Hummeler *et al.*, 1970).

Dales has marshalled the strongest arguments in favor of virus infections being initiated by nonspecific engulfment (1973). Dales and Pons (1976) observed that enveloped viruses such as influenza can be infectious under conditions of neutralization or aggregation in which specific attachment and fusion to membrane surfaces was unlikely. A similar conclusion was drawn earlier in studies by Mandel (1967) which showed that neutralized poliovirus retained ability to penetrate HeLa cells, even though the released viral RNA was abnormally labile. Ultrastructural studies of a rhabdovirus suggested that enveloped virions were swallowed in pits at the cell surface which are believed to be coated by a sticky substance that traps particulates (Simpson *et al.*, 1969).

The molecular aspects of viral genome penetration from within phagosomes has not been fully elucidated, but except in the case of diplornaviruses which have a nuclease-resistant genome, the role of lysosomal hydrolases is questionable (Dales, 1973; Choppin, 1976). Even without formation of a phagolysosome, evolution of the phagosome by dehydration may increase permeability of the limiting membrane up to 100-fold (Jacques, 1975). Free adenovirus DNA appears to penetrate the plasma membranes of KB cells (Groneberg *et al.*, 1975) and recent investigations of DNA bacteriophage provide a basis for speculating that an amphiphilic virus coat protein could become the carrier to draw nucleic acid polyectrolytes through the hydrophobic fluid-lipid bilayer of host membrane (Marco *et al.*, 1974). This parallels the "permions" concept which postulates a class of large globular membrane proteins forming energetically favorable channels lined by polar residues within the lipid bilayer to facilitate permeation of charged macromolecules (Rothschild and Stanley, 1972).

In considering the biological and functional significance of various sets of experimental observations on virus entry mechanisms, limitations of tissue culture and other systems *in vitro* which have been widely employed for these investigations should be fully appreciated. Each experimental approach and method of examination is subject to its own set of arbitrary conditions and limited means of observation, introducing a biological "uncertainty principle." Cells in culture may gain or lose susceptibility to particular viruses (Holland, 1961) and changes induced by serum concentration (Allison, 1971) or temperature shifts conventionally employed during virus adsorption have no obvious parallel in the natural host. Furthermore, in the "unnatural universe of the laboratory," propagated virus strains may con-

ceivably lose specific genetic characteristics which would be essential to their survival in nature (Reanney, 1974). Except for hematogenous dissemination, the physicodynamics of virus–cell interactions in the intact animal must differ remarkably from events in monolayer or suspension cultures. Direct cell–cell contacts and localized concentrations of virus particles in cell processes can be expected to amplify the efficiency of virus transfer in solid tissues by an exponential factor (Cohen, 1963; Grimley and Friedman, 1970a; Iwasaki and Koprowski, 1974). This can be observed even in organ cultures (Leestma *et al.*, 1969).

C. Molecular Mechanisms of Virus Attachment to Cell Membranes

Direct contact and eventually irreversible attachment of inoculum particles to the host cell surface, its projections or invaginations, is an essential and probably universal first step in virus infections (Kohn and Fuchs, 1973; Lonberg-Holm and Philipson, 1974; Dales *et al.*, 1976). When virus particles and host cells are bathed in a generous volume of fluid and free to follow random Brownian movements, the kinetics of collision are predictably proportionate to relative concentrations. The rate of binding to cell surfaces, however, is exponentially lower than values expected if each virus–cell contact were to produce a firm attachment. Taken alone, these observations provide circumstantial evidence for the existence of specific virus-binding mechanisms, requiring steric orientation of macromolecules in addition to electrostatic forces (see Cohen, 1963). Attachment also can be significantly influenced by pH (Mooney *et al.*, 1975; Schloemer and Wagner, 1975), ionic strength (Pierce *et al.*, 1974), and other conditions modifying the charge environment (Vogt, 1967; Hochberg and Becker, 1968; Cartwright *et al.*, 1970; Miyamoto and Gilden, 1971).

Virus attachment is mediated by an interaction of natural cell membrane components ("viroceptive" molecules) with complementary elements of the virus envelope or capsid ("cytotropic" subunits). While some viroceptive molecules may represent common phospholipid or glycolipid constituents of animal cell membranes, others represent genetically specified host proteins. The genetic control of viroceptor specificity for picornaviruses has been elegantly revealed in experiments with cell hybrids (Medrano and Green, 1973). Since poliovirus type 1 can infect primate cells but not murine cells, Miller *et al.* (1974) exploited human–mouse heterokaryons with observed deletions of human chromosomes to assign the gene for the viroceptor of poliovirus to human chromosome 19.

Cytotropic subunits of the virion are obviously specified by virus genes. Mutations involving cell-attachment ability have been recognized for in-

fluenza virus (Palese *et al.*, 1974) and noninfectious Rous sarcoma virus (DeGiuli *et al.*, 1975).

The specificity of virus–cell interaction varies. Similar neuraminic acid-bearing viroceptive molecules may be recognized by more than one major virus group (Mori *et al.*, 1962). At the same time cross-competition (an attachment interference assay) and cell hybridization experiments have demonstrated the existence of distinct viroceptive molecules for individual virus serotypes within major virus subgroups (Philipson *et al.*, 1968; Chardonnet and Dales, 1970; Crowell, 1976). Such observations prompt the concept of virus receptor families (Lonberg-Holm and Philipson, 1974). Biologically, the species and tissue distribution of virus receptors can be an important determinant of host range and possibly even disease predilection (see Crowell, 1976; Weiss, 1976). Phenotypically mixed viruses, i.e., with mixed envelope antigens, may exhibit a host range similar to that of both parental strains (Weiss, 1976). Nevertheless, host susceptibility also involves genetically regulated intracellular factors unrelated to virus binding (Tucker and Docherty, 1975; Rey *et al.*, 1976).

The concept of a virus "receptor" complex ("viroceptor") is useful in the abstract, recognizing that the dynamics of virus attachment involves intramembranous mobilization of individual viroceptive molecules which aggregate and cross-link in loci subjacent to the virus particle (Howe *et al.*, 1970; Tillack *et al.*, 1972; Philipson *et al.*, 1976). Indeed, independent saturation of the cell surface by more than one class of virus particle indicates the formation of discrete viroceptor domains. Conceivably, more than a single species of viroceptive molecule may mobilize to provide a multivalent viroceptor configuration (Lonberg-Holm and Philipson, 1974; Choppin, 1976). Differences in affinity of molecules could account for experimental observations of relatively "loose" and "tight" degrees of virus particle binding (Pierce *et al.*, 1974). Evidence for viroceptor mobility was provided in experiments of Birdwell and Strauss (1974b); when Sindbis virus was adsorbed to glutaraldehyde-fixed cells, the particles counted by electron microscopy of surface replicas were evenly distributed; however, in unfixed cells the virus clustered in arrays of variable size, and lateral diffusion of viroceptive molecules evidently occurred even at 4°C.

Destruction of viroceptors by nonpenetrating treatment of intact cells with surface-active enzymes (see Kohn and Fuchs, 1973) such as neuraminidase (Tillack *et al.*, 1972), subtilisin (Philipson *et al.*, 1968), or trypsin (Levitt and Crowell, 1967), supports a model of asymmetric viroceptive molecules projecting outward from the cell surface (Tiffany and Blough, 1971; Marchesi *et al.*, 1976). Presumably, it is only peripheral moieties of these molecules which actually engage the cytotropic subunits of the virus particles.

Ultrastructurally, the intramembranous portions of viroceptive molecules

for myxoviruses appear as 75-Å diameter particulates within frozen-cleaved erythrocyte surfaces (Tillack *et al.*, 1972). These are typical of the IMP mentioned in Section III,C. Estimates of viroceptor numbers, based upon saturation of the cell surface with virus particles at low temperature, range from 10^3 per HeLa cell exposed to poliovirus type 1 (Lonberg-Holm and Philipson, 1974) to 10^4 per HeLa cell exposed to adenovirus type 2 (Philipson *et al.*, 1968) and 10^5 for chicken embryo cells exposed to Sindbis virus (Birdwell and Strauss, 1974b). From the latter figure, Birdwell and Strauss (1974b) calculated 20–160 Sindbis virus receptors for each μm^2 of cell surface. In view of probable aggregation dynamics, the actual numbers of individual viroceptive molecules should be severalfold greater than the numbers of attached particles, depending upon the virus circumference and valency (Philipson *et al.*, 1976).

Idiosyncracies in the attachment properties of enveloped viruses are as striking as their similarities and much remains to be learned. For example, some myxoviruses bind to artificial membranes (liposomes) only in the presence of sialylated fetuin (Tiffany and Blough, 1971), while Sendai virus and arboviruses bound to protein-free liposomes (Haywood, 1974, Mooney *et al.*, 1975). In contrast, removal of neuraminic acid from the surface of mouse cells actually increased the adsorption and infectivity of a rhabdovirus (Schloemer and Wagner, 1975). The host cell neuraminic acid evidently marks viroceptor molecules or prevents the formation of auxillary electrostatic bonds. Since rhabdoviruses have a remarkably wide host range, they presumably attach to a cell membrane component of relatively high frequency. Although the identity of the viroceptive molecules is not known, some functional properties were explored by Schloemer and Wagner (1975) in experiments in which goose erythrocyte or mouse L cell receptors for VSV were effectively blocked by fully sialylated fetuin or purified VSV oligoglycopeptides. If small VSV glycopeptides were generated by excessive trypsinization, the inhibition of hemagglutination was less effective. Either the viroceptor cites recognized sialoglycoprotein only in a restricted size range or cross-linking of sites—mimicking attachment of whole virus—is necessary for inhibition.

Attachment of nonenveloped viruses also involves a specific interaction with the cell surface which may be quite rapid (Mackay and Consigli, 1976; Philipson *et al.*, 1976). The viroceptors for adenoviruses and picornaviruses appear to be lipoproteins (McLaren *et al.*, 1968; Philipson *et al.*, 1968). When nonenveloped virus is adsorbed at low temperature, it is common for a large proportion of bound particles to elute spontaneously at 37°C (Crowell, 1976). This phenomenon has offered an advantageous tool for dissecting the interaction of cytotropic subunits and viroceptors, since the complex is often sufficiently firm to extract one or the other from its foothold in

the membrane. Thus, cells lose their capacity for agglutination by fresh virus and the virus is rendered noninfectious (Mandel, 1967; Crowell and Philipson, 1971). Polyacrylamide gel electrophoresis of eluted picornavirus particles demonstrates loss of a rapidly migrating polypeptide VP 4 which is located on the surface of the virion (see Crowell, 1976) and presumably represents the cytotropic subunit. This is consistent with inactivation by removal of the VP 4 in low ionic strength solutions (Cords *et al.*, 1975). Further evidence of the firm attachment between nonenveloped viruses and host viroceptors comes from studies of adenoviruses and papovaviruses. Dissociated virions undergo a shift in buoyant density which may be explained by extraction of a cell-bound cytotropic subunit or addition of an excised segment of the lipoprotein viroceptor from the host plasma membrane (Philipson *et al.*, 1968).

Ultrastructural studies have suggested that an antennalike fiber in the capsid penton of adenoviruses represents the cytotropic subunit which anchors to host viroceptors (Chardonnet and Dales, 1970). Blockage of adenovirus attachment by purified fiber preparations supports this contention (Philipson *et al.*, 1968). The adenovirus fiber is a 183,000 MW polypeptide complex with two glycosylated chains (Dorsett and Ginsburg, 1975).

Cytotropic subunits in the envelopes of myxoviruses, rhabdoviruses and arboviruses also provide specific moieties for attachment to the cell surface. The envelope of myxoviruses and of paramyxoviruses is rich in neuraminidase (Kendal and Kiley, 1973; Scheid and Choppin, 1974). In paramyxoviruses, the hemagglutinating and neuraminidase activity are combined in a single molecule (HN) on the virus envelope (Scheid, 1976) and are represented in the virus spikes visible by negative staining and in thin sections. This macromolecule is evidently the cytoropic subunit which combines with sialic acid-bearing viroceptor components on the cell surface. In contrast, the hemagglutinating and neuraminidase activities of myxoviruses are associated with separate proteins of the virus envelope (see Kendal and Kiley, 1973). Palese *et al.* (1974) suggest that influenza neuraminidase may prevent self-aggregation of virus inoculum particles or progeny, while Scheid (1976) postulates a role in the inactivation of molecules which might compete for attachment cites.

The envelope of a rhabdovirus, vesicular stomatitis virus, is formed from lipids and glycolipids of host cell origin and contains two viral proteins (cf. Shimizu and Ishida, 1975; Atkinson *et al.*, 1976; Knipe *et al.*, 1977a). One of these is a sialoglycoprotein associated with the envelope spikes visualized by negative staining. Selective hydrolysis of the sialic acid moieties with neuraminidase almost totally eliminates infectivity, but activity can be restored by resialylation (Schloemer and Wagner, 1974). Sialoglycolipids of host derivation found in the virus envelope did not appear to be involved in

virus attachment. Schloemer and Wagner (1975) confirmed that the positively charged polycation DEAE-dextran enhances the adsorption of VSV (Cartwright *et al.*, 1969) by twofold, but this did not increase infectivity and the sialoglycoprotein of the virus was still essential. Their results indicated that the virus neuraminic acid functioned as a true cytotropic subunit in the attachment process and did not merely influence the charge environment.

The biochemical nature of herpesvirus and poxvirus cytotropic proteins or viroceptors has not yet been extensively investigated. Inactivation of herpes simplex virus by the plant lectin concanavalin A (Ito and Barron, 1974) suggests the presence of a cytotropic glycoprotein.

D. *Fusion of Enveloped Viruses with the Cell Surface*

There are many examples of complex biological systems arising by evolutionary superimposition (e.g., Silverstein, 1964) and it should not be surprising if the long course of virus evolution in animal cells (see Kurstak and Maramorosch, 1974) had accumulated a plurality of potential mechanisms for critically vulnerable steps in the transmission of virus. Virus entry into host cells by fusion of the virion envelope with the host surface membrane possibly represents an evolutionary superimposition upon the more generalized route of particulate entry by engulfment. As one example, enveloped herpesviruses display a relatively sophisticated capacity to fuse selectively with host cells (Miyamoto and Morgan, 1971), yet herpesvirus cores or even free nucleoproteins also may initiate infection, presumably by the mechanisms of endocytosis or phagocytosis (Spring and Roizman, 1968; Abodeely *et al.*, 1970). Nucleocapsids of a rhabdovirus evidently also retain cytotropic subunits distinct from those on the virus envelope (Cartwright *et al.*, 1969).

The formation of multinucleate syncytia during infections by enveloped viruses such as influenza and varicella long suggested that these viruses might produce a factor which promotes membrane fusion. In 1962, Hoyle reported evidence for the incorporation of myxovirus envelopes into the cell surface using radiolabeled virus particles. Direct observation of the fusion of enveloped viruses with the cell surface was later achieved by electron microscopy of thin sections (Morgan and Howe, 1968; Heine and Schnaitman, 1971; Miyamoto and Morgan, 1971; Granados, 1973). This mechanism might be particularly advantageous for RNA viruses such as the myxoviruses, rhabdoviruses, and oncornaviruses in which the virion genome is complementary to the messenger RNA involved in virus polypeptide synthesis, so that a virion associated transcriptase is essential for initiation of the replicative cycle (Fenner *et al.*, 1974). Direct "transfusion" of relatively large and intact nucleocapsid structures into the host cytosol would minimize the risk of exposure to potentially damaging lysosomal nucleases.

Several elegant studies with hybrid antibody have demonstrated that specific virus envelope proteins marked by a ferritin label are actually engrafted and diffused into the host surface within minutes after virus attachment (Heine and Schnaitman, 1971; Wagner *et al.*, 1971; Bächi *et al.*, 1973) (Fig. 6). Consistent with virological and biochemical studies of virus attachment, this process is temperature independent. There is now substantial evidence that a specific glycoprotein of myxoviruses, distinct from the hemagglutinin or neuraminidase, is responsible for fusion of virion envelopes to the cell surface (Scheid, 1976). While fusion is dependent on an intact mechanism for virus attachment (Scheid and Choppin, 1974; Seto *et al.*, 1974), treatment with specific antibodies directed against individual virus envelope compo-

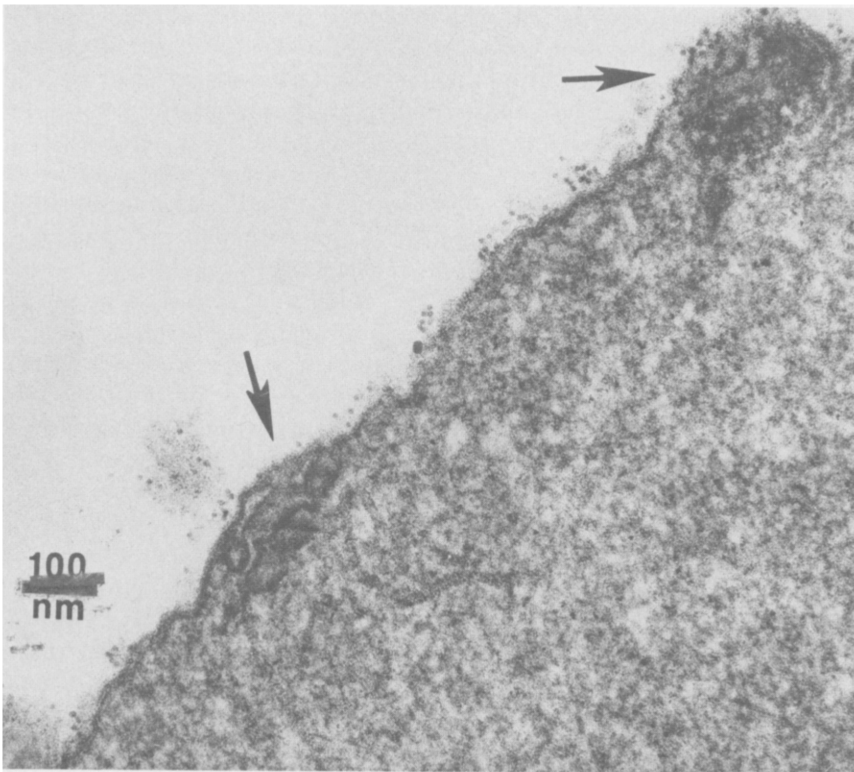


Fig. 6. Human erythrocyte, exposed to a paramyxovirus (Sendai) for 2 min, then reacted with ferritin-labeled antiviral antibody. Two aggregates of viral nucleocapsid material (arrows) indicate regions in which fusion of virions with cell surface occurred. Note that viral antigens have apparently diffused into the host membrane during the incubations. ×100,000 (from Bächi *et al.*, 1973; courtesy of Dr. Thomas Bächi).

nents confirms a functional dissociation of the attachment and fusion steps (Seto *et al.*, 1974).

E. *Transmembranous Penetration and Uncoating of Nonenveloped Viruses*

The complexity of steps in virus uncoating varies. Release of infectious materials from some of the small RNA viruses occurs almost simultaneously to translocation across the cell membrane. This may involve a relatively simple configurational rearrangement of the capsid molecules (Luria and Darnell, 1967; Philipson *et al.*, 1976) which allows them to unravel and release the contained genome. While this process is temperature dependent it may not require energy generation, active host cell metabolism, or new protein synthesis (Luria and Darnell, 1967; Morgan *et al.*, 1969). Changes in the symmetry of adenovirus nucleocapsids in transit through the cytoplasm have been interpreted as a reflection of this "configurational" uncoating process (Morgan *et al.*, 1969; Brown and Burlingham, 1973). Experiments with picornavirus suggest that uncoating and attachment can have a common locus on the plasma membrane, although they are sequentially distinct steps. Uncoating is selectively inhibited by low pH, glutathione or a microsomal factor (Roesing *et al.*, 1975).

Uncoating of virus nucleic acid is measured by release of acid-soluble polynucleotides prelabeled with ^{32}P (Chan and Black, 1970) and by their increased sensitivity to nucleases. This can be gauged by the proportion of soluble counts remaining after treatment of ^{32}P-labeled virus with TCA. The TCA soluble material is nuclease sensitive, indicating release from the protective nucleocapsid casing or protein "shell" of the virus (Chan and Black, 1970; Roesing *et al.*, 1975). The intracellular fate of released virus genetic material can also be pursued with radiolabeled inoculum (Dahl and Kates, 1970).

V. Membrane-Dependent Steps in Virus Maturation

A. *Membrane Complexes in Replication of Viral Nucleic Acids*

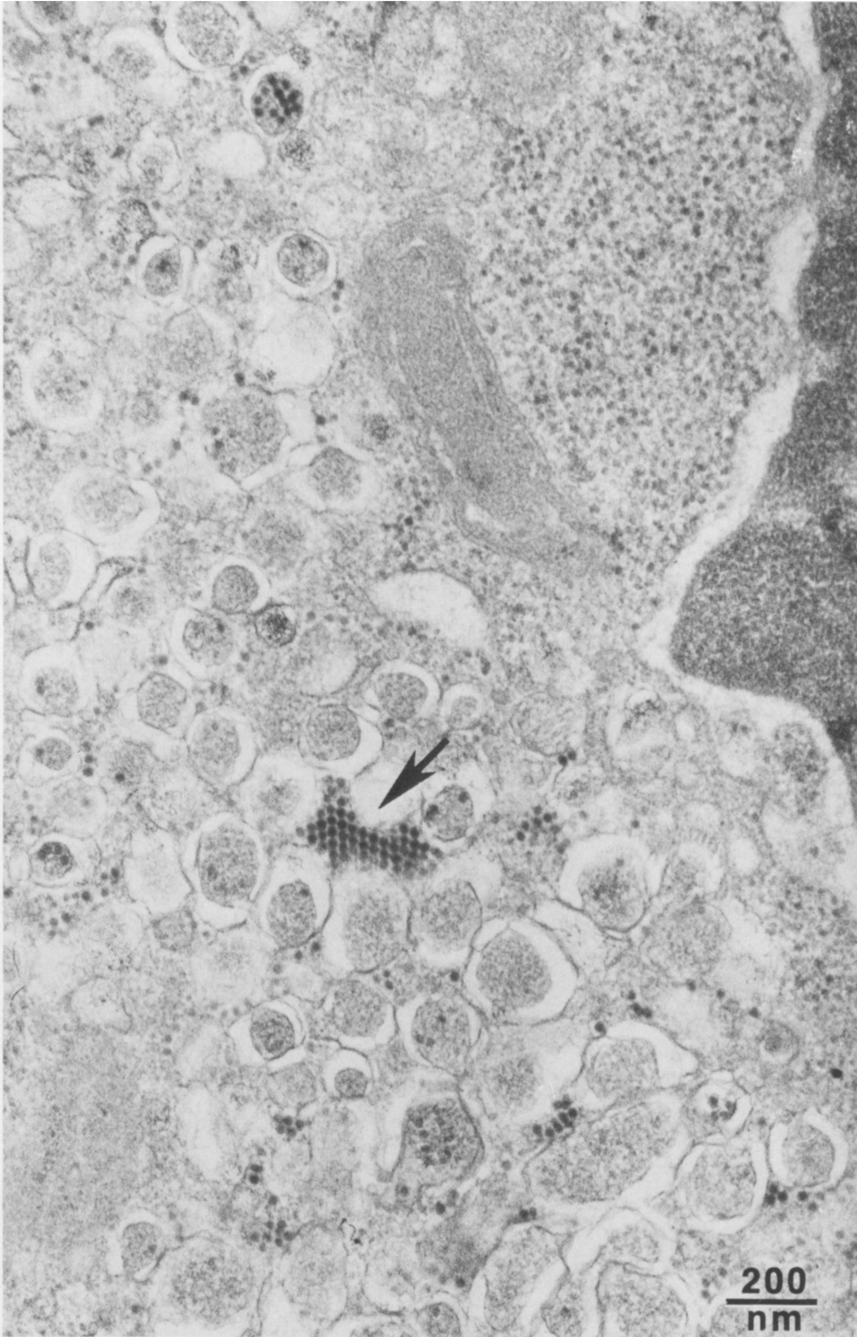
Production of virus progeny after penetration of parental virus into a host cell commences with transcription of the nucleic acid genome or its translation to polypeptides. In the case of intranuclear DNA viruses which have a large capacity to code for polypeptides (see Portner and Pridgen, 1975), the processes of DNA replication and RNA synthesis may parallel those of unin-

fected mammalian cells and expression of the viral genome may be similarly regulated by complex control mechanisms (see Sambrook, 1977). The cytoplasmic RNA viruses employ two major strategies for synthesis of viral polypeptides and replication of the genome (a) the genome RNA can itself be messenger RNA which is directly translated to viral polypeptides or act to replicate new genomes through an intermediate RNA strand with complementary base sequence; (b) the virion contains a transcriptase enzyme which enables its RNA genome to be transcribed to a messenger RNA of complementary base sequence. Complementary RNA strands then also serve as template for synthesis of new genome. The manner in which function of any one complete transcript is determined, i.e., message for viral protein synthesis or employment as a replicative intermediate, remains unresolved (see Portner and Pridgen, 1975; Spector and Baltimore, 1975).

Both picornaviruses and togaviruses employ a strategy in which virion genomes encode message and can be translated directly to polypeptides by host ribosomes and transfer RNA. For reasons not yet clear, replicative events in these infections also share an intimate and possibly unique association with internal cell membranes. The cytomembranes evidently provide a stable orientation for nascent viral enzymes, nucleic acids or proteins, thereby increasing the efficiency of interactions and facilitating assembly into nucleocapsids. At the ultrastructural level, this is manifested in a proliferation of smooth cytoplasmic membranes in picornavirus infections (Dales *et al.*, 1965; Amako and Dales, 1967b; Skinner *et al.*, 1968) (Fig. 7) and the morphogenesis of novel membrane structures in arbovirus infections (Blinzinger, 1972; Grimley *et al.*, 1972) (Figs. 8–11).

The development of poliovirus RNA begins in association with sedimentable cytoplasmic structures termed a *replication complex* (Girard *et al.*, 1967). Caliguiri and Tamm (1970a) dissected this process by biochemical analysis combined with subcellular fractionation. The membrane fractions were obtained with a modification of the technique developed by Bosmann *et al.* (1968) for isopycnic centrifugation in discontinuous sucrose density gradients. Pulse-labeling of nascent viral RNA with [^{3}H]uridine and of nascent viral proteins with [^{3}H]leucine demonstrated that replication of the poliovirus RNA genome and the transcription of genomes acting as messenger occurred in association with physically separable membrane elements (Caliguiri and Tamm, 1970a) resembling those described as membranous proliferations in thin sections (Fig. 7), whereas translation was associated with membrane-bound polyribosomes (granular ER). Smooth membrane iso-

Fig. 7. Proliferation of smooth cytoplasmic membranes and membrane-limited channels in cultured human cell infected by a picornavirus (polio type 1). Cluster of virus nucleocapsids in cytoplasmic matrix is prominent (arrow). ×60,000 (from Dales *et al.*, 1965; courtesy of Dr. Samuel Dales).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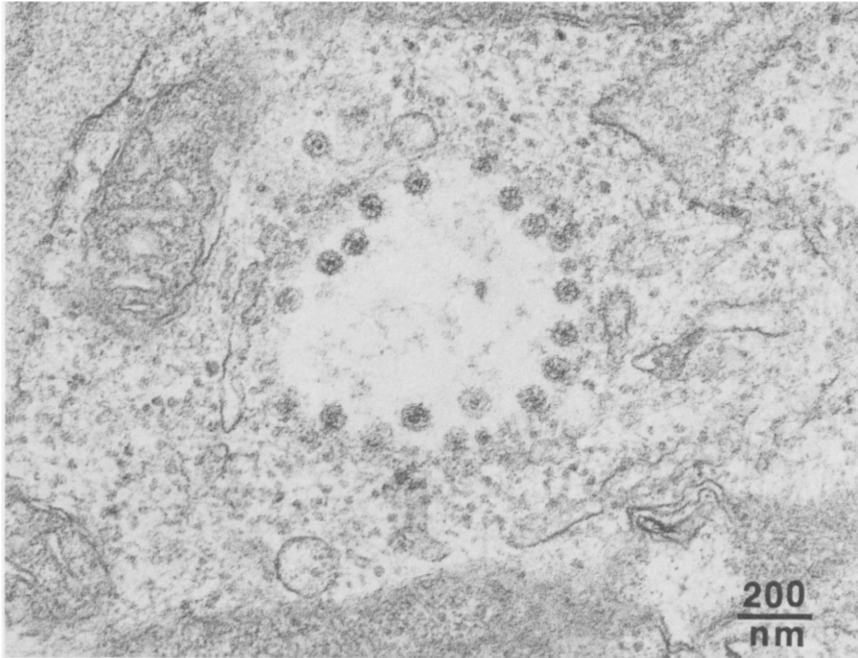
lates in the density range of 1.12–1.18 gm/cm^3 contained RNA species identified by velocity sedimentation and acrylamide-agarose gel electrophoresis as single-stranded virion RNA, and as replicative forms with complementary strands (Caliguiri and Tamm, 1970b). Most of the viral RNA polymerase activity also is associated with the smooth microsome fraction (Caliguiri and Mosser, 1971), and it has more recently been shown that solubilized poliovirus RNA polymerase spontaneously associates with phospholipid membrane bilayers *in vitro* (Butterworth *et al.*, 1976). Further experiments suggest that initiation of virus particle assembly in the form of "procapsids" is closely coupled to the membrane-associated replication complex (Caliguiri and Mosser, 1971). Assembly *in vitro* of poliovirus also can be mediated by membranes isolated from infected HeLa cells (Perlin and Phillips, 1973).

Picornavirus infection dramatically stimulates cellular incorporation of [^{3}H]choline into membrane lipid (Amako and Dales, 1967b; Mosser *et al.*, 1972b). In cells infected by mengovirus, Plagemann *et al.* (1970) reported a doubling or tripling of choline incorporation into membrane phosphatidylcholine. Using high-resolution autoradiography after pulse-labeling with [^{3}H]choline, Amako and Dales (1967b) localized this lipid precursor over the smooth membrane proliferations. Biochemical analyses of Mosser *et al.* (1972a) disclosed a higher phospholipid to protein ratio in smooth membrane produced after poliovirus infection and a corresponding decrease of the enzyme NADH diaphorase which normally is associated with the endoplasmic reticulum. This indicated an alteration of protein constituents in nascent membranes produced during virus infection. Presumably, viral replicase is inserted into these membranes (Caliguiri and Mosser, 1971).

The mechanism for regulation of new membrane synthesis in picornavirus infection is not known, but studies of Mosser *et al.* (1972a) suggest a shift of lipid precursors from the host rough endoplasmic reticulum (RER) to new smooth membranes. This seems analogous to formation of new smooth membranes from the RER in stimulated hepatocytes (Higgins, 1974). The control of membrane biosynthesis during virus infection evidently involves a complex interaction between virus and host (see Blough and Tiffany, 1975). Since picornavirus-induced membrane proliferation proceeds in the presence of actinomycin D (Caliguiri and Tamm, 1970a), it apparently does not rely upon transcription of new host message. Neither is the membrane proliferation necessarily coordinated with the rates of viral RNA (Mosser *et*

Fig. 8. (Top) Cytoplasmic vacuole with membranous spherules (CPV-1) in a chicken embryo cell culture infected for 8 hr with the alpha togavirus, Semliki Forest virus. ×48,000.

Fig. 9. (Bottom) Nucleocapsids of an alpha togavirus (Semliki Forest virus) surrounding sarcoplasmic reticulum in mouse skeletal muscle cell infected for 12 hr. Note some mature virions within cisternae. ×60,000.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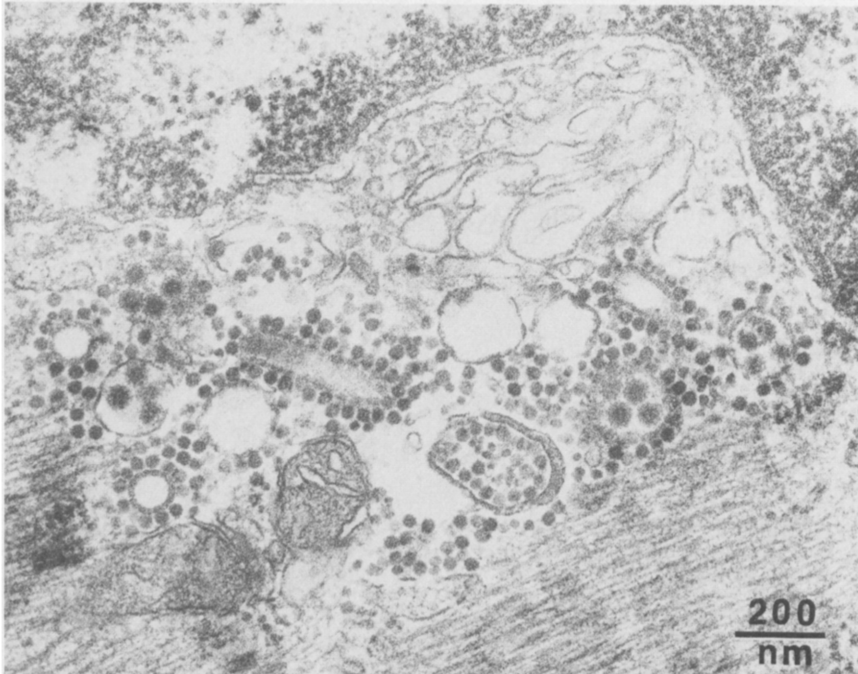
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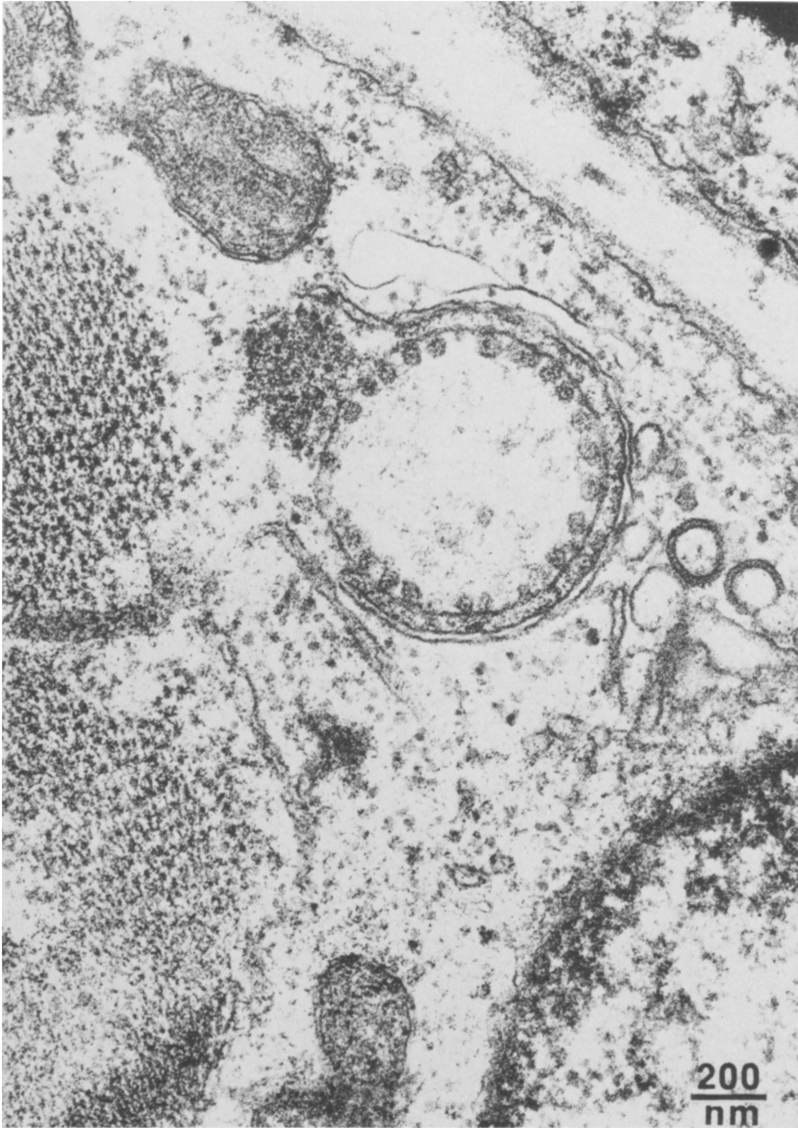
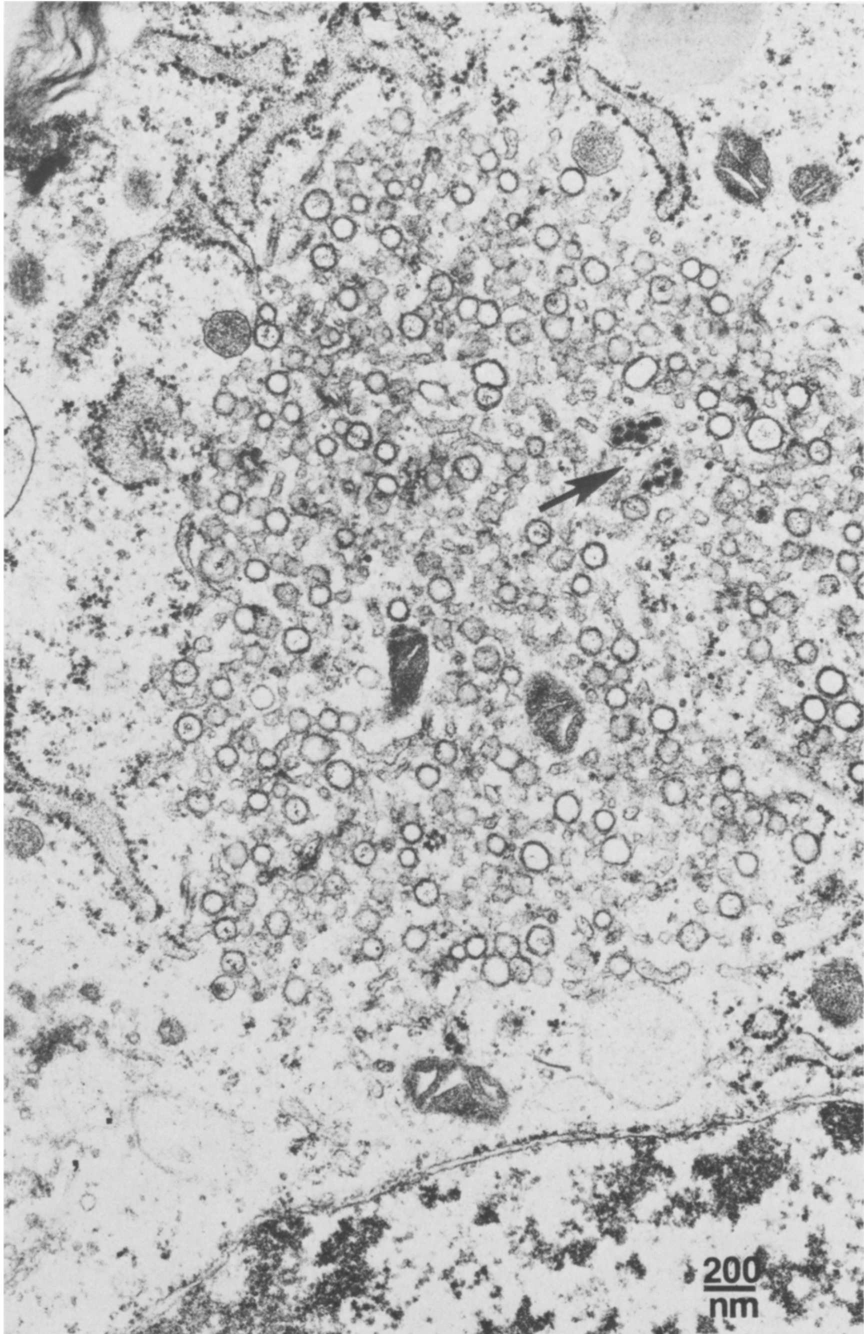


Fig. 10. Formation of CPV-1 in a mouse skeletal muscle cell at 7 hr after infection with an alpha togavirus (Semliki Forest virus). Note encircling profile of endoplasmic reticulum. Virus nucleocapsids are not evident at this time. ×48,000.

Fig. 11. Mosquito cell (*Aedes albopictus*) infected with a flavivirus (yellow fever). Tangential plane through the rough endoplasmic reticulum exposes numerous membranous spherules. Note mature virions within cisternae (arrow). ×33,000.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al., 1972b) or protein synthesis (Plagemann *et al.*, 1970). Conceivably, the interference of picornavirus infection with normal direction of cellular protein synthesis by host messenger RNA (see Spector and Baltimore, 1975) permits a selective proliferation of membranes, uninhibited by normal control mechanisms. A precedent for this concept is the evidence that smooth membrane proliferation in hepatocytes can be enhanced by low concentrations of actinomycin D, probably just sufficient to "derepress" normal controls (Orrenius and Ericsson, 1966).

Membrane structures also play a vital role in the development of togaviruses (arboviruses), although a net stimulation of membrane biosynthesis as noted in picornavirus infections may not be observed (Waite and Pfefferkorn, 1970b; Vance and Lam, 1975). The earliest ultrastructural evidence of infection with alpha togaviruses is often the appearance of 50-nm diameter membranous spherules with a fine central density (Fig. 8). These spherules are clearly distinguished from the smaller and denser virus nucleocapsids of enveloped virions (Fig. 9) and they become most numerous during the period of exponential virus growth (Grimley *et al.*, 1972). The spherules may be observed both at the cell surface and within large cytoplasmic vacuoles designated CPV-1 (Grimley *et al.*, 1972). The origin of CPV-1 membrane is uncertain. Thorotrast tracer from the growth medium may be included in CPV-1 suggesting origin by surface invagination. Cytochemical studies show the presence of acid phosphatase similar to that observed in the Golgi region (Grimley *et al.*, 1972), but another Golgi enzyme, TTPase was absent. The CPV-1 lack IDP associated with membranes of the RER, but they are frequently surrounded intimately by profiles of ER (Fig. 10). Freeze-fracture of CPV-1 membranes shows a lack of normal intramembranous particles within cleavage planes (Virtanen and Wartiovaara, 1974).

The possibility that membranous spherules represent a defective form of alpha togavirus was excluded by a series of experiments with alpha togaviruses passaged under different biological conditions and inoculated at low multiplicities of infection (Grimley *et al.*, 1972). The CPV-1 arise in tissue culture cells from several sources including human fibroblasts and HeLa cells and in mouse brain (Grimley and Friedman, 1970a) or striated muscle (Grimley and Friedman, 1970b).

Several lines of experimental evidence point toward a direct participation of CPV-1 in togaviral RNA replication. A large proportion of input virion RNA binds to host membranes within 1 hr (Friedman and Sreevalsan, 1970; Sreevalsan, 1970). Sedimentable membranous structures are associated both with nascent virus RNA (Friedman and Berezesky, 1967) and virus-induced viral RNA polymerase (Sreevalsan and Yin, 1969). When the cytoplasmic membranes of cells infected with an alpha togavirus were subjected to fractionation utilizing the approach of Caliguiri and Tamm (1970a), CPV-1 sepa-

rated with smooth membranes and mitochondria at a density of 1.16 gm/cm^3. This fraction was enriched in viral RNA forms and viral RNA polymerase (Friedman *et al.*, 1972). High-resolution autoradiography after pulse labeling of infected cells with [^{3}H]uridine during the time of exponential virus growth showed development of silver grains over CPV-1 (Friedman *et al.*, 1972). Membranous spherules were decreased in numbers or absent in cells infected by temperature-sensitive alpha togavirus mutants grown at a temperature where RNA synthesis was restricted to no more than 24% of normal levels (Tan, 1970).

The role of membrane structures in group B togavirus (flavivirus) infections has been less fully documented, but Qureshi and Trent (1972) reported that a membranous structure with an average sedimentation coefficient of 250 S associated with viral RNA forms, RNA polymerase and viral-specific proteins in St. Louis encephalitis infection. Stohlman *et al.* (1975) reported localization of dengue virus RNA synthesis to the rough endoplasmic reticulum, and suggested a role of smooth membranes in virus capsid maturation. Boulton and Westaway (1976) also noted a predominance of Kunjin virus RNA and protein synthesis on internal cytomembranes. Membranous spherules measuring 100–120 nm in diameter are prominent in the ER of cells infected with flaviviruses (Fig. 11) and their morphogenesis typically precedes the appearance of mature virions (P. M. Grimley and N. A. Young, unpublished). These intracisternal spherules have been found in flavivirus-infected mammalian or arthropod cell cultures (Filshie and Rehacek, 1968; Matsumura *et al.*, 1971), and brain tissue (Boulton and Webb, 1971; Blinzinger, 1972). The similarity to spherules lining CPV-1 in alpha togavirus infections is intriguing and suggests homologous functions but there is yet no direct evidence.

In flavivirus infections, formation of new membrane structures may be associated with increased incorporation of [^{3}H]choline (Zebovitz *et al.*, 1974). Surprisingly, this does not occur in alpha virus infection. Waite and Pfefferkorn (1970b) showed that Sindbis virus infection of chick embryo cells causes a progressive and indiscriminate reduction of phospholipid synthesis. Temperature shift experiments with a temperature-sensitive Sindbis mutant showed that limited replication of RNA at the nonrestrictive temperature was essential for inhibition of choline incorporation, but that structural proteins of the virus were not involved. Their experiments with chick embryo and baby hamster kidney (BHK) cells indicated that the virus inhibition of phospholipid synthesis paralleled effects of the metabolic inhibitors actinomycin D and cycloheximide. Vance and Lam (1975) showed that Sindbis virus infection inhibits incorporation of choline into cellular phospholipids and inhibits an enzyme involved in biosynthesis of phosphatidycholine. It can only be concluded that the striking membrane changes which occur at

the ultrastructural level in alpha togavirus infections must involve a highly selective redirection of residual membrane biosynthesis or a reorganization of preexisting membrane macromolecules. The latter was suggested by continued formation of CPV-1 for up to 3 hr in the presence of cycloheximide (Grimley *et al.*, 1972). This problem requires further investigation with individual cell membrane fractions.

B. Membrane-Associated Synthesis of Viral Proteins

As obligate intracellular life forms, viruses rely totally upon host cell support for translation of the viral genetic code and synthesis of viral polypeptides. The latter includes structural polypeptides which are incorporated into progeny virions and nonstructural polypeptides such as viral enzymes employed during nucleic acid transcription or steps in progeny virion maturation (i.e., polypeptide phosphorylation or posttranslational macromolecular cleavages). Size of the viral genome determines its capacity to code for polypeptides. For example, the relatively large DNA genomes of herpesvirus (82×10^6 MW) can theoretically code about 50 polypeptides of which over half have actually been identified (see Portner and Pridgen, 1975). Most RNA viruses have a more limited coding capacity (6–10 polypeptides). Thus, they provide relatively simple models to investigate the dynamics of membrane-associated protein synthesis, membrane integration, and virion maturation. Virus polypeptide biosynthesis can be specifically traced by introducing radiolabeled precursors at times after infection when host protein synthesis is restrained or totally inhibited by metabolic competition.

The site of messenger RNA translation may be on polysomes either within the cytosol or attached to membrane (RER). In uninfected mammalian cells, membrane-bound polyribosomes are typically engaged in synthesis either of proteins for secretion or of glycoproteins which become integrated into cell membrane. Lodish and Froshauer (1977) have reviewed evidence which suggests that attachment of ribosomes to the ER is mediated by the amino terminal sequence of nascent polypeptides. This "leader" may be very hydrophobic, so that the completed protein macromolecule immediately penetrates into the membrane bilayer near its origin of synthesis. Strong support for this train of events comes from studies of Sindbis virus (an alpha togavirus) by Wirth *et al.* (1977). In this very elementary model, the mature virion contains only three proteins: an internal core protein (C) and two envelope glycoproteins (E_1 and E_2). All three proteins are encoded by a single virus RNA (26 S), and synthesized by the same ribosome. The core protein is translated first in the cytosol. Enzymatic cleavage then exposes the amino terminus of protein E_2 which interacts with a proximate leaflet of ER and thereby binds the ribosome. After protein E_2 is cleaved it remains

embedded in the membrane, becoming an integral protein of the future virus envelope. Synthesis of protein E_1, also membrane-associated, can then begin. Protease treatment of membrane vesicles isolated with the newly synthesized Sindbis proteins, suggests that protein E_2 has a cytoplasmic tail (carboxy terminus), thus totally traversing the membrane bilayer.

Studies of vesicular stomatitis virus have also been of special interest, since the rhabdovirus employs a more complex mechanism in the synthesis of envelope membrane proteins. Nascent chains of a protein penetrating the virion envelope (G) are closely associated with membrane-bound polyribosomes, whereas a nonpenetrating, matrix protein (M), associated with the inner aspect of the virion envelope, is evidently synthesized in the cytosol (David, 1977; Knipe *et al.*, 1977b). These G and M proteins may be considered analogous to the integral and peripheral membrane proteins defined in the fluid-mosaic concept (see Fig. 3). Again, linkage of ribosomes to membrane of the ER in rhabdovirus infection is mediated by the nascent G polypeptide (Lodish and Froshauer, 1977).

In addition to providing templates for insertion of virus gene products and ultimate conversion to virion envelopes, host cytomembrane systems exert a remarkable role in the biosynthesis of virus envelope glycoproteins. Small viruses lack sufficient genetic endowment to direct synthesis of the multiple enzymes which would be required for this task in the absence of host cell aid (Sefton, 1976). Thus, differences in the sugar composition of a togavirus (Sindbis) and a rhabdovirus (vesicular stomatitis) grown in different cell lines reflect disparities in host cell ability to complete the glycosylation of virus-coded polypeptides (Etchison and Holland, 1974; Keegstra *et al.*, 1975). Even more striking was the finding by Stollar *et al.* (1976) that a togavirus grown in cells from a potential mosquito vector lacks sialic acid, whereas the same virus grown in vertebrate cells contains sialylated protein. This was not associated with any detectable biological or antigenic differences.

In paramyxovirus infections, the host cell asserts a role in maturation of a fusion-related glycoprotein of the virion. This glycoprotein arises by cleavage from a larger precursor (Scheid, 1976). After replication in bovine kidney (MDBK) or mouse L cells virions emerge with abundant precursor but little active protein. Such virus adsorbs to host cells but is incapable of causing infection and fails in tests for hemolysis or cell fusion. Virions maturing in the chick embryo allantoic sac have little precursor and abundant cleavage product. They are fully capable of infection and manifest both cell fusion and hemolytic activities. Treatment of the MDBK-grown virus with trypsin *in vitro* can induce infectivity, evidently by cleaving the fusion factor precursor (Scheid and Choppin, 1974).

From the standpoint of virus replication, the role of transferase enzymes in glycosylation of membrane-associated proteins is the major focus of inter-

est; however, viruses with a large genetic endowment, such as herpesvirus (Kaplan *et al.*, 1975) and poxvirus (Moss *et al.*, 1971b) may in fact produce nonstructural glycoproteins which conceivably modify cell behavior or interactions and thus play a pathobiological role in the infectious process at a tissue or organ level (Spear, 1975).

C. *Origins of Virus Structural Membrane*

A number of biochemical and ultrastructural studies, cited earlier (Section III,A), have provided evidence for the biosynthesis of membrane elements in the cell interior with peripheral transport of prefabricated units to renew the cell surface or to form the envelope of secretory products. The kinetics of viroceptor regeneration on the cell surface after removal by enzymatic treatment indicate that receptor macromolecules are integrated with newly synthesized membrane in the cell interior, then move centrifugally (Zajac and Crowell, 1965; Levitt and Crowell, 1967; Marcus and Schwartz, 1968; Philipson *et al.*, 1968). Time-lapse cinematography and phase microscopy convincingly document the intermittent motion of endoplasmic reticulum in cultured cells with reversible connections, subdivisions, and regroupings of the tubular or pancake-shaped compartments (Buckley, 1965).

Biochemically, the endoplasmic reticulum evidently comprises a patchwork of preexisting and newly formed elements (see Higgins, 1974). Virus-induced membrane proliferations appear to be similarly constructed (Amako and Dales, 1967b; Mosser *et al.*, 1972a). An orderly succession of molecular modifications presumably accounts for specific differences observed in the protein distribution and enzymatic activities of interior and peripheral cytomembranes (cf. Heath, 1971; Hirano *et al.*, 1972; Meldolesi and Cova, 1972). For example, some monosaccharide residues are attached to secretory glycoproteins almost concurrently with termination of the polypeptide core; however, glycosylation is consummated in a stepwise process, and the sequential addition of monosaccharides also appears to propel secretory glycoproteins (such as immunoglobulin) through the Golgi region toward peripheral cites of exocytosis (Melchers, 1973). This progression is predetermined by the localization of specific glycosyltransferases which are concentrated in specialized membrane elements.

Evidence for a very similar progression in protein maturation has emerged from investigations of the "template viruses" which utilize internal cytomembrane elements of the host as a scaffold for addition of virus-specific products. The former include most groups of the RNA viruses and the DNA herpesviruses. In certain virus groups, covalent sulfation of membrane glycoproteins also involves multiple steps beginning in the rough endoplasmic reticulum, and continuing through the smooth membranes or even at the cell surface (Nakamura and Compans, 1977).

Thermodynamically, the direct transfer of newly synthesized membrane protein from membrane-bound polyribosomes to nascent segments of phospholipid (Higgins and Barnett, 1972) would appear to be more efficient than a release of proteins from polyribosomes and cytoplasmic diffusion to loci of membrane incorporation. A direct transfer process appears to characterize formation of the integral envelope proteins in several RNA virus infections (Hay, 1974; Klenk *et al.*, 1974; David, 1977; Katz *et al.*, 1977; Wirth *et al.*, 1977). Other proteins, considered equivalent to the interior peripheral proteins shown in Fig. 3, associate with the envelope just before virion emergence and evidently pass through a membrane-free cytosol phase (David, 1977; Knipe *et al.*, 1977b).

The rhabdovirus, vesicular stomatitis virus, contains five structural proteins, two of which are associated with the envelope. A glycosylated protein (G) penetrates the lipid bilayer (Katz *et al.*, 1977) and forms the outer envelope spikes. After a brief radioactive pulse, G protein is associated both with a smooth membrane fraction and with a fraction containing RER (Wagner *et al.*, 1972). Glycosylation evidently occurs only during association of the G protein precursor with membrane (David, 1977; Knipe *et al.*, 1977a). A second membrane protein (M) is associated with the inner aspect of the virus envelope, arrives after the G protein and apparently is synthesized on free polysomes (David, 1977; Knipe *et al.*, 1977b,c). This protein can bind strongly to isolated plasma membranes (Cohen *et al.*, 1971) and is conceivably responsible both for anchoring the G protein spikes and stabilizing the internal helix of ribonucleoprotein (Blough and Tiffany, 1975). In contrast to G and M proteins, the nucleocapsid ribonucleoprotein (N) is not membrane associated (Wagner *et al.*, 1970) and apparently is synthesized on free polyribosomes prior to complexing with viral RNA in the cytoplasm.

Protein constituents of myxovirus envelopes are more numerous. The envelope spikes represent four glycoproteins. The largest glycoprotein (HA) functions as a hemagglutinin and may cleave during maturation (Lazarowitz and Choppin, 1975). Myxovirus glycoproteins are similarly localized in the RER shortly after synthesis begins, and subsequently with smooth membrane (Compans and Caliguiri, 1973). Individual sugar residues on the viral glycoproteins are incorporated stepwise with glucosamine being added in the RER and fucose being added on smooth membrane (Compans, 1973). The precursors of the envelope hemagglutinins and the envelope neuraminidase polypeptides, both are synthesized in close association with the RER and at least partially glycosylated there (Hay, 1974; Klenk *et al.*, 1974). The macromolecular precursor of hemagglutinin is cleaved to yield two polypeptides sometime during the traverse of smooth membranes to the cell surface. The M protein is incorporated as one of the last steps in envelope assembly (Nagai *et al.*, 1976), thus very simialr to the sequence in rhabdovirus envelope formation discussed above.

The sequence of myxovirus antigen association with cell membranes has also been traced by immunoelectron microscopy. This shows labeling of the RER within 4 hr after infection (Hoshino *et al.*, 1972; Ciampor *et al.*, 1974) and of the smooth membrane or cell surface at later times (Ciampor *et al.*, 1974; Lampert *et al.*, 1975). In studies of surface budding viruses, viral antigens typically localize in discrete patches on the plasma membrane exterior, corresponding to a zone of internal membrane thickening where the nucleocapsid attaches (cf. Howe *et al.*, 1969; Aoki and Takahashi, 1972; Coward *et al.*, 1972; Bächi and Howe, 1973). This may also be seen directly as formation of a "fuzzy coat" on the cell surface exterior, corresponding to glycoprotein spikes visualized by negative contrast or in freeze-fracture preparations (Bächi *et al.*, 1969).

D. Virion Assembly, Emergence and Release

In analyzing the terminal events of virus maturation, virions formed by budding can be divided conceptually along lines suggested by Blough and Tiffany (1975).

1. Nucleocapsids are fully assembled and tightly organized before budding into a tightly conforming envelope (e.g., herpesviruses, togaviruses).
2. Assembled nucleocapsids align beneath the cite of budding but are only loosely cloaked in an envelope of variable proportions (e.g., myxoviruses).
3. Final organization of assembled nucleocapsids occurs during emergence from the cell (e.g., rhabdoviruses).
4. Assembly of the nucleocapsid is concerted with emergence from the cell (e.g., oncornaviruses).

In the infections which form preassembled nucleoprotein, interaction of the cores with the inner membrane leaflet at a site of emergence appears to be the event which initiates outgrowth of a virus "bud" (Brown *et al.*, 1972; Dubois-Dalcq *et al.*, 1976a,b; Hashimoto *et al.*, 1975; Knipe *et al.*, 1977c). Intramembranous fluidity within this region is reduced (Sefton and Gaffney, 1974; Moore *et al.*, 1976) and the IMP normally detected by freeze-fracture typically disappear (Brown *et al.*, 1972; Sheffield, 1974; Dubois-Dalcq *et al.*, 1976a,b; Demsey *et al.*, 1977). At the same time, projecting spike proteins or globules characteristic of the mature virion envelope can be recognized ultrastructurally (Bächi *et al.*, 1969; Demsey *et al.*, 1977). In the case of togaviruses, myxoviruses, and rhabdoviruses, these projections represent the integral envelope-penetrating glycoproteins discussed in Section V, C. They typically arrive at the cell surface as long as 20 min in advance of virion emergence (cf. Lenard and Compans, 1974; Atkinson *et al.*, 1976), while posttranslational macromolecular alterations such as sulfation (Nakamura and

Compans, 1977), sialylation (Knipe *et al.*, 1977a), or cleavage (Lazarowitz and Choppin, 1975; Hay, 1974; Jones *et al.*, 1977) may still be in progress.

In myxovirus and rhabdovirus infections, attachment of a nonpenetrating "M protein" sets the stage for binding, positioning or shaping of the core (Blough and Tiffany, 1975; Shimizu and Ishida, 1975; Atkinson *et al.*, 1976). Recent studies of temperature-sensitive rhabdovirus mutants (Knipe *et al.*, 1977c) are consistent with this hypothesis. Mutations thought to involve the penetrating spike protein block membrane binding of both the nucleocapsids and M protein. On the other hand, mutations which cause failure in assembly of virus nucleocapsid do not block insertion of spike protein into membranes of the ER and migration to the cell surface. When the integral spike proteins are not stabilized by attachment of the peripheral M protein, structures of virion density fail to form. In myxovirus infection, the binding of M protein localizes to membrane cites in which penetrating hemagglutinin macromolecules are already present (Hay, 1974), while M protein is almost immediately incorporated into virions after attachment to the cell surface. Spike proteins are shed into virus from a larger pool of membrane-associated macromolecules and at a slower rate (Hay, 1974; Knipe *et al.*, 1977b).

A parallel disparity in the rates of shedding and pool sizes of newly synthesized core proteins and virion envelope precursors has been noted in oncornavirus infection (Witte and Weismann, 1976). An important mechanism in oncornavirus assembly is a terminal processing of large polyprotein intermediates (e.g., env-pr 85) to the macromolecular forms present in the virion envelope—gp 69/71, p15(E). This may involve sialylation and proteolytic cleavages (cf. Shapiro and August, 1976; Van Zaane *et al.*, 1976; Witte *et al.*, 1977).

Interaction of virus cores with envelope glycoproteins need not be specific. Virions of mixed phenotype (pseudotype particles) have been shown to emerge in a number of coinfections (Závada, 1972). In phenotypically mixed particles, spike glycoproteins of a paramyxovirus could be identified on virions with the morphology typical of a rhabdovirus (McSharry *et al.*, 1971). In this circumstance, however, the nonglycosylated M protein of the rhabdovirus segregated with the bullet-shaped nucleocapsids, further implicating an architectural interaction during rhabdovirus emergence.

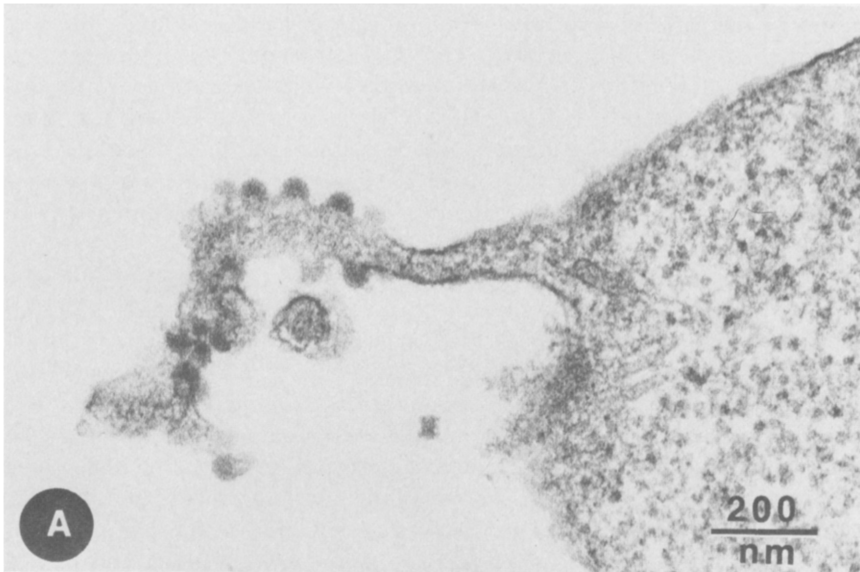
Since arrival of M protein and nucleocapsids can occur just minutes prior to emergence and release of a mature virion (Atkinson *et al.*, 1976; Knipe *et al.*, 1977b), it remains difficult to explore the temporal sequence of organization. Evidence thus far can be interpreted to suggest: (a) a primary conformational or phase change in a membrane domain which promotes interaction with physically abutting core material (Hay, 1974; Lenard and Compans, 1974; Rifkin and Quigley, 1974; Blough and Tiffany, 1975; Brown and Smith, 1975; Dubois-Dalcq *et al.*, 1976a; Jones *et al.*, 1977; Witte *et al.*, 1977); (b) an active intermediation of "director" molecules such as the M

protein to initiate the binding process (Shimizu and Ishida, 1975; Atkinson *et al.*, 1976; Knipe *et al.*, 1977c); and (c) a concerted "nucleation" of macromolecules triggered by physical contact and intrinsic properties of the nucleocapsid (Garoff and Simons, 1974; Hashimoto *et al.*, 1975). Interestingly, major shifts in the nucleci acid core composition of oncornavirus are not incompatible with budding of particles identified by density and negative staining (Levin *et al.*, 1974) and budding of Visna virus without "dense cores" has been observed (Coward *et al.*, 1972).

It is clear that any model of virion assembly must rest heavily upon the internal fluidity of membranes and lateral movements of viral or cellular proteins within and subjacent to the membrane (Birdwell and Strauss, 1974a; Hay, 1974; Blough and Tiffany, 1975). This assumption best explains observations that virus cores of different genotype and phenotype can emerge from physically proximate regions on the cell surface as observed by electron microscopy (Lunger and Clark, 1972; Grimley *et al.*, 1973). Furthermore many oncornaviruses incorporate host-specified glycoproteins within their relatively small and tightly fitted envelope. This is demonstrated both by immunoelectron microscopy and the capacity of purified virions to inhibit cytotoxic activity of various anti-H-2 sera. A curious aspect of this phenomenon, recently noted by Bubbers and Lilly (1977), is a selective incorporation of H-2 antigenic determinants into Friend leukemia virus, although the average proportion of such host surface molecules in each virion envelope probably is quite small (Aoki and Takahashi, 1972; Dorfman *et al.*, 1972). In general, experimental observations indicate that cell membranes of uninfected cells are far from saturated with integral proteins, and are thus able to accommodate virus-specified envelope glycoproteins by a simple process of lateral displacement perhaps involving steric segregation (Garoff and Simons, 1974). This is consistent with extensive "bare" regions in the phospholipid bilayer (see Section III,B), contiguity of host membrane and viral proteins observed in macrovesicles (Heine and Roizman, 1973), and quantitative persistance of host glycoproteins (Spear *et al.*, 1970; Birdwell and Strauss, 1973; Hay, 1974).

Depending on the virus group, virion emergence, and thereby envelopment, may proceed primarily at the inner nuclear membrane, through membranes of the ER, into cytoplasmic vacuoles, or at the cell surface (Fig. 12). While a functional interaction between the virus core and cell membrane at a cite of emergence depends upon complex local factors already discussed, general host cell conditions or the time after infection may also

Fig. 12. (A) Virions budding preferentially from filopodial projection on surface of a mouse L cell infected with an alpha togavirus (Semliki Forest virus). ×70,000. (B) Virions budding into channels of endoplasmic reticulum in neuron of a mouse infected with an alpha togavirus (Semliki Forest virus). ×60,000.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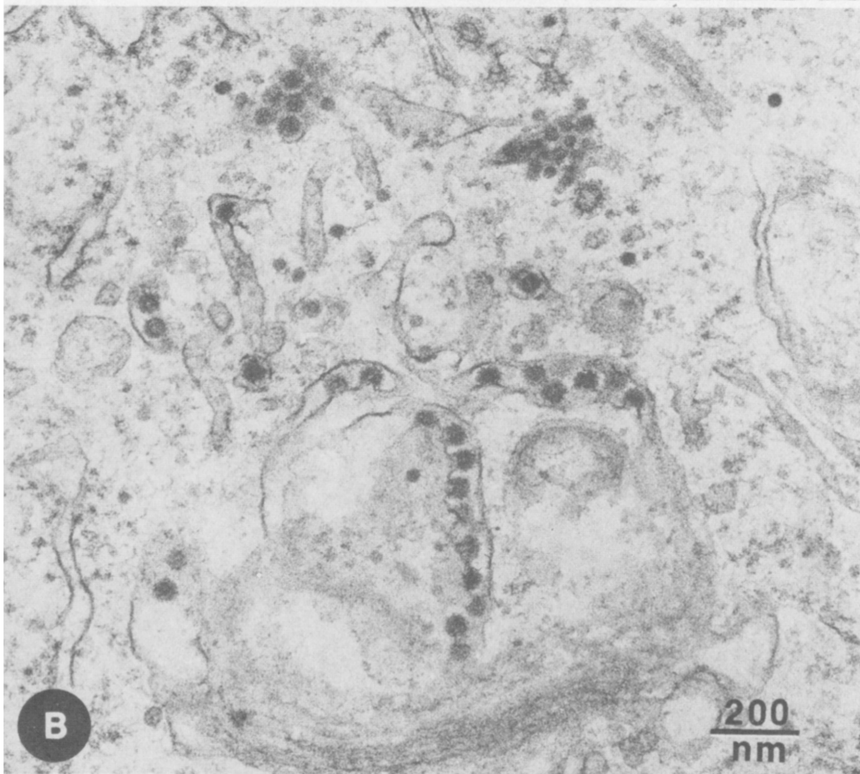
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influence the pattern of virion emergence (Grimley and Friedman, 1970a,b; Gliedman *et al.*, 1975) or ability to emerge from carrier cells (Dubois-Dalcq *et al.*, 1976a; Ray *et al.*, 1976). Although physical collision of nucleocapsids with membranes probably is a relatively random process (Wong and MacLeod, 1975) microtubular movements or cytoplasmic flow dynamics may produce vectorial forces which direct virus emergence into surface projections (Fig. 12) or cell body extensions (Grimley and Friedman, 1970a; Birdwell *et al.*, 1973; Dubois-Dalcq *et al.*, 1976b).

Normally, the nucleocapsid alignment with specific envelope components at a membrane cite of emergence proceeds within minutes (Witte and Weissmann, 1974; Knipe *et al.*, 1977a) and the terminal steps in virion release may occupy no more than 20 sec (Waite and Pfefferkorn, 1970a). Some transitional events in this process can be dissociated in temperature-sensitive virus mutants or by experimental treatments. In cells infected with alpha togavirus, temperature-sensitive cleavage of one of the viral envelope proteins (PE_2) appears to be essential for the budding process (Jones *et al.*, 1977), and the preassembled virus cores do not bind to the plasma membrane in cells infected at nonpermissive temperature (Brown and Smith, 1975). Exposure of cells infected with togavirus to media of reduced ionic strength results in subplasmalemmal accumulation of nucleocapsids (Waite *et al.*, 1972). In studies of leukemia virus mutants, Wong and McCarter (1974) observed a stage in which virus partially emerged from the cell surface but where final constriction of the bud and release was not possible at the restrictive temperature. They ascribed this to a reversible defect in protein conformation.

A constriction mechanism presumably triggers the final steps of membrane fusion at the base of each particle (Brown *et al.*, 1972; Blough and Tiffany, 1975), and ultrastructural studies with freeze-etching indicate separate fusions of inner and outer membrane leaflets to complete the virion envelope (Brown *et al.*, 1972). Release from the cell surface apparently depends on the ionic or mucoprotein environment (Grimley and Friedman, 1970b; Waite *et al.*, 1972) as well as the proximity of viroceptive molecules. Ability of myxovirus progeny to separate from the cell surface may be mediated by the virion neuraminidase (Palese *et al.*, 1974; Scheid, 1976) and can be inhibited by antibody to viral surface antigens (Dowdle *et al.*, 1974).

VI. The Nuclear Envelope in Virus Infection

A. *Segregation and Replication of the Virus Genome*

Segregation and amplification of functions by means of internal membrane systems accompanied the evolution of eukaryocyte organization and genetic

regulation (see Watson, 1976). The nuclear membrane separates the major genetic reservoir from the bulk of synthetic machinery which resides in the cytoplasmic compartment, and undirectional movement of informational transcripts (RNA) from the nucleus to the cytoplasm is basic to cellular control of protein synthesis (Goldstein, 1974; Watson, 1976). Intercompartmental flow of cations and polypeptides can also be vectorial (Goldstein, 1974). For example, histones and other nascent nucleoproteins preferentially enter the nucleus after synthesis in the cytoplasm and a rapid influx of molecules from the cytoplasm is associated with external stimulation of nuclear functions (Goldstein, 1974; Johnson *et al.*, 1974).

Intranuclear microenvironmental conditions which facilitate regulation of the cellular genome and processing of messenger RNA transcripts presumably sustain analogous functions during infection by viruses with a nuclear phase (cf. Honess and Roizman, 1974; Sambrook, 1977). Formation of nucleoprotein complexes occurs in several DNA virus infections (Chantler and Stevely, 1973; Seebeck and Weil, 1974; Meinke *et al.*, 1975; Gautschi *et al.*, 1976; Su and DePamphilis, 1976) and intranuclear assembly of viral nucleocapsids must depend upon appropriate physicochemical conditions for interaction of the viral proteins and nucleic acid (Gautschi *et al.*, 1976; Iida and Oda, 1975). Indeed, pseudovirions may contain host DNA (Qasba *et al.*, 1974).

A direct role of the nuclear membrane in initiating replication of viral nucleic acid, analogous to the role of cytoplasmic membranes discussed in Section V, A, has been debated for several years. In uninfected mammalian cells there appears to be no specific relationship of DNA replication to the nuclear membrane (Comings and Okada, 1973); however, the nuclear membrane does provide a major locus for orientation of DNA strands (see Comings, 1974; Franke and Scheer, 1974) and may selectively bind native DNA or synthetic polynucleotides (Kasper, 1974). In monkey cells infected by a papovavirus (SV40), LeBlanc and Singer (1974) found about 85% of DNA synthesis to be associated with a nuclear membrane fraction during a brief pulse-labeling with [^{3}H]thymidine. The mature viral DNA was evidently released after completion of replication. In adenovirus-infected human cells, Shiroki *et al.* (1974) found both parental virus DNA and nascent DNA in a nuclear membrane fraction. They identified DNA synthesized on nuclear membranes *in vitro* as a viral form by DNA–DNA hybridization. Almost coincidentally, Yamashita and Green (1974) reported the finding of new polypeptides closely associated with the isolated nuclear membranes of adenovirus-infected cells. These were interpreted as early viral gene products synthesized before replication of the adenovirus DNA. They appeared similar to DNA-binding polypeptides isolated from adenovirus-infected cells by Van der Vliet *et al.* (1975). Thus a nuclear membrane replication complex

for initiation of adenovirus synthesis with a DNA polymerase capable of transcribing viral DNA sequences (Ito *et al.*, 1975) appears to exist.

B. Function of the Nuclear Pore Complex

Despite some specific differences, both inner and outer nuclear membranes are fundamentally similar to the ER in terms of intrinsic macromolecular composition and enzymatic functions (Kasper, 1974; Berezney, 1974). Thin sections reveal that the nuclear envelope consists of paired membranes separated by a perinuclear cisternum which is intermittently continuous to cisternae of the granular ER (Blackburn, 1971). The outer nuclear membrane may have attached ribosomes and can participate in the synthesis of secretory proteins (Leduc *et al.*, 1968). The inner nuclear membrane appears asymmetrically thickened by a closely applied 300–600 Å deep layer of electron-dense material. This peripheral lamina consists primarily of polypeptides and is considered to provide a firm skeleton which supports and orients the nuclear pores (Aaronson and Blobel, 1975).

Nuclear pores or "channels" are the most significant microanatomic feature of the nuclear envelope (Fig. 13) since they probably represent the major pathway for nuclear–cytoplasmic macromolecular exchanges (Goldstein, 1974). Using the freeze-fracture technique and a computer program for calculation of topographic distributions, Maul *et al.* (1971) estimated a relatively constant pore to pore spacing, and the total numbers of nuclear pores were related to DNA content of the nucleus. Detailed fine structural studies of nuclear pores including negative staining of isolated nuclear envelopes indicate an orifice of relatively fixed diameter (ca. 60–80 nm) framed on inner and outer faces by annuli composed of symmetrical subunits which may be associated with fine fibrils (Franke and Scheer, 1974; Aaronson and Blobel, 1975). The center of the pore typically contains dense material or a granule (Blackburn, 1971). Electron images suggesting active extrusion of nuclear materials through pores have been obtained (Franke and Scheer, 1974), and some of the material occupying pore channels is evidently enriched in RNA, possibly representing messenger transcripts or ribosomal RNA in passage (Franke and Scheer, 1974; Goldstein, 1974). Migration is evidently restricted to the central part of the channel with a particle size range of 95–140 Å for

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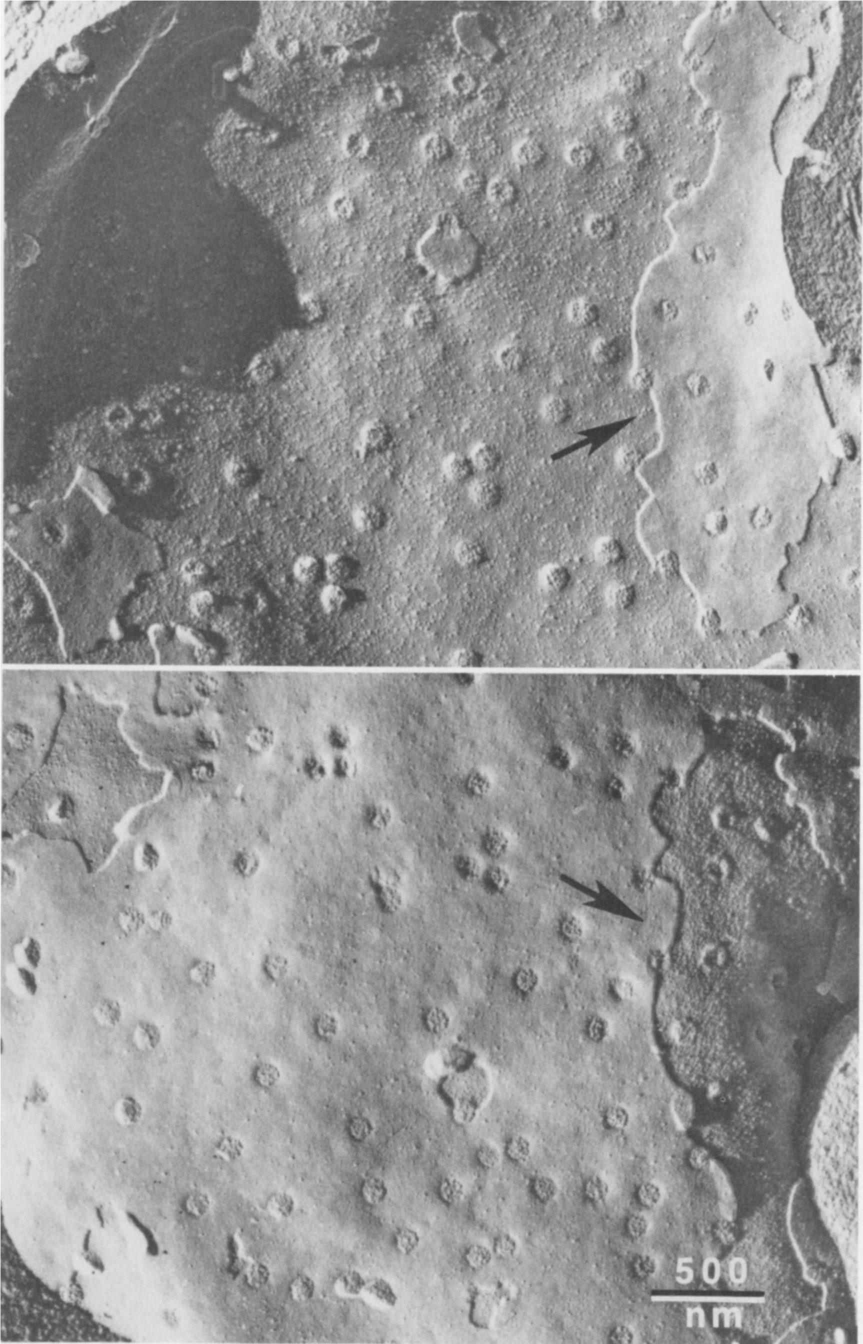
Fig. 13. Nuclear pores shown in complementary freeze-fracture replicas of the nucleus of an STU-Eveline cell. Most of the membrane surface revealed in the bottom micrograph is the EF surface of the outer nuclear membrane, although a step down to the PF surface of the inner membrane can be seen (arrow). The top micrograph reveals the apposing surfaces: i.e., mostly PF surface of the outer membrane, with some EF surface of the inner membrane (arrow). ×32,000 (from Demsey *et al.*, 1974).


500
nm

ferritin and gold respectively (Feldherr, 1972). This is sufficient for egress of papovavirus nucleocapsids (Maul, 1976).

As in normal cellular processes, the replication of intranuclear DNA viruses also depends upon an active exchange of materials between the nucleus and the cytoplasmic compartments. This begins at the time of virus entry when cores of engulfed herpesvirus, adenovirus, or papovavirus rapidly approach the nuclear envelope (Morgan *et al.*, 1969; Barbanti-Brodano *et al.*, 1970; Hummeler *et al.*, 1970; Dales and Chardonnet, 1973; Mackay and Consigli, 1976). Final uncoating of the infectious nucleic acid genome may occur on the cytoplasmic outlet of the nuclear pore complex (Dales and Chardonnet, 1973) or within the nucleoplasm (Morgan *et al.*, 1969; Barbanti-Brodano *et al.*, 1970), possibly involving active transport (Dales and Chardonnet, 1973). The subsequent movement of viral RNA transcripts back into the cytoplasm is apparently regulated (Kozak and Roizman, 1974), and the proteins of DNA viruses which are synthesized on free or membrane-bound cytoplasmic polysomes must finally return to the nucleus for assembly of virus progeny (Ben-Porat *et al.*, 1969; Velicer and Ginsburg, 1970; Mark and Kaplann, 1971). This can be a selective process. In herpesvirus infection, capsid proteins required for intranuclear particle assembly processes evidently are released into the cytoplasmic sap, whereas glycoproteins destined for the cell surface can pass vectorially through or reside in membranes of the ER (Kaplan *et al.*, 1975). Similarly, in the case of the intranuclear nucleocapsids of the RNA myxoviruses, proteins destined for the virus envelope do not appear in the nuclear inclusions (Maeno and Kilbourne, 1970). Under certain experimental conditions viral proteins normally expected in the nucleus may fail to migrate (Duff *et al.*, 1970; Ishibashi, 1970). This could be due to a defect in active transport at the level of nuclear pore complex (see Blackburn, 1971).

C. Ultrastructural Pathology

Gross configurational changes in the nuclear envelope occur in the course of normal cell growth, division, and maturation. Electron microscopy has shown that prophase dispersal of the nuclear envelope leads to formation of multiple individual membrane-bound cisternae and vesicles which lose asymmetry and become structurally indistinguishable from other constituents of the ER. Chromosomes are the organizing units responsible for reunification of the nuclear envelope during late anaphase and telophase when nuclear pores also reform (Franke and Scheer, 1974). Fluctuations in the numbers and distribution of nuclear pores also indicate that the nuclear envelope is a dynamic structure (Maul *et al.*, 1972).

Ultrastructural alterations of the nuclear membranes often are quite dra-

matic during herpesvirus infections and maturation of these viruses occurs to a large degree through the nuclear envelope. In fungal cells, herpes-type virus is temporarily enveloped by both inner and outer nuclear membranes in the passage from nucleoplasm to cytoplasm. These membranes are degraded in the cytoplasm and a final envelopment occurs by budding into cisternae of the Golgi apparatus or into cytoplasmic vacuoles (Kazama and Schornstein, 1973). Budding of herpesvirus nucleocapsids through the inner nuclear membrane can be readily observed in thin sections of vertebrate cells infected with herpes simplex (Darlington and Moss, 1969), herpes zoster, varicella (Achong and Meurisse, 1968), cavine herpes (Fong and Hsuing, 1977), Epstein-Barr virus (Glaser *et al.*, 1976), and cytomegaloviruses (Berezesky *et al.*, 1971). Herpes virions can traverse the nuclear membrane individually or in groups presenting a "peas in the pod" appearance (Fig. 14). The latter evidently segment into individual particles, since visualization of more than one nucleocapsid per virion envelope is very rare. The route of transfer from the perinuclear cisternum to the cell surface can only be surmised from thin-section observations. Before cytolysis or during infections by less virulent members of the herpes group, virions evidently can be transported to the cell surface within the system of ER cisternae and Golgi vesicles which eventually open to the exterior (Darlington and Moss, 1969). Nii *et al.* (1968) suggested that maturing nucleocapsids with a dense nucleic acid core bud preferentially through the nuclear envelope. On the other hand, Schaffer *et al.* (1974) observed that nucleocapsids of a temperature-sensitive herpes simplex mutant (ts022) with empty and partial cores could be preferentially enveloped at the restrictive temperature.

While relatively little is yet known about the molecular interactions at cites of nuclear virus budding, a freeze-fracture study (Haines and Baerwald, 1976) suggests events similar to those at the cell surface with loss of intramembranous particles (see Sections V, D). The envelope of herpes simplex virus contains new species of glycoproteins, but no appreciable loss of host membrane proteins is noted (Heine *et al.*, 1972). These observations suggest lateral displacement of host membrane proteins as discussed above (Section IV,E). Experiments of Ben-Porat and Kaplan (1971) indicated that herpes-type virions can assemble from newly synthesized segments of the inner nuclear membrane.

Breakdown of the nuclear envelope may occur early in herpesvirus replication, even prior to extensive disruption of normal cellular organization (Fig. 15). One possible explanation is a viral induction of host cell DNA synthesis (Melvin and Kucera, 1975) leading to an abortive prophase condition in which the nuclear envelope begins to fragment. Nucleocapsids then escape through gaps and mature at membrane surfaces in the cytoplasm rather than at the nuclear envelope (e.g., Fong and Hsuing, 1977). Studies of

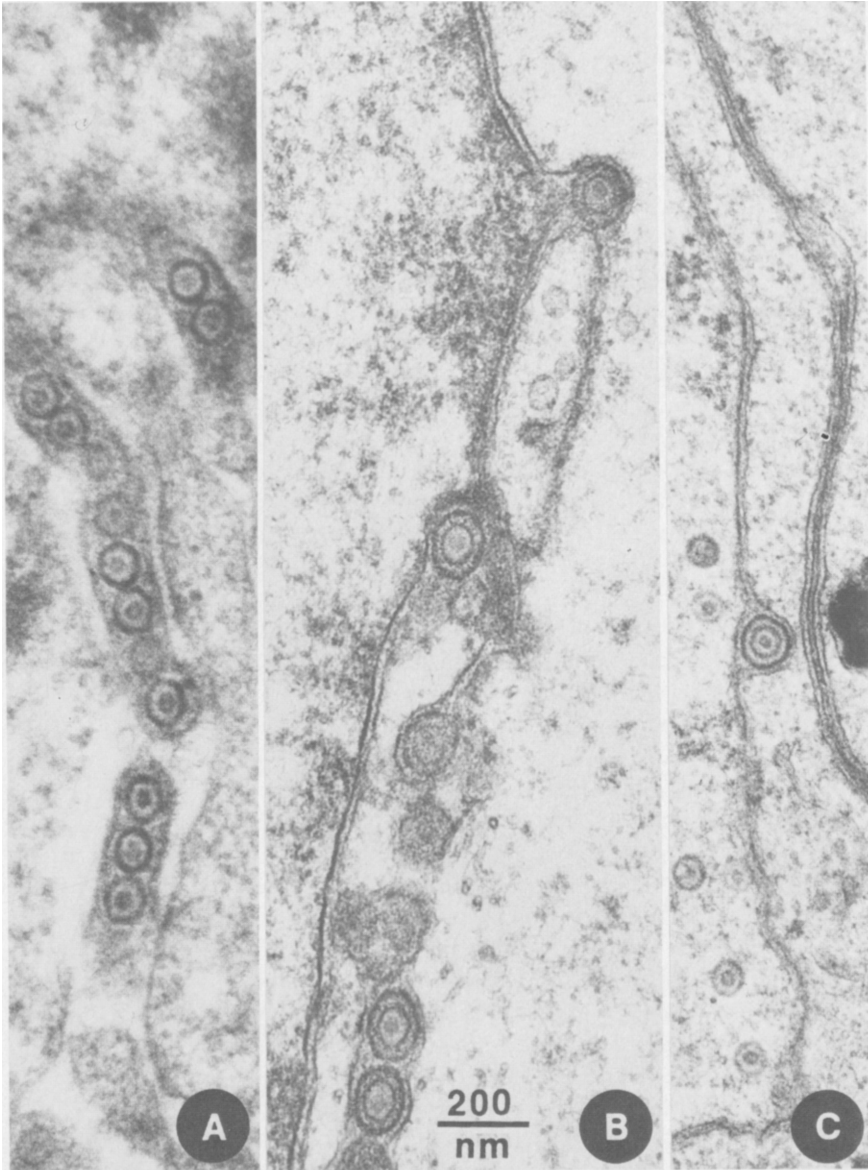


Fig. 14. Human lymphoma cells infected with herpes simplex virus. (A) Immature nucleocapsids extruded from nucleus in common outpouchings of the inner nuclear membrane. (B) Enveloped nucleocapsids which have budded through the inner nuclear membrane lie within endoplasmic reticulum cisternae connected to the perinuclear cisternum. (C) Comparison of immature intranuclear nucleocapsids and mature virion in the perinuclear cisternum. Reduplicated lamella of nuclear envelope appears to the right. ×60,000.

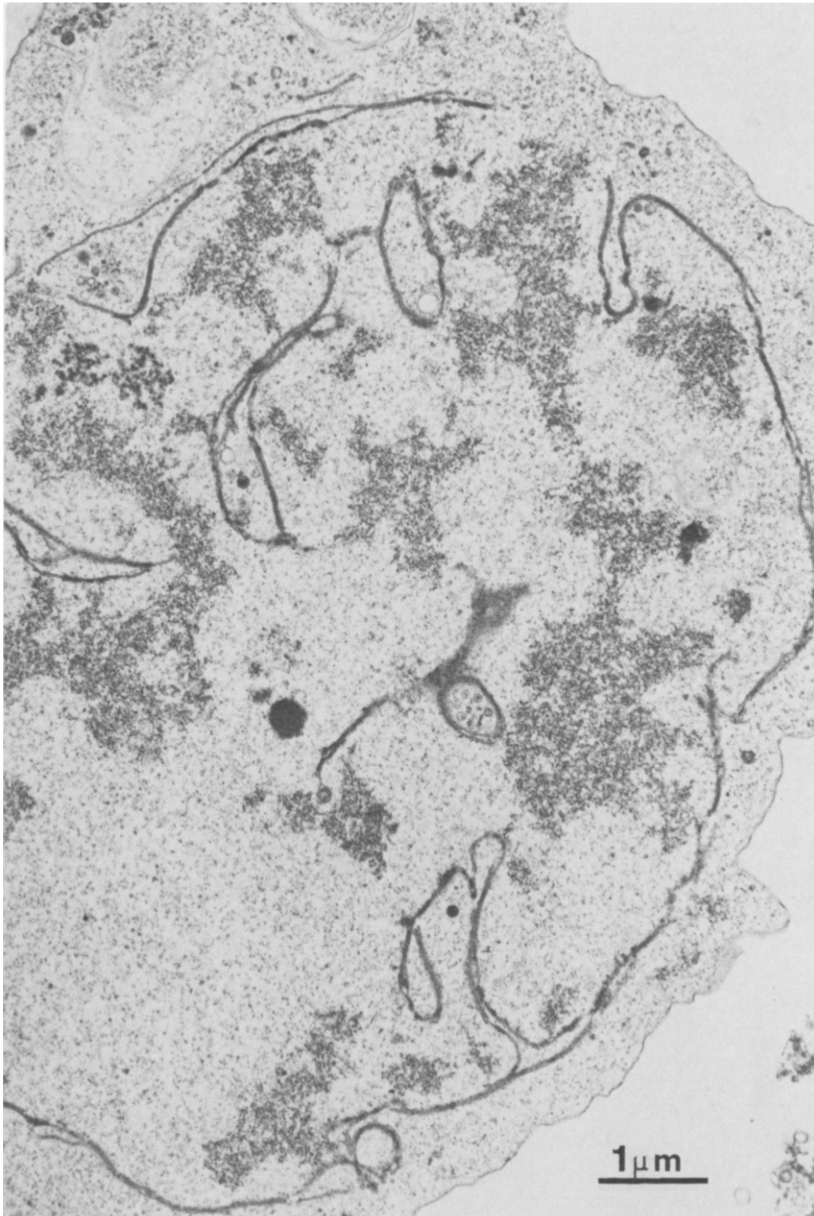


Fig. 15. Human lymphoma cell infected with herpes simplex virus. Nuclear envelope shows large discontinuities and abnormal reduplications of limiting membranes. Pattern of nuclear densities suggests chromosomal condensations. ×14,000.

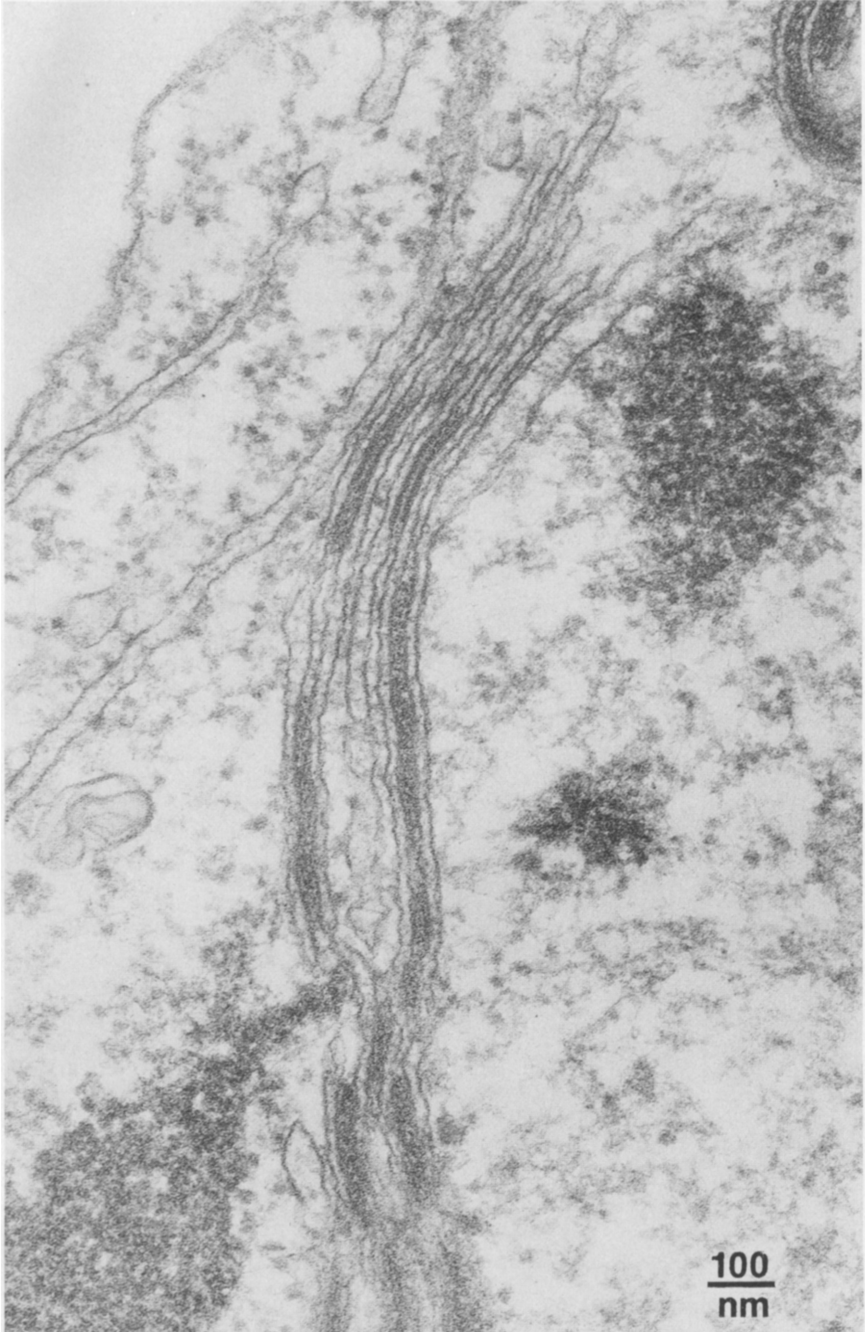


Fig. 16. Reduplications of nuclear envelope in human lymphoma cell 48 hr after infection with herpes simplex virus. ×78,000.

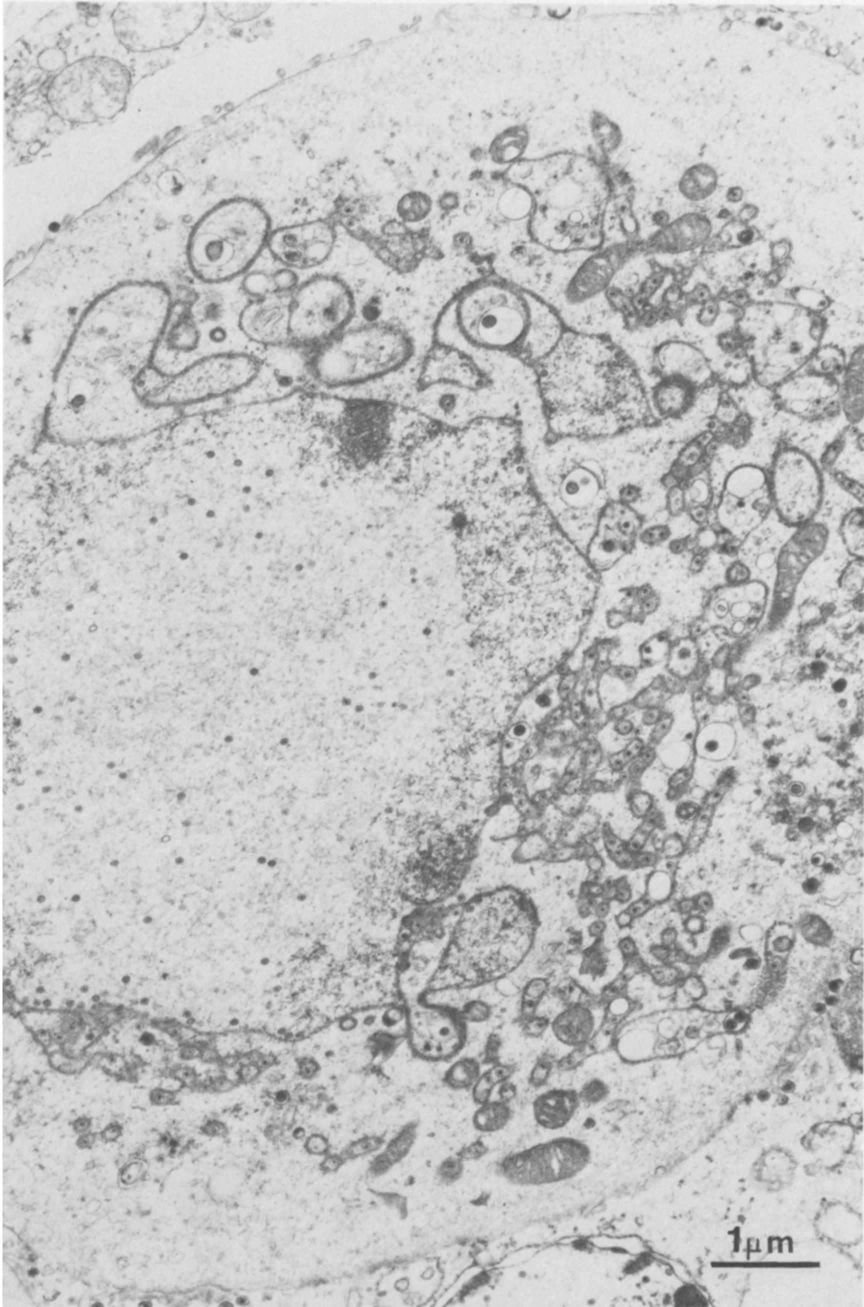


Fig. 17. Tortuous protrusions and reduplications of nuclear envelope in monkey kidney cells 24 hr after infection with herpes simplex virus. ×14,000.

a rat cytomegalovirus showed extensive accumulations of nucleocapsids in the cytoplasm of cells with apparently intact nuclear envelopes (Berezesky *et al.*, 1971). These nucleocapsids often lacked a dense core and were surrounded by an osmiophilic matrix. Some of them, however, appeared to represent complete nucleocapsids and budding into cytoplasmic vacuoles was observed. Similar observations have been made in other cytomegalovirus infections and could be explained by a maturation process in which a temporary nuclear envelope disintegrates (Kazama and Schornstein, 1973).

An almost pathognomonic feature of herpesvirus infection is a remarkable pairing or redundant stacking of nuclear membranes and the extrusion or drawing out of the nuclear envelope in tortuous projections, "blebs", and extended lamellae. Some of the envelope nuclear extensions may represent "short-circuit" bridge connections between regions of the outer nuclear membrane as described by Franke and Scheer (1974). In part, however, the ultrastructural appearance indicates that the inner nuclear leaflets and an associated 100–150 Å layer of dense granulofibrillar material or heterochromatin accompany the projections (Fig. 16). At low magnification, the overall pattern often suggests an elaborate lacework (Fig. 17). While generally related to an active process of virus envelopment, this membrane activity can also occur in abortive infection (Schaffer *et al.*, 1974; Glaser *et al.*, 1976). The finding of active phospholipid metabolism in pseudorabies virus-infected rabbit kidney cells (Ben-Porat and Kaplan, 1971) strongly suggests that the morphological changes reflect stimulated nuclear membrane synthesis analogous to the cytomembrane proliferations observed in picornavirus infections (Section V, A). Fusion or reduplications of paired envelope cisternae is noted also in adenovirus infection (Gregg and Morgan, 1959). These phenomena may be manifestations of pathobiological cytomembrane interactions due to synthesis of viral cytotropic or fusion proteins (see Sections IV, C and D) which insert indiscriminately. Redundancy of the nuclear envelope also occurs under conditions of active protein synthesis (Mollo *et al.*, 1969). Conversely, cell differentiation, as in spermatogenesis or leukopoiesis, often involves a contraction of the nuclear mass (Merriam, 1962) with formation of redundant nuclear envelope. Extreme examples of the latter are the polymorphonuclear leukocytes. Separation of envelope sheets in the form of annulate lamellae also can rapidly decrease nuclear surface area (Guylas, 1971). Formation of annulate lamellae noted in adenovirus-infected cells by Merkow *et al.* (1970) appeared to originate in close proximity to the nucleus within 1 hr after infection.

The frequent occurrence of nuclear bridges, projections, or pockets in lymphocytes (Pope *et al.*, 1968) is of some relevance to the discussion of

herpesvirus infections, since members of this virus family, notably EBV, are lymphocytotropic and have been implicated in the pathogenesis of lymphomas (Miller *et al.*, 1977). No direct correlation should be drawn between these characteristics of lymphocyte nuclei, and the changes observed in experimental herpesvirus infections although they may appear superficially similar. Nuclear "pockets" projections or "blebs" are common in normal human leukocytes (Huhn, 1967; Smith and O'Hara, 1968) as well as in atypical and leukemic cells (Ahearn, 1967; Mollo and Stramignoni, 1967). Anomalous nuclear projections also have been described in a chromosomal triplication disorder (Lutzner and Hecht, 1966). These observations merely reinforce the concept that the nuclear membrane responds in an active manner to a variety of normal and pathological stimuli including virus infection.

VII. Membrane-Related Cell Reactions

A. *Activity of Lysosomes*

The disposition of hydrolytic enzymes and associated "inflammatory proteins" contained within primary lysosomes is governed by the limiting membranes of these microvesicular organelles. Fusions of the lysosomal membranes with phagosomes containing engulfed virions initiates a process of intracellular digestion similar to that observed with other particulate nucleic acids and proteins (see Friend *et al.*, 1969; Ericsson and Brunk, 1975). Intact virions may persist in phagolysosomes for several hours, but they are ultimately degraded and infectivity is abolished (Ogier *et al.*, 1977). As discussed in Section IV,B, infective virions probably escape the lytic pathway entirely, or liberate their nucleic acid within phagosomes before evolution to phagolysosomes. The proportion of inoculum virions which is shunted into the lytic pathway appears to vary with the virus group and even amongst serotypes (Dales, 1973; Ogier *et al.*, 1977). Only phagosomes with engulfed diplornaviruses appear to fuse preferentially with primary lysosomes (Dales, 1973). Since the diplornaviruses contain a nuclease-resistant (double-stranded) genome, lysosomal degradation can be advantageous in releasing their infectivity. Presumably, the single-stranded genomes of most other viruses are at least partially sensitive to lysosomal nucleases (Ogier *et al.*, 1977).

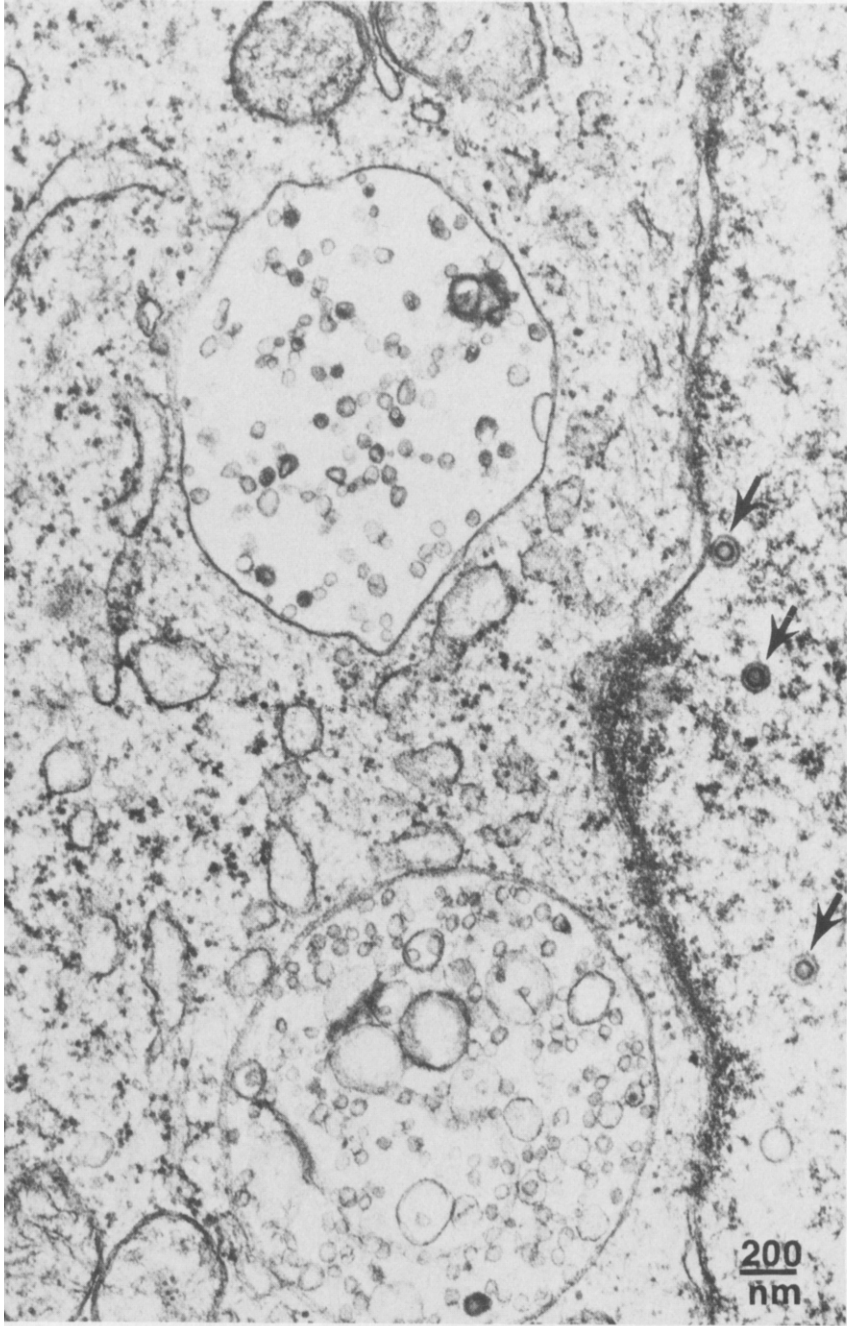
Evidence for an increase in lysosomal enzyme activity during virus infection comes from histochemical, cytochemical, and biochemical studies (Ruebner *et al.*, 1966; Allison, 1971; Greenham and Poste, 1971; Reeves and

Chang, 1971). During some virus infections, large numbers of multivesicular bodies appear (Fig. 18). These membranous structures are apparently related to the lysosomal system (see Anteunis, 1974) and may be particularly common in cytomegalovirus infections (Berezesky *et al.*, 1971).

In specialized defensive cells such as granulocytes and macrophages, lysosomal hydrolases and associated proteins accumulate and concentrate within secretory vesicles which transport them to the cell surface. Fusion of these lysosomal membranes with the plasma membrane is the normal route for egress. When release of these products is provoked under appropriate conditions, local destruction of foreign materials or microorganisms and stimulation of the inflammatory response can be beneficial to the host. When the release of lysosomal enzymes is disproportionate to the noxious stimulus or even inappropriate, harmful destruction of host tissues may ensue. Such over-reaction may propagate chronic inflammatory or connective tissue diseases (Hamerman, 1966). In certain virus infections, an increased production and extracellular secretion of lysosomal enzymes probably occurs (Ruebner *et al.*, 1966; Greenham and Poste, 1971; Reeves and Chang, 1971). In laryngotracheitis virus infection of fowl, this leads to a destructive chondrolysis in the turbinate cartilage beneath infected nasal mucosa (Schultz and Bang, 1977). Such extreme effects are probably rare; however, increased contact of lytic enzymes with the exterior of virus-infected cells may be an important factor in the process of cell to cell fusion (see Greenham and Poste, 1971).

A large number of experimental studies have been directed toward measuring changes in the permeability of lysosomal membranes during virus infection (see Allison, 1971; Tamm, 1975). This can lead to significant shifts in the intracellular distribution of acid hydrolases. In normal processes of cell division or differentiation, controlled and selective release of hydrolytic enzymes conceivably facilitates specific biochemical processes or moderates the environment for gene regulation (Allison, 1967). Pathological implications of incontinent hydrolytic enzyme release into the cell sap range from subtle effects on chromosomes (see Allison, 1967) to obvious weakening of the cell framework and cytolysis. In virus infections, particularly infections by nonbudding nucleotropic viruses, lysis of host membrane from within may facilitate the escape of progeny virions (see Tamm, 1975). A number of experiments have indicated that virus cytopathic effects occur more rapidly than expected on the basis of direct metabolic inhibition with drugs (see Tamm, 1975). The cytopathic effects in picornavirus infection appear to depend upon a virus-directed function and may even be retarded by treatment with metabolic inhibitors during the early stages of virus infection

Fig. 18. Multivesicular bodies in perinuclear cytoplasm of rat kidney cell infected with rat strain of cytomegalovirus. Note intranuclear herpes-type nucleocapsids (arrows). ×40,000.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(Amako and Dales, 1967a; Guskey and Wolff, 1974). Perhaps the most likely explanation for these phenomena is modification of lysosomal membranes by insertion of viral proteins (Allison, 1971); however, molecular mechanisms which account for labilization of lysosomal membranes by virus proteins remain to be elucidated.

B. *Intracellular Membranous Proliferations*

Proliferation of smooth membranous elements associated with the ER or the nuclear envelope is a common subcellular response to virus infections. As previously detailed in Sections V,A and VI,C, some membrane alterations are integrally related to specific events in the virus replication cycle, including nucleic acid transcription, nucleocapsid assembly, or virion maturation. In this section, we draw attention to membranous proliferations which evidently represent a secondary subcellular reaction in virus-infected cells and characteristically appear after the onset of virion maturation. The functional significance, if any, of such cytopathologic phenomena remains obscure.

On an ultrastructural basis, nonspecific membrane proliferations which occur at a relatively late stage of virus replication may be subdivided into two general groups:

1. Arrays of smooth endoplasmic reticulum which closely resemble those observed in drug-treated hepatocytes (Hutterer *et al.*, 1969). The membrane profiles are relatively heterogeneous in dimensions (200–400 nm) and do not assume any organized pattern. This type of proliferation was observed by Heine and Dalton (1974) in cultured fibroblasts infected with varicella-zoster virus. We have observed similar smooth membrane elaborations in cultured human lymphoblasts or primate kidney cells infected with herpes simplex or cytomegalovirus.
2. Arrays of membranous tubules which share a relatively uniform dimension 200–300 nm), occupy dilated cisternae contiguous to or within the rough ER, and typically assume organized patterns. Membranous tubules which assume a reticular pattern (Fig. 19) typically occur in togavirus infections. They can be observed *in vitro* and *in vivo*. Membranous tubules with a more compact pattern are exemplified in Tana poxvirus infection (España *et al.*, 1971).

Of related interest is a family of membranous inclusions comprised of relatively electron-dense tubular elements with a similar range of dimensions (200–300 nm). These may be subdivided into compact and tubuloreticular forms (Schaff *et al.*, 1973). In contrast to any of the mem-

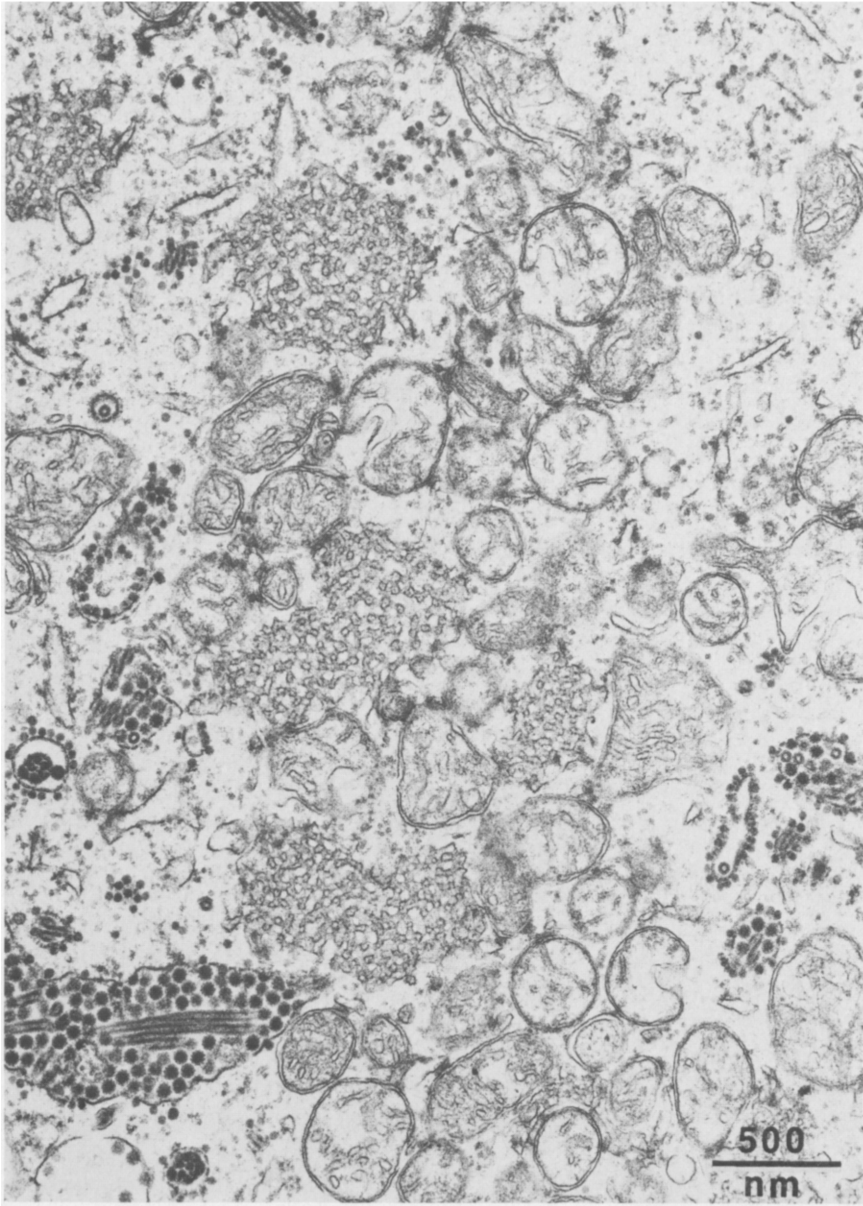


Fig. 19. Proliferation of anastomosing membranous tubules within a neuron of a suckling mouse infected with an alpha togavirus (Semliki Forest virus). Virus nucleoids surround profiles of endoplasmic reticulum. ×33,000.

branous proliferations described thus far, compact tubular inclusions and tubuloreticular inclusions are not usually found within the same cell sections as replicating virus. Rather, they appear to arise in cells adjacent to foci of infection, possibly in cells with occult virus. One hypothesis is that the tubular inclusions reflect a systemic or local immune response to virus. A comprehensive review of this subject has lately been published (Grimley and Schaff, 1976) and an experimental model of tubuloreticular inclusions has been developed in Burkitt lymphoma cells treated with halogenated pyrimidines (Hulanicka *et al.*, 1977).

C. Membrane Fusions

Observation of multinucleated cells is a classical histopathological clue to the presence of a virus infection. With development of tissue culture techniques for growth of viruses *in vitro*, it quickly became obvious that this phenomenon was due primarily to aggregation and fusion of infected cells rather than to repeated endomitosis. In recent years the molecular basis of this phenomenon has been elucidated by ultrastructural, biochemical, and biological studies. In all cases, cell to cell fusion has been related to an insertion of virus gene products into host cell membranes.

There are two basic mechanisms for cell to cell membrane fusions—fusion "from within" and fusion due to external attachment of virions (Bratt and Gallaher, 1972). In myxovirus infections, the same molecules are involved in both processes and represent the cytotropic subunits of the virion which engage viroceptive molecules on the cell surface to create a firm attachment (see Section IV,C). Cell to cell fusion can be blocked by specific antibodies to envelope glycoproteins (Seto *et al.*, 1974) or by lectin binding (Ludwig *et al.*, 1974).

As discussed in Sections V, B and C, the cytotropic subunits responsible for cell fusions are viral proteins synthesized on the endoplasmic reticulum and translated to the cell surface by continuous peripheral movements of recycled or new membrane segments. Final maturation of these molecules requires cleavage of a larger glycoprotein precursor near or at the cell surface (Scheid, 1976). The delayed "activation" may prevent premature sticking of paired internal membranes before they can evert by fusion to the cell surface. Once at the cell surface, and before assembly into virions, cytotropic subunits can engage viroceptive molecules on adjacent uninfected cells. This may result in pathological fusions (Feldman *et al.*, 1968; Iwasaki and Koprowski, 1974) or cell aggregations (Larke *et al.*, 1977). In the central nervous system, cell to cell fusions induced "from within" may create a privileged pathway for spread of virus without exposure to humoral or cell-mediated

immunological defenses (Iwasaki and Koprowski, 1974). In virus carrier cells which do not permit deployment of viral antigen at the plasma membrane, bud formation is defective and cell fusion is minimized (Dubois-Dalcq *et al.*, 1976a). The efficiency of fusion may also be subject to extracellular conditions such as pH (Gallaher and Bratt, 1974). Greenham and Poste (1971) suggest that activation of lysosomes is an important prelude to cell fusion and that acid hydrolases prepare the cell surface for attachment—perhaps by attenuating the glycocalyx.

In herpesvirus infections, membrane fusions are evidently mediated by nonvirion glycoproteins which lavishly coat interior cell membranes as well as the plasmalemma (Roizman and Kieff, 1975). Indeed, these virus gene products may be produced in such excess that they ultimately accumulate on the cytoplasmic aspect of cell membranes. A resultant "stickiness" probably explains the frequency of internal membrane fusions (Figs. 20 and 21). In contrast to myxovirus infections, hyperimmune serum directed against the virion antigens fails to inhibit polykaryocytosis (Ludwig *et al.*, 1974).

Poxvirus infection can also induce cell to cell fusion by production of a nonvirion protein. This evidently reaches the surface with mature virions by a unique mechanism. The virions become enwrapped by paired membrane elements in the Golgi membrane. The outer membrane element fuses to the cell surface while the inner membrane ruptures to effect release of the virion (Dales *et al.*, 1976). Poxvirus strains which produce hemagglutinin fail to induce fusion and vice versa. Dales *et al.* (1976) suggest that the fusion protein may actually contain the same polypeptide core as the hemagglutinin but lack an acceptor region for terminal glycosylation.

Cell to cell fusion mediated externally by viruses is probably of little consequence under natural biological conditions. Its main interest is as an experimental and diagnosic tool. In the field of cell biology, introduction of inactivated myxovirus (Sendai) for the purpose of producing heterokaryons had an almost revolutionary impact since it provided a reproducible working method applicable to a large variety of cell types (see Sidebottom, 1973). Fusion of XC cells by murine leukemia virus provides an important biological assay. A heat-labile virion protein appears to activate the syncytium formation (Johnson *et al.*, 1971).

The process of cell to cell fusion probably is essentially similar to that described during virion adsorption and entry into cells (Section IV,E). Despite a number of efforts to examine these processes at an ultrastructural level, however, the fine mechanism by which approximated continuous membranes open and reunite remains unclear (see Sidebottom, 1973; Okada *et al.*, 1975). New technical and experimental approaches will be required to resolve this question.

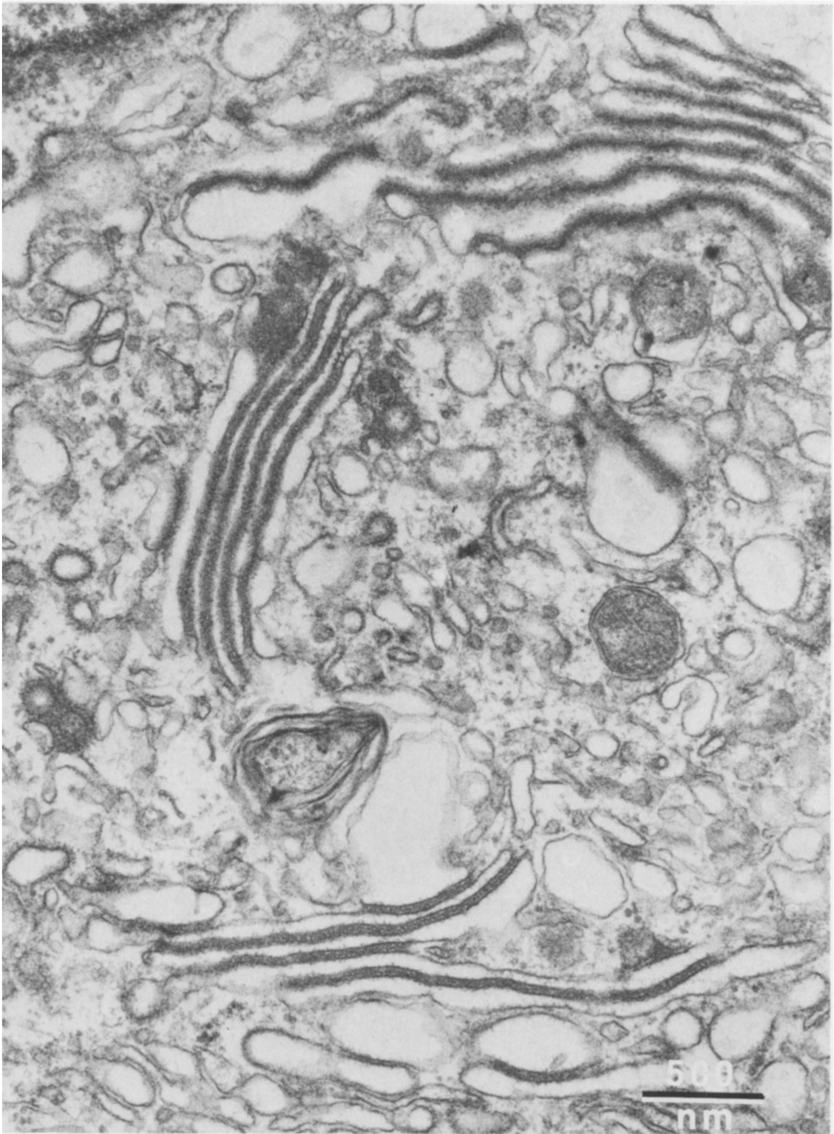
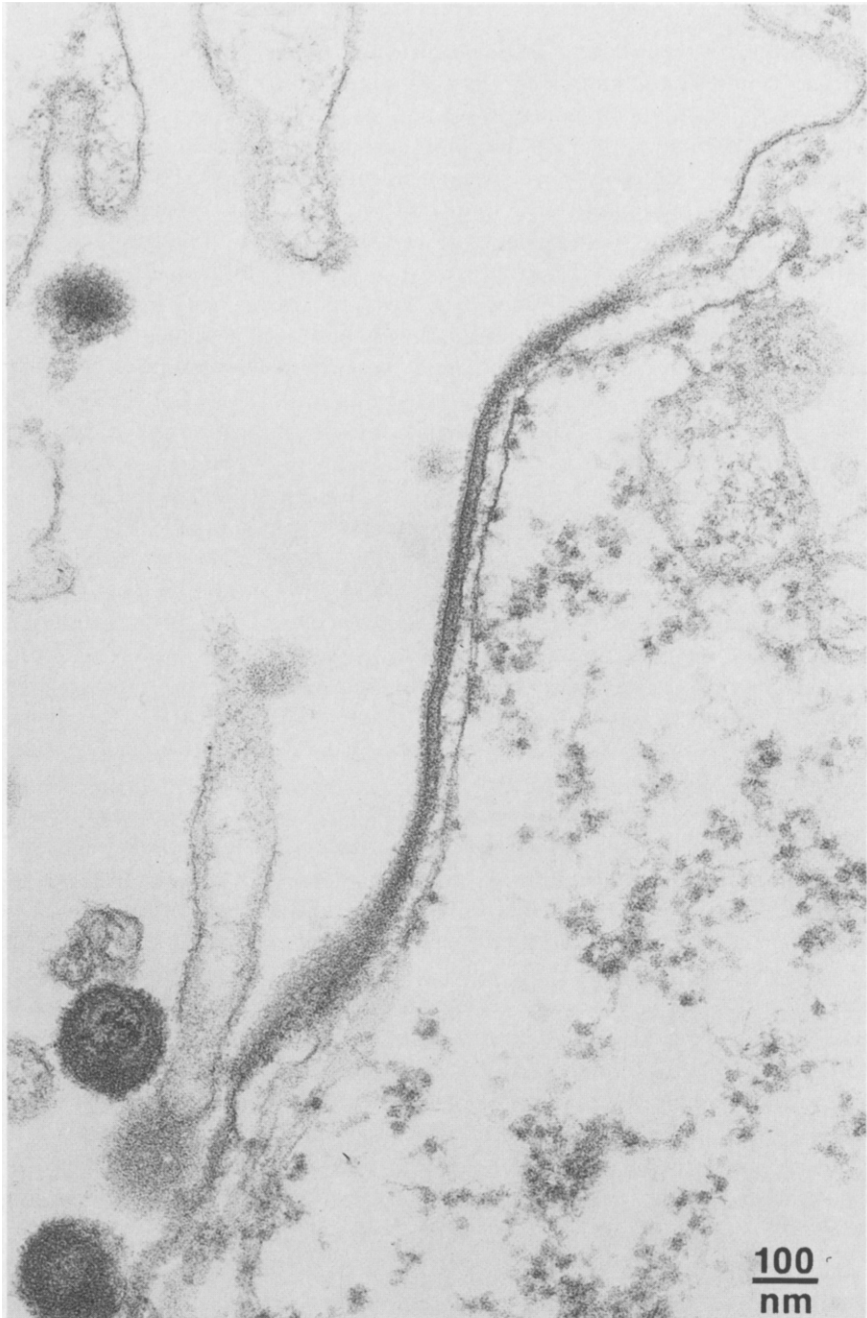


Fig. 20. Fusions of Golgi membranes in rat kidney cell infected by rat strain of cytomegalovirus. ×33,000.

Fig. 21. Fusion of endoplasmic reticulum (ER) with plasmalemma in human lymphoma cell infected by herpes simplex virus. Note ribosomes on cytoplasmic aspect of ER, and fuzzy coat (presumably virus glycoprotein) exterior to zone of fusion. ×82,000.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D. *Effects at the Cell Surface*

Insertion of virus-coded proteins into membranes which form the cell surface is an integral step in the maturation of most enveloped viruses (see Sections V, B and C). In contrast, changes in the pattern of host membrane lipid composition appear to be minimal during active virus infections (Blough *et al.*, 1977). A more profound reorganization of the cell surface, involving host membrane glycolipid patterns as well as glycoproteins, accompanies the process of phenotypic transformation by oncogenic viruses (see Glick *et al.*, 1974; Brady, 1975; Hynes, 1976), and may change the strength of normal cellular antigens (see Ting and Herberman, 1971). Acquisition of a neoplastic potential under these conditions involves a relatively stable change in cellular behavior, and the surface configuration also may reflect a more active metabolic state (see Sheinin, 1974). For example, in cells actively producing C-type virions surface amplification is typical (Fig. 22), but the extent of surface irregularity appears to depend upon a complex interaction of proviral and cellular genes (see Perecko *et al.*, 1973). The gene interactions may even result in an independent expression of virus envelope glycoprotein without concordant production of the virion core elements (Bilello *et al.*, 1974; Ledbetter *et al.*, 1977). From this perspective, the subject of cell surface reorganization after oncogenic virus transformation extends well beyond the scope of present discussion. Our attention is confined to just two subjects previously addressed by Allison (1971), both wih a potential influence on the pathobiology of active virus infections: (a) surface changes conditioning "social behavior" of cells; and (b) surface changes conditioning the immune response.

In the broadest sense, the "social behavior" of cells includes responsiveness to growth controls as well as cell movements and local interactions (Sheinin, 1974). Surface effects of active virus infection in tissues are limited to the latter phenomena. Relatively little is known of the immediate effects on membrane mobility, although scanning electron microscopy suggests increased activity at the cell surface in virus-producing cells (Fig. 22) and retraction or extension of cell processes may be observed in infected cell cultures. Considerably more is known about the effects of virus on local interactions. This has been treated in Section VII,C, as it relates to intercellular fusions of fixed cells and even aggregations of free-flowing platelets (Larke *et al.*, 1977). Effects of these processes on the pathobiology of infections, particularly chronic diseases of the central nervous system (Dubois-Dalcq *et al.*, 1976a), remain an important area for future investigation. Two

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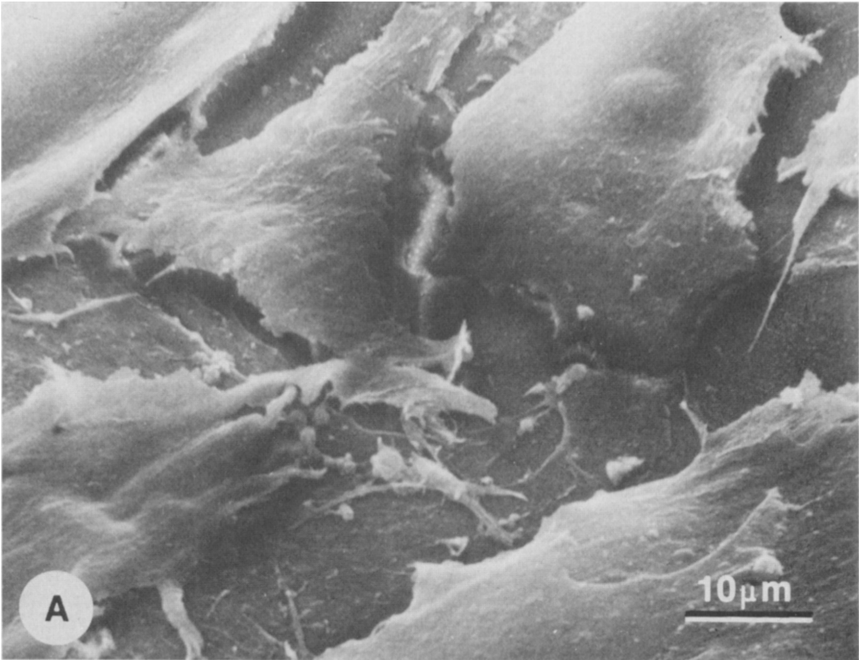
Fig. 22. Surface features of mouse cells in secondary electron scanning of samples prepared by the critical point method. (A) Primary culture of mouse embryo fibroblasts with relatively flat surfaces. ×1680. (B) Established mouse line $JLSV_9$ transformed by Rauscher virus. ×4000.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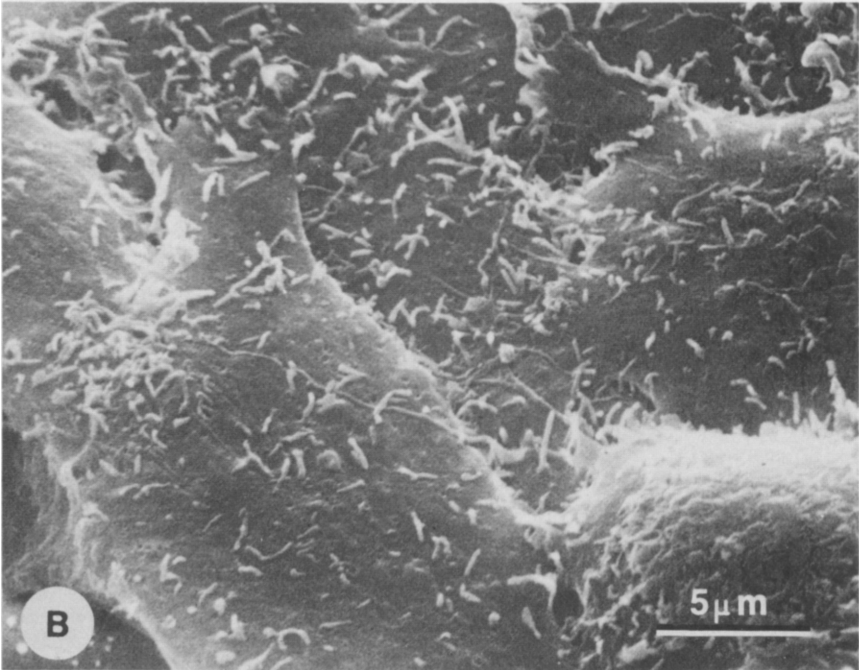
10µm


B
5µm

DNA viruses which produce an abundance of nonstructural glycoproteins appear to be capable of inducing changes in cell interactions under control of the virus genes. In poxvirus infection, there is an inverse relationship between stimulation of host cell fusion activity and induction of a surface hemagglutinin (Dales *et al.*, 1976). Strains of herpes simplex virus also differ in their ability to induce cell fusion or cell agglutination and these phenomena have been associated both with the quanity and quality of proteins associated with smooth membranes of the host cell (Roizman and Kieff, 1975; Spear, 1975).

Interest in the role of membrane macromolecular interactions on the immune response to virus infection has been growing rapidly. Both the cell type and virus are determinative factors (Brandt and Russell, 1975). There is now experimental evidence for an interaction of virus antigens and cellular histocompatibility determinants in cell-mediated immune cytolysis (Zinkernagel and Oldstone, 1976). Clinically, the cellular immune response to vaccinia virus immunization appears related to HLA type (De Vries *et al.*, 1977). Edelman (1976) postulates the formation of physical complexes between mobile histocompatibility antigens and other antigenic molecules (such as viral polypeptides) to form adaptor–antigen complexes which are recognized *in toto* by cytotoxic lymphocytes. Thus effects of viruses on the density of surface histocompatibility antigens may have important biological implications (see Weiss, 1977). It has been known for some time that maturing virions may incorporate histocompatibility antigens into the virus envelope, even on a selective basis (Bubbers and Lilly, 1977), while at the same time an absolute decrease in these antigens may occur on the infected cell surface (Ting and Herberman, 1971; Hecht and Summers, 1974). In oncornavirus infection, expression of host histocompatibility-antigens and antigens determined by the proviral genome may be closely interrelated (Cikes and Friberg, 1971).

Another rather intriguing phenomenon is the unexpected appearance of F_c receptors on nonlymphoid cells infected by herpesviruses (Costa and Rabson, 1975; Westmoreland *et al.*, 1976). This has been demonstrated by binding of iodine-labeled purified IgG to the surface of cells infected by herpes simplex or cytomegaloviruses (Westmoreland *et al.*, 1976). The nature of the binding site or protein has not been resolved; however, a biological advantage for the propagation of herpesvirus infection has been postulated (Costa and Rabson, 1975). This may relate to findings of Stevens and Cook (1974) suggesting that antiviral IgG influences the maintenance of latent herpesvirus infection.

Thus we terminate this chapter on the rapidly advancing and converging frontiers of virology, membrane biology, and cellular immunology. Membrane-associated glycoproteins are proving to be strategic elements in

each of these areas and further understanding of their structure, biosynthesis, and immunogenicity will provide important keys to unlocking the secrets of virus pathology and pathogenesis across the spectrum of active as well as oncogenic infections.

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