Early Events in Cell-Animal Virus Interactions

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INTRODUCTION

The impact of the classical experiments demonstrating injection of deoxyribonucleic acid (DNA) by the tailed bacteriophages firmly implanted in the minds of virologists the concept that nucleic acid is the only prerequisite for transmission of infection by all agents of prokaryotes and eukaryotes. This notion was strengthened when clear-cut evidence showed the infectiousness of ribonucleic acid (RNA) derived from picornaviruses (60). These considerations must have been a powerful factor in fostering skepticism about our original observations demonstrating that animal agents carry the infectious moiety into cells as a complex subviral entity or even as a quasi-intact particle (44, 52). Today it can be accepted without much reservation that components other than nucleic acid are required and transferred as a unit to the site of genome function. This review will attempt to communicate to the reader the subtlety, complexity, and variety of events associated with early cell-virus interactions. Earlier observations on this subject, encompassing work dating to about eight years ago, were documented in a previous survey (47). Since that time, sufficiently extensive new information has been published about the nature of animal viruses and their modes of penetration to provoke a re-evaluation of this field of study. Hopefully this article will succeed in informing the general readership about the current status of this branch of animal virology, evoke from the specialists fruitful ideas, and instigate further experimentation to answer many of the questions remaining.
INTERPRETATION OF ELECTRON MICROSCOPY IMAGES AND THE PROBLEM OF ARTIFACTS

In my view the complexity of intracellular events during virus penetration precludes complete, meaningful analyses dependent strictly on the methods of biochemistry and molecular biology. The exquisite sensitivity provided by the electron microscope permits the investigator to literally account for individual intracellular virions, making it an indispensable aid for localizing inoculum particles and subviral components. When coupling the morphological approach with chemical analysis, satisfactory and detailed information can be generated regarding the mechanisms of uncoating as related to the expression of genome functions. Unfortunately, the methodology of electron microscopy, like any other, is fraught with artifacts: as a result a number of controversies have arisen regarding the "true pathways of penetration," especially those concerning behavior of the inoculum particles at the plasma membrane, where entrance is gained into the interior of the cell. Such divergent opinions among different investigators may explain the reason for the skepticism prevailing about the validity of morphological evidence of this type. The presumed inadequacy of electron microscopy requires, first of all, a consideration of the difficulties and pitfalls in the preparation of specimens and evaluation of the electron microscopy images.

Specimen Preservation

The commonest artifact develops as a result of inadequate preservation of membranes. In our experience this difficulty is especially evident when cultured cells are fixed initially as a monolayer by glutaraldehyde and then are scraped and postfixed with osmium tetroxide (OsO$_4$). The disruption because of scraping and inadequate, as yet unexplained, osmication affects the sought-after sharpness of the lines delineating a phase separation by membranes between cellular compartments and around some organelles such as the mitochondria and lysosomes. An example is shown in Fig. 1 and 2, relating to the uptake of reovirus by L cells. In this comparison, the preservation of material from replicate cultures infected under essentially identical conditions was the same in every respect, with the exception that a briefer post-fixation with OsO$_4$ was used when membranes were inadequately preserved. Thus, the inoculum particles in Fig. 2 appear as if they were lodged free in the cytoplasmic matrix, whereas in fact they are enclosed by an inconspicuous membrane around a phagocytic vacuole.

Interpretation of Images from Sections

It may not be universally appreciated that routinely prepared thin sections are usually in the range of 500 to 1,000 nm in thickness, which is equal to or greater than the width of many animal viruses. It is to be anticipated, therefore, that in random slices through cell-virus complexes, some particles that are attached at the surface where invagination has occurred (Fig. 3) may occasionally appear as if they have lost morphological integrity at the site of contact and have merged with the plasma membrane (185), as is illustrated with vesicular stomatitis virus (VSV) and diagrammatically (Fig. 4, 5, and 6). In this instance the thick coating of the cytoplasmic face of the membrane is frequently connected with the sites of preferential attachment and sometimes is evident as an interlocking network of ring structures, presumably homologous with the so-called "coated" vacuoles (Fig. 5). Incidentally, such coated membranes deserve further attention in the future, for they are regions where often the inoculum is both attached and engulfed, as has been observed with several animal agents including influenza, adenovirus, and VSV (36, 141, 185).

Another problem of electron microscopy image interpretation concerns virus particles that appear to transgress the plasma membrane as if they were "melting" their way into the cytoplasmic matrix. The geometric relationship between the virus and cell surface can be analysed by means of the tilting and rotating specimen stage of a Phillips EM300 electron microscope. When thin sections are subjected to such an analysis, it can be demonstrated that virions "melting" their way through the plasma membrane are, in fact, externally situated particles, as is illustrated with an adenovirus in Fig. 7 A, B, and C and 8 A and B.

Nature of the Inoculum

With certain agents possessing relatively flexible lipoprotein membranes, damage to the envelope may occur at some stage in purification or handling of the material. Inocula with ruptured envelopes (Fig. 9) may attach as readily to the host as intact virions. If attachment should occur at the point of rupture, the electron microscopy images may be misinterpreted as demonstrating a dissolution of the envelope at the site of contact with the host (Fig. 10). Likewise, extrusion through a rup-
tured envelope of the nucleoprotein core of paramyxoviruses, such as Newcastle disease virus (NDV), is sometimes encountered in the purified inoculum, even among virions that become attached (Fig. 11). In viewing such images one may erroneously surmise that a process of uncoating is being visualized at the surface.

**Quantitation of the Process**

The sensitivity of the electron microscope in detecting individual virions can be used to advantage in quantitating the early events in penetration. However, because disposition of the cell-associated inoculum usually varies from cell to cell and in different regions of any one particular cell, an objective assessment cannot be based on a few images selected arbitrarily by the investigator. The best evaluation of the course of events in penetration is obtained by enumerating in standard replicate samples the disposition of inoculum particles. For this purpose we usually evaluate samples of 50 to 100 thin sections containing an average of 2 to 10 particles per cell profile and have found this method to give reproducible data. In studying the fate of specific isotopic tracers in the inoculum by autoradiography, it is desirable to employ a similar quantitative approach.

**FUSION, LYSIS, AND REPAIR OF CELLULAR MEMBRANES**

The network of cellular membranes, emanating from or circumscribed by the plasma membrane and terminating at the nuclear envelopes, compartmentalizes the cell interior, thereby acting as a barrier to virus penetration. The dynamic nature of membranes and their capacity for being lysed, reformed, and fused with one another are properties fundamental to the exclusion or internalization of the penetrating inoculum. For this reason, it seems appropriate at this juncture to consider the nature of membranes as they are influenced by perturbations evoked with specific agents including viruses.

Numerous studies have clearly established that animal viruses can be syncytiogenic. Those of the paramyxovirus type, especially the so-called hemagglutinating virus of Japan (HVJ) or Sendai are the most active and nondiscriminating inducers of fusion (7, 71, 87, 146, 147, 148, 151, 169, 208). A large variety of cell types, including nucleated and mammalian erythrocytes, can participate in the process of fusion and hemolysis. Potential for syncytogenesis is possessed by Visna virus (75), some variants of NDV (66, 113, 133), poxvirus (59, 97), herpesvirus (173), and other viruses. With some agents polykaryocytes develop regardless of productive multiplication, as with Visna virus (75), which is equally syncytiogenic for the productive sheep choroid plexus or the abortive BHK-21F hamster fibroblast host cells. With other agents, such as the paramyxovirus simian virus 5 (SV5), BHK-21 cells are rapidly and extensively fused, whereas another host, the monkey kidney cells, remains mononucleate and viable for prolonged periods (41). In some cases a particular agent may possess hemolytic activity after propagation in one host cell and not another. Thus, Sendai virus derived from L cells has low infectivity and hemolytic activity, whereas the same agent propagated in chicken eggs pos-
Fig. 3. Viropexis of VSV particle by an L cell is evident 10 min after initiation of penetration. The section was probably cut normally (A-A, direction in the diagram, Fig. 6), clarifying the separation between the virion and enveloping membrane. x140,000.

Fig. 4 and 5. The same preparation as in Fig. 3 illustrating images resulting from tangential sections (cut in B-B1 direction in the diagram, Fig. 6). In Fig. 5, the coating on the inside can be resolved into groups of interconnecting ring-like elements (arrows). x140,000.

Fig. 6. Diagrammatic representation of the tangential or longitudinal sections that can be made through a virus-cell complex of the type shown in Fig. 3-5.

Sesses these activities at a greatly enhanced level (83). Potentiation of the fusing and hemolytic capacities of the L cell-grown virus is obtained by trypsinization of the isolated particles, implicating a proteinaceous inhibitor from mouse cells which masks the two activities.
involved. Within a particular group of closely related viruses, such as NDV, some elicit fusion soon after the virus is adsorbed to the surface by a so-called fusion from without (23, 66), whereas others are syncytogenic at the time or after assembly and emergence from the surface, involving fusion from within (23). The intactness of virions is not required for eliciting fusion by HVJ, as is evident from the activity of subviral membrane fragments prepared by sonicating the virus (84, 179), which emphasizes an obligatory requirement for attachment of the virion surface component to the cell membrane. A great deal of information, reviewed by Poste
(164), is available regarding the parameters involved in the fusion process. Nevertheless, much remains to be learned about the cell-fusing factor and its site and mode of action. For fusion of Ehrlich ascites and other cells in vitro by Sendai virus, a minimal critical number of virions (about 1,300) is required (148, 149). However, agglutination of virions by low concentrations of neutralizing antibody, which doesn’t abolish infectivity, enhances cell-fusing capability over that of individual virions. But, if sufficient antibody is added to abolish infectivity without preventing attachment, fusion is totally suppressed (150), revealing that fusion demands an intimate contact with the cell surface. Sendai-induced fusion is dependent upon both temperature (66, 146) and the metabolic energy of host cells (147, 151). The lytic or hemolytic principle can be extracted or dissociated from the virion by enzymatic treatment without necessarily suppressing infectivity or hemagglutinating activity (11, 71, 85), although close coupling of cell-fusing factor with neuraminidase activity of Sendai virus has been reported (89). The site for fusion between cells may be the bridges established by virions, as is suggested by the direct fusion observed between virions and erythrocyte membranes (7, 89). On the other hand, evidence has been obtained suggesting that, although the fusing factor may be released at the site of interaction between the virions and the plasma membrane, it elicits its effect at some distant point from the site of attachment (7, 84).

It is known that certain lysolipids, contained in the virion envelope, can lyse cellular membranes. This has led to the suggestion that they or other phospholipids are the factor for polykaryocytosis (11, 17, 113). It is also believed that phospholipases may elicit fusion (11, 211), although such enzyme activities have not been demonstrated conclusively in virions. Recently, Hosaka et al. (84, 179) were able to break apart and reconstitute fragments of HVJ envelopes, whose fusion or hemolytic activity was restored from dissociated components by mixing the protein and glycolipids with either the natural or equivalent synthetic lipids (84–86). Because none of the lysolipids was apparently included in these reformed membranes, it was concluded that lysolipids do not play a fundamental role in the fusion process (86). However, much indirect evidence supports the possibility that lysolipids are, at least in some instances, concerned with membrane fusion. Poole et al. (163) suggest that the formation and removal of lysolipidic at localized regions of the membrane may be closely controlled by endogenous enzymes. Lysolipid, when contained in dispersed droplets of the lipid phase in an aqueous emulsion, can under controlled conditions enhance cell-hybrid formation, although usually such intercellular fusion is limited, difficult to control, and frequently results in complete cell lysis (2, 124). Uncontrolled lysis can be diminished by using lysolipidic incorporated in micro-droplets of glycerol trioleate, thereby obtaining enhanced homo- or heterokaryons (124). Glycerol monooleate increases at 4 to 7 times the rate of cell-hybrid formation, perhaps because there is not the extensive membrane destruction observed by use of lyssolecithin (124). It also has been suggested that lyssolecithin may be the precursor of lecithin at the site of formation of phagocytic vacuoles from the plasma membrane, or fusion between vacuoles and lysosomes, or in the process of exocytosis of granules (57).
In more general terms, a theoretical evaluation of the fusion process has been formulated by Lucy (123). He proposes that, although most biological membranes are organized as bimolecular leaflets of lipid complexed with specific proteins, parts of these membranes can exist at any given instant as globular micelles of lipid. At the site of fusion a relatively high proportion of phospholipid is converted into the micellar configuration. Thus, the lipids originating from the two interacting membranes must assume an appropriately compatible configuration for them to be moved or exchanged between the membranes. Agents which bring about phase changes favoring the micellar configuration in the lipids of one or both interacting membranes can enhance or facilitate fusion, and the syncytogenic viruses may be among such agents.

ATTACHMENT

Experience has shown that with most animal virus-host cell systems, under physiological conditions, the early steps of penetration occur rapidly, within minutes after attachment. To synchronize these events as much as possible, advantage may be taken of the temperature independence of adsorption on the one hand and close temperature dependence of penetration from the surface on the other. Thus, after mixing cell and virus in vitro at temperatures of 0 to 4 C, the rate of virus attachment resulting from random collisions has been found to be proportional to the concentration of host cells in suspension. For example, in the case of adenovirus 2 attachment of physical and infectious units occurs at the same rate and is proportional to the cell density in the mixture (156). With papova agents, such as SV40 and polyoma virus (12, 65), binding to cell receptors is evident within minutes after mixing and is complete by 2 h when 50% of the input virus becomes cell associated (12). More rapid adsorption occurs with Sindbis virus, because 40 to 60% of the particles attached ultimately are bound within 1 min (168). Similar rapid-attachment rates have been reported with other agents including reovirus (120, 182), herpesvirus (80, 140), and picornavirus (31, 82, 127, 129, 130). Variability in rates of attachment to mouse L cells was related to plaque-type mutants in the case of Mengo virus (127). Unfortunately, in most instances the optimal physiological conditions for adsorption have not been established, so that it is not possible at the present time to provide a rational explanation for the observed differences cited.

It is a common experience with many virus-host cell systems that after adsorption in the cold and upon elevating the temperature, a fraction of the inoculum becomes eluted from the surface. This phenomenon, initially reported for poliovirus by Joklik and Darnell (100) has been encountered again with poliovirus (82, 130), reovirus (120, 182), herpesvirus type 1 (80), and adenovirus type 2 (156). The intimacy of initial binding to receptors presumably determines the degree of elution. Concerning the particles that are internalized tight coupling must occur when SV40 attaches to host receptors, for upon isolation of the inoculum by disruption of the membranes, the freed particles acquire a lower buoyant density than does input virus, implying that a segment of the receptor and plasma membrane is dislodged as a complex with the virion. Similar observations have been reported regarding adenoviruses 2 and 5 after attachment to HeLa cells (36, 122). By contrast, the closely related adenovirus 7 fails to bind tightly to the cell surface (36, 158).

Attachment sites for viruses occur on the rigid, highly organized, and differentiated regions of membrane covering cilia and microvilli as well as on the flexible portions of the plasma membrane and intracellular membrane network. Holland (82) has presented information concerning microsomal and plasma membrane attachment sites for poliovirus. Avian leukosis and sarcoma viruses attach preferentially to receptor sites possessing the morphology of a dense reticulum (49). Other agents, particularly VSV, show predilection for coated membranes and associate with them at the rounded end (185; Fig. 3). Sendai and influenza viruses attach firmly and with equal efficiency to either the ciliary or other segments of surface of tracheal or oesophageal epithelium of guinea pig and ferret nasal-mucosa cells after exposure of fragments of organ culture to these viruses (55, 70; Dourmashkin, personal communication).

Integrity of host cells is not required for attachment, because isolated plasma membranes possess the receptors and interact efficiently with poliovirus (31). Likewise, WSN influenza virus adsorbs at low temperature to isolated chicken embryo cell membranes and the virus attaches exclusively to the external surface (38). The poxviruses exhibit indiscriminate attachment to a large variety of cell types and even to any appropriately charged surface (3, 165). For example, Molluscum contagiosum adsorbs to nonpermissive chicken embryo cells at the same rate as vaccinia virus and may later interfere with the replication of vaccinia, for.
which these same cells are permissive (165, 171). The nature of the receptors and virion components reacting with them appear to vary with each virus type involved. A mucous-covering substance on the plasma membrane becomes the initial site of interaction with the myxoviruses (70). Contact is established via the projections or spikes present on enveloped agents including the myxovirus, paramyxovirus, and rhabdovirus groups (6, 90, 185). Some spikes contain neuraminidase activities whereby, after adsorption, the mucous or mucoprotein layer on the surface may be locally dissolved to allow more intimate and firmer attachment onto the plasma membrane itself. The receptor sites, whether on erythrocytes or host cells, contain neuraminic acid residues upon which the virion enzyme acts after attachment. Pretreatment of cells with a receptor destroying enzyme or by exposure to virus having neuraminidase activity eliminates or decreases subsequent virus adsorption (211), at least until the receptors have been regenerated. For a more detailed review of this subject the reader should consult the articles by Cohen (40) and Philipson (155).

There exist about 10,000 specific receptors on each KB cell for attachment of adenovirus 2 (158). This number agrees approximately with the calculated number of adenovirus type-5 particles that can become attached to HeLa cells (37). Extracellular adenovirus 2 attaches to the plasma membrane receptors with a half-life of 15 min and is subsequently internalized (122). The virion-receptor complex which is amenable to disruption or release by sonic oscillation involves the fiber protein. Exposure to a lytic substance, subtilisin, inactivates the receptors which can be regenerated upon incubation for 4 to 8 h in the absence of subtilisin from the medium. Either complete or partial interference with attachment of adenovirus type 2 and 5 can be effected by pre-exposure to preparations of pure fibers, whereby 10^5 to 10^6 fiber units per cell can completely abolish adsorption, presumably by masking all available receptors for the virion-associated fibers (158). Shielding of receptors for an adenovirus does not affect whatsoever attachment of poliovirus type 1, indicating that these two agents at least possess independent sets of binding sites on a particular host cell.

With some agents the intact virions are required, and with others subvirial particles can attach. In the case of Sindbis virus, the envelopes are necessary for the isolated nucleoprotein core particles to adsorb to host cells (19). By contrast, both enveloped virions and cores of herpesviruses, including equine abortion virus, attach readily to their respective host cells (1, 51, 190). Subviral particles of VSV, in the form of tightly coiled skeletons of nucleoprotein and created by stripping the envelope with detergent treatment, can be attached to host cells and possess some infectivity (30). Heat treatment and neutralization by specific antibody do not necessarily affect adsorption in all cases, as has been demonstrated with vaccinia (50), influenza (R. R. Dourmashkin, personal communication), and polioviruses (94, 129). Attachment of both whole virions (135, 201, 204) and subviral particles (30) can be greatly enhanced by polycations such as diethylaminoethyl(cellulose) (DEAE)-dextran, whereas negatively charged polyanions such as heparin bring about a release of virions of herpes simplex virus from the surface of host cells (80). The above observations correspond with information reviewed by Cohen (40) and Philipson (155) about the requirements for attachment of animal agents in general, which information indicates that an involvement of electrostatic interactions brings about binding to surfaces of host cells.

Evidence, particularly relating to the picornaviruses, has been obtained indicating that externally situated virions can undergo profound structural rearrangements of their capsids (100), so as to render their genomes susceptible to ribonuclease (RNase) hydrolysis. In the case of poliovirus complexed with isolated plasma membranes, about 30% of the RNA becomes spontaneously solubilized if the virus-membrane complex is allowed to remain at 0 C for a period of 2 h. The remainder of the RNA becomes specifically accessible to dual hydrolysis by trypsin and RNAses when temperature is elevated to 37 C (31), implying that after adsorption the capsid conformation is rearranged at 0 C and moreso upon elevation of the temperature to 37 C. With echovirus 7, irreversibly complexed with erythrocyte receptors, the high-molecular-weight RNA is freed from an inaccessible into an RNase-susceptible state upon elevation of the temperature (157). The degree of intactness of poliovirus capsids attached to HeLa cell membranes can be determined by exposure to 6 M LiCl or 8 M urea which rupture hydrogen bonds. These salts normally do not affect either the integrity of the capsid or infectivity of native particles. However, after attachment to either whole cells or isolated plasma membranes, treatment with these salts irreversibly abolishes infectivity.
interaction with membrane receptors.

MECHANISMS FOR INTERNALIZATION OF THE INOCULUM: PHAGOCYTOSIS OR FUSION

Survey of the literature to 1965 (47), including work from this laboratory, indicated that in most cases animal viruses are internalized by viropexis. Since the time of the previous review and from numerous publications on this subject, it would appear that viropexis is not as universal as believed heretofore. Unfortunately, many among the publications under consideration are the results of electron microscopy studies and are subject to just the types of difficulties in evaluation and interpretation that have already been mentioned in the first section of this article. In my judgment it now has to be accepted that with certain agents there prevail alternative mechanisms or a variation of a single process for internalizing the virions. In addition to viropexis fusion between envelopes of the inoculum and the plasma membrane as a process for incorporating directly the nucleoprotein moiety must now be recognized. Among the rarely proposed additional routes for ingress are (i) the development of breaks or dissolution of the plasma membrane (135) and (ii) direct transgression whereby intact or quasi-intact virions "melt their way" through the membrane into the cell proper (56, 141). The up-to-date electron microscopy information on penetration has been summarized in Table 1 and will now be considered according to the respective virus types.

Herpesviruses

Several studies cited previously (47) and published more recently show evidence for rapid clearance from the surface of attached inoculum particles (1, 51, 67, 95, 136, 140). Some workers report either exclusive or almost exclusive transfer from the surface by viropexis of both enveloped virions and the nucleoprotein cores (1, 51, 95). Others propose that transfer of cores into the cytoplasm may occur as a consequence of either the digestion or fusion of both the virus envelope and contiguous plasma membrane or by phagocytosis (136, 140). In rare instances the mode of entry was cited as exclusively by fusion (210). Naked, infectious nucleocapsids, which can be prepared in a pure form, are adsorbed readily and penetrate by viropexis into host cells (1, 95), as well as by comparable enveloped particles possessing a much higher infectivity (1). Within a few minutes after engulfment, the external coats of herpes simplex virus disappear in phagocytic vacuoles, perhaps in the stage preceding release of free nucleocapsids into the cytoplasmic matrix (95). With this agent rapid clearance of virions by means of viropexis from the cell surface coincides temporally with an equally rapid onset of resistance of the infectious material to specific neutralizing antibody (92), suggesting that viropexis is the most likely route of infectious particles (51, 95). A contrary opinion, favoring fusion as the mode of entry leading to productive infection, has been enunciated (140). The view is expressed that herpesviruses possess a capability after contact to rapidly digest their own and host cell membranes, whereas release of the nucleocapsids from phagocytic vacuoles is considered to be either a very rare or non-existent phenomenon (140). Unfortunately, there is no experimental evidence supporting the idea of a virus-mediated dissolution of membranes.

Poxviruses

Heretofore, viropexis was shown to be the process of entry into host cells. Our earlier studies with vaccinia virus (45) were corroborated with both permissive and restrictive host cells as in the penetration of vaccinia into mouse macrophages (180) and Mollusca contagiosa into human epidermal and chicken embryo fibroblast cells (167, 171). Hemocytes circulating in the lymph of insects, in which invertebrate poxviruses multiply, can also engulf the inoculum (54; R. R. Granados, personal communication). Deliberate clumping of inoculum virions, which have previously been inactivated by heating, ultraviolet (UV) irradiation, or methyl methane sulfonate apparently enhances reactivation (111), most probably because such aggregates are phagocytized as a unit and efficiently converted into cores within the cytoplasm of the host. By contrast with viropexis, it was recently demonstrated (R. R. Granados, personal communication) with an insect poxvirus that penetration can also be initiated via fusion of envelopes of adsorbed virions with the membrane of microvilli (Fig. 12). With vaccinia virus we have encountered in a few rare instances a similar, apparent fusion between the virion and plasma membrane of mouse L cells (Fig. 13), but only in cell-virus complexes sampled 3 to 6 h after inoculation, long after the time when replication commences in this system. Although the information on an insect poxvirus clearly demonstrates that fusion
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<th>Mechanism</th>
<th>Virus type</th>
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<td>Melolontha</td>
<td>Insect hemocytes</td>
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<td>Adenoviruses:</td>
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<tr>
<td></td>
<td>Amsacta pox</td>
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with the microvilli and movement of cores into the cell proper can occur, perhaps as part of the normal infectious process, in the case of vaccinia, fusion of the type illustrated in Fig. 13 is most probably an aberrant stage associated with a few particles remaining immobilized at the cell surface.

### Nuclear Insect Agents

As noted above with an insect poxvirus, other insect agents have been observed to penetrate into host cells by both viropexis (116, 197, 209, 210) and fusion of virion envelopes with microvilli (109, 192, 193). Once again, cells that possess deformable surfaces acquire the virus by engulfment, whereas the mechanism associated with differentiated, nondeformable surfaces is fusion.

### Adenoviruses

Recent studies using more highly synchronized systems confirmed in every respect the previous conclusion (47) that adenoviruses are internalized by viropexis (35, 36, 64). Because viropexis can be completely arrested at temperatures of 0 to 4 °C, only after elevating the temperature does the inoculum pass into vacuoles. Quantitative results from electron microscopy counts reveal that sojourn in phagocytic vacuoles is very brief (35). Adenovirus 5 is highly efficient at penetrating out of vacuoles at 37 °C and gains access into the cytoplasmic matrix very rapidly. However, at intermediate temperatures of 12 or 20 °C, the proportion of inoculum found within phagocytic vacuoles increases rapidly with time (Fig. 14; 35), but that of free virions does not, indicating that viropexis is a relatively more efficient process than is release from vacuoles. It appears that active virions possess the capacity to free themselves, perhaps by lysing the vacuolar membranes. This notion is supported by our studies with heat-denatured inocula that are actively phagocytised but not released as efficiently (S. Dales and Y. Chardonnet, unpublished data). Some evidence has been presented favoring the idea that in addition to viropexis, adenovirus 7 may

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*For other, older references, see a previous review of this subject by Dales (47).*
FIG. 12. Penetration of Amsacta moorei pox into a microvillus of intestinal epithelium cell of Estigmene acrea larva. Fusion between the virion and plasma membrane must have occurred prior to release of the core (C) and separation of the lateral bodies (L). ×85,000 (photomicrograph courtesy of R.R. Granados).

FIG. 13. Unusual interaction between vaccinia and L-cell plasma membrane. Lysis at the point of contact appears to have occurred between the virion envelope and cell membrane before the engulfing membrane (arrows) was sealed into a phagocytic vacuole. The lateral bodies (L) separated from the free core (C). Such rarely encountered images were found in samples taken 3 to 6 h after inoculation, when replication was already in progress. ×120,000.
pass directly through the plasma membrane into the cytoplasm of the host (141). In this study, paucity of images purporting to demonstrate such a phenomenon is explained by presuming that the process is unusually rapid or uncommon.

**Papovaviruses**

There is a consensus of opinion that papovaviruses, small icosahedral agents including polyoma and SV40, enter cells by viropexis (12, 65, 96, 132). Uptake of either individual units or small groups occurs within minutes, and thereafter virus in vacuoles is moved rapidly either towards the nuclear border or into lysosomes. In one reported situation, inoculum of polyoma that enters nonpermissive BHK-21 cells is retained in cytoplasmic vacuoles for prolonged periods and may be transmitted to daughter cells in such packets for at least six generations (65).

**Reoviruses**

Reoviruses, including type 3, are phagocytized efficiently and rapidly by the host cells (48, 182). Virtually all of the inoculum can be accounted for at either the cell surface, in phagocytic vacuoles, or ultimately in lysosomes during the 1st h after inoculation (182). Loss of particles from the external surface is mirrored by their appearance in vacuoles or lysosomes.

**Rhabdoviruses**

Conflicting data have been published concerning the mechanism by which rhabdoviruses, especially VSV, penetrate into host cells. In the case of synchronous infection of either stationary or suspension cultures by rabies and VSV, rapid and efficient viropexis appears to be the exclusive mode of entry (88, 94, 185; unpublished data). Apparently the envelopes, obligatory for fusion, are not strictly required for infectivity of VSV, as has been demonstrated by employing for inoculation stripped particles that were converted by means of detergent into nucleoprotein "skeletons" (30).

Another procedure which has been employed for initiating rapid cell-virus contact is sedimentation of the inoculum with host cells at high centrifugal forces. As a consequence, an apparent fusion of virion envelopes with cell plasma membranes seems to occur, albeit not to
the exclusion of viropexis (77). In this study no evidence was provided for the anticipated presence at the plasma membrane of remnants of virion envelopes that had become fused prior to discharging the nucleoprotein. However, in a subsequent investigation by the same workers (78), immunological and related evidence were presented to show the occurrence of VSV envelope glycoproteins at the cell surface. It remains to be shown whether the apparent discrepancies between the different studies cited stem from problems in the interpretation of electron microscopy images or are due to the application, in one case, of high-gravity forces.

**Myxoviruses**

In addition to the previously cited work (47), several studies have focused on the penetration of influenza virus. As with other animal agents, viropexis is readily observed with influenza type A interacting with Ehrlich ascites cells (6), type WSN and BHK-21 hamster cells (unpublished data), and influenza interacting with the chori- oallanotic membrane (R. R. Dourmashkin, personal communication). In the last-cited study, evidence was obtained showing dismemberment of both virion and vacuole membranes 10 to 30 min after initiation of penetration, indicating that release of subviral components into the cytoplasm may occur at this time. The purely morphological evidence for viropexis of influenza is supported by the experimental work with closely related fowl plague virus, in which the inoculum carrying a neutral, red-dye label was permitted to uncoat in chicken embryo cells (105). Release of the RNA genome and, hence, separation from the dye can be monitored by a reduction in photosensitivity of the infectious component, whereas removal from the surface of the virions is determined by acquisition of antiserum sensitivity and loss of hemagglutinin. The evidence obtained is consistent with the view that fowl plague virions become uncoated internally rather than at the cell surface. Influenza virus adsorbed to nondeformable membranes, such as those of erythrocytes or of cilia in guinea pig tracheal epithelium, fails to penetrate or become fused (55). Some published electron microscopy images have been interpreted to show fusion of the virus with the plasma membranes of other host cells (16, 139); in one of these studies (139), viropexis also was found; whereas in the other (16), it was never encountered. An electron microscopy analysis by means of a tilting specimen stage revealed that particles attached to the cell surface, which appeared to be fusing, were in fact superimposed onto the cell membrane in thin section (unpublished data; R. R. Dourmashkin, personal communication). In other studies, whole, chicken embryonic cells or isolated plasma membranes from them were complexed at low temperature with influenza A particles and examined by electron microscopy at intervals after elevation of temperature to 37 C. Evidence was claimed for release of the nucleocapsid as a result of the mutual disintegration of virion and contiguous plasma membrane segments (38). By monitoring release of the nucleoprotein G antigen and by using complement fixation tests on the same material, supportive data were also obtained (114). Appearance of increasing amounts of free G antigen was related to the duration of the incubation and implies release of the nucleoprotein moiety from the membrane-associated viirions.

In the reviewer's opinion, unequivocal demonstration of fusion of myxoviruses with host cells has not been made, and definitive results demonstrating whether initiation of infection commences by fusion or viropexis have yet to be published.

**Paramyxoviruses**

This group includes agents with the most powerful cell-fusing and hemolytic factors (the evidence was reviewed in detail recently by Poste; 164). Nevertheless, some types, among them NDV and SVs, appear to penetrate either exclusively by viropexis (41, 87, 183) or concomitantly by viropexis and fusion (57, 133, 138). Variants within the NDV group exhibiting the fusion from without characteristics (39) may possess the cell-fusing factor on the virion. NDV uptake by either process is envisaged as a two-step mechanism culminating in the release of the nucleocapsid helices, which is followed by a freeing of the RNA in preparation for replication (57). Penetration and uncoating are both temperature-dependent events facilitated by the presence of serum in the medium (57). With another agent, Sendai virus interacting with Ehrlich ascites tumor cells, the virions may be initially phagocytized and subsequently ruptured to release their nucleocapsids into the cytoplasm. Transfer of the nucleoprotein helix need not necessarily occur at the site where syncytigenesis is initiated (36). Although cell-to-cell fusion under the influence of Sendai inoculum can be demonstrated readily (89, 138), it is not certain that the bridges for initiating fusion are formed directly by the attached virions themselves (71, 164). Sendai particles that are adsorbed to the nondeforma-
ble membranes of human erythrocytes (89) and cilia (55) appear to fuse and inject their nucleocapsids from the surface. The significance to the infectious process of this mode of release remains to be elucidated.

**Picornaviruses**

Although several contradictory lines of experimental evidence have been published about the penetration of Picornaviruses, electron microscopy data are meager (56). The original work of Holland (82) provided evidence of an early, irreversible eclipse of poliovirus at or near the surface of permissive cells. Other observations indicate that attached virions at the surface are altered prior to uncoating: when complexed for some time at 37 C to isolated plasma membranes poliovirions become leaky-to-hydrolytic enzymes as if uncoating had occurred at the surface prior to internalization (31).

Comprehensive studies of Mandel (128, 130) clearly established by a variety of experimental techniques that penetrating virions of poliovirus type 1, which are first removed from the cell surface, acquire antibody resistance without becoming grossly changed in their structure or losing their integrity or infectivity. Later, in a second stage of penetration, after the capsids are irreversibly altered, the RNA genome is released and can enter the cytoplasm. Mandel’s experimental evidence, indicating internalization of polio by means of viropexis, has been entirely confirmed by electron microscopy, by using an identical system (unpublished data; Fig. 15-17). When the intact host cells are involved, viropexis may be the obligatory event preceding disaggregation of the capsid and uncoating. Direct penetration through the plasma membrane, as reported by others (56), could not be confirmed.

**Oncornaviruses**

Experiments using a combination of electron microscopy, autoradiography, and biochemical procedures to follow the fate of the inoculum in a synchronous infection of chicken embryo cells by avian sarcoma and leukosis viruses revealed that the fraction of inoculum that is capable of penetrating does so exclusively by viropexis within 30 min after elevating the temperature (49). The individual virions or groups of them inside vacuoles are moved rapidly towards the vicinity of the nucleus where transfer of the genomes is presumed to occur. The mammary tumor agent of mice employed in a less-well-synchronized system was shown to enter gradually by viropexis into mouse embryo cells (177). Uptake and disappearance of virions from vacuoles was observed over a period of several days. Some images of inoculum at the surface were interpreted in this study to indicate very occasional fusion between the virus envelope and plasma membrane. Entirely different and novel mechanisms for penetration are proposed in the case of Rauscher leukemia virus interacting with mouse embryo fibroblasts (135). Three types of interaction are described at the site of attachment: (i) dissolution of virus envelopes without alteration of the plasma membrane; (ii) simultaneous dissolution of virus envelopes and plasma membrane allowing for the direct passage of nucleoids into the cytoplasm, and (iii) dissolution of the plasma membrane without affecting the virions, which then pass directly into the cytoplasm. Uncoating is presumed to occur intracytoplasmically. In no case was fusion between the virion envelope and plasma membrane encountered in this system. The true significance of these observations remains to be evaluated.

**Summary and Conclusions**

In this reviewer’s opinion, viropexis appears to be a predominant, but not exclusive, mechanism for internalizing many animal agents. Despite the voluminous literature to the contrary, only in the instance of some insect viruses that invade epiphiel cells after attachment to the rigid microvilli and in the case of Sendai virus, one among the paramyxoviruses, has it been demonstrated unequivocally that fusion between the virion envelopes and plasma membrane of the host and other cells occurs frequently. This does not mean that other agents, including poxviruses and adenoviruses, lack the capacity for rupturing membrane barriers. In the case of poxviruses, it was shown that after viropexis the inoculum can simultaneously lyse at the point of contact of the virion’s own envelope and that of the enclosing vacuole (45). One should not be surprised to find that on rare occasions a similar fusion event may occur at the surface after only partial engulfment by the host membrane (Fig. 13), for presumably, the requisite conditions exist for eliciting membrane-to-membrane lysis. The aberrant situation encountered occasionally with vaccinia exemplifies the fusing capability of at least some enveloped animal viruses which permits them to lyse both their own and host cell membranes so as to effect release into the cytoplasmic matrix of the inner components.
containing the nucleoprotein moiety. Whether this release occurs at the cell surface or after viropexis may depend upon the nature or expression of the virion-fusing factor involved. In the case of Sendai virus, fusion of attached virions apparently commences very soon after elevation of the temperature and permits the nucleocapsid to enter the matrix directly through the channel created by membrane-to-membrane lysis at the point of contact (138). However, it should be remembered that even with Sendai virus not all particles behave in this manner, for some are phagocytized. In the case of other agents, the phagocytic response on the part of the host cell precedes fusion and ensures that the virions are internalized before expression of the lytic function comes into play, as appears to be the case with vaccinia, influenza, NDV, and other enveloped-type viruses. Thus, determination as to whether fusion or viropexis occur externally may be the consequence of a race between the expression of the lytic principle of the virus and the phagocytic response of the host cell to the presence of a foreign, macromolecular complex on its surface. Such a view can explain why, on one hand, agents possessing powerful lysins can fuse with host membranes, particularly where the membrane encloses erythocytes, cilia, and microvilli and is relatively nondeformable, and why, on the other hand, the same agents become readily phagocytized at regions where the host membrane is more plastic.

**ACTIVITIES OF VIRION-ASSOCIATED ENZYMES AND THEIR EFFECT ON THE HOST**

Existence of enzymatic activities superficially positioned on the envelopes of several animal virus types has been known for many years (61, 79, 199, 208). In some cases these activities, such as the NA⁺⁻, K⁺⁻dependent, ouabain-sensitive adenosine triphosphatase (ATPase) may be derived from the membrane of the host cell. Others, like the neuraminidases of myxoviruses and paramyxoviruses, are virus specified and have been shown to be connected with the phenomena of attachment or elution from specific mucoprotein membrane receptors (79) (see review by A. Cohen [40]).

Observations on the penetration of poxviruses reveal the transformation of the viruses within...
become the cytoplasm into complex core structures which become ruptured or uncoated to release the DNA as long as there is protein synthesis. From this observation, speculation arose that poxviruses and other viruses can regulate their own uncoating prior to the initiation of genomic functions (46, 47, 99, 104). Discovery of a DNA-dependent RNA polymerase activity in vaccinia virus cores (102, 104, 144), catalyzing transcription from the coated core into some early messenger RNA (mRNA), provided an explanation for the generation of information by a coated core for an uncoating factor. Since the time of this fundamental discovery many additional agents of vertebrates and invertebrates were shown to contain RNA polymerase activities, including other poxviruses (162, 178), reoviruses and closely related agents (10, 33, 166, 181), influenza viruses (14, 15), the paramyxoviruses (172), and rhabdoviruses (9). In the case of oncornaviruses, their single-stranded RNA genome is transcribed into DNA in the reverse sense by an endogenous activity. In turn, the DNA transcript can be copied most probably by the same enzyme or enzyme complex into double-stranded DNA, perhaps in preparation for integration into the host cell genome (8, 196).

The complexity of the process related to initiating genome functions must have determined that during evolution those agents possessing the RNA polymerase also acquired certain other necessary enzymatic activities. Among those found thus far are the nucleotide phosphohydrolases, identified in poxvirus, reovirus, and frog (FV3) viruses (5, 20, 69, 106, 143, 162, 178). These activities are generally found to be present in the core and are capable of hydrolysis of the terminal phosphate of both deoxy- and ribonucleotide triphosphates (69, 178). Although the actual function of these phosphohydrolase activities has not been elucidated, it has been suggested that, at least in part, they are involved in transporting out of the core the nascent mRNA (69), for such transfer appears to be an energy-dependent process (103). Recently these and other agents have also been shown to possess phosphokinase activities for the transfer of PO4 from adenosine triphosphate (ATP) to acceptor proteins either in the virion or related to the host (154, 189). The role of such kinases is not clear at present. In the case of the oncornaviruses, many or all of the virion polypeptides can be phosphorylated (189), but with a poxvirus, selective phosphorylation of only one or two polypeptides is found (154). In more general terms, it has been suggested that protein kinases function in the nucleus in gene activation. The virion kinases acting in a similar role may prepare the genome for transcription or replication.

Reovirus cores contain a third activity which catalyzes ribonucleoside-triphosphate-dependent pyrophosphate exchange reaction (202). Occurrence of this exchange activity in reovirus provides presumptive evidence for the presence of guanine residues at the 5' end of the transcriptase product, because when individual nucleotides are tested, only guanosine triphosphate supports the exchange reaction (202).

It has been demonstrated repeatedly that inoculum particles of several animal agents are cytotoxic for the cells they penetrate. With these viruses expression of the cytotoxicity is not contingent upon replication and may be effected by virions inactivated by means of UV irradiation or other procedures (73, 91, 101, 117, 121). In addition to suppressing rapidly and profoundly host cell protein synthesis (121), these agents also suppress DNA and RNA metabolism of the host (28, 31, 93, 101, 161), even at temperatures that are nonpermissive for virus replication (72) and usually in proportion to the multiplicity of infection employed (72, 101). In the case of FV3, the decreased activity of the host cell RNA polymerase II parallels the inhibition of RNA synthesis, although the ability of the chromatin of cell nuclei to act as a template is not affected by the infection (28). Similar results are obtained with HeLa cells infected by vaccinia viruses. Loss, due to heating of vaccinia virus, of the capacity for cytotoxicity (50, 101) is most probably related to a denaturation of a surface component required to effect the release of the core (50). The cytotoxic principle, at least in the case of adenovirus 5, may be either the fiber or hexon protein, or both, for these moieties can bind noncovalently to either virion or host DNA so as to inhibit DNA replication and transcription (117).

The presence of nuclease activities within virions provides in some instances a more facile and direct explanation for the cytotoxic effects observed. A number of virus types have associated with them endo- and exonucleolytic activities, which act upon either single or double-stranded DNA (25, 26, 106, 159, 162, 178), and either single or double-stranded RNA (106, 153). An endonuclease activity of SV40 virions which nicks the circular genome and converts it into a linear molecule, has been reported (107, 110). This activity may be either virion or host specified, and its absence from preparations of purified empty capsids indicates a localization
in the virus core (110). Nucleases of FV₃ are believed also to reside in the core (106). These activities, when introduced with either active or UV-irradiated purified inoculum, are highly effective at shutting off host cell nucleic acid synthesis (4, 28, 72). Furthermore, in double-infection experiments with FV₃ and vaccinia virus, the cytoplasmic DNA replication of the latter is also effectively blocked after superinfection with FV₄ (200). These results are analogous to those obtained with vaccinia and other poxviruses that have been tested to date (101, 161), which possess acidic exonuclease and neutral endonuclease activities with specificity for single-stranded DNA (159, 162, 178). Both enzymes are carried into the host cell along with the virus core (160, 161). The enzymes can be activated in vitro after controlled digestion of the lateral bodies, which may be the inhibitor in the virion for these enzymes (101, 159). This type of in vitro activation suggests that in the normal infectious process, separation of the lateral bodies from the core (45) likewise activates these DNases intracellularly. Although the DNases are late functions, synthesized at the time of virion assembly (160), they become active early during infection, perhaps because they participate in replication of the viral DNA. Intracellularly released DNase, particularly the neutral endonuclease, can be identified for a brief period in the soluble fraction from cytoplasm (160, 161). A close temporal relationship between solubilization of this enzyme and an abrupt cessation of host DNA synthesis is suggestive evidence for direct action in hydrolysis of the replicating DNA in the nucleus. In fact, the vaccinia endonuclease has been identified in the nucleus and shown to inhibit the host DNA polymerase (B. G. T. Pogo and S. Dales, manuscript in preparation). Therefore, pre-existing virion enzymes, when carried into the host as part of the core, can suppress promptly host-associated syntheses.

In some cases nucleases of animal agents are not located in the core, as with adenoviruses type 2 and 12, in which an endonuclease specific for double-stranded DNA occurs either in or near the penton structure (25, 26). This activity is absent from adenovirus type 5, and its function in relation to infection is not fully understood. This activity can introduce several breaks in the virion DNA in vitro to yield large fragments with sedimentation values reduced from 31 to 20, or less. Because a similar size reduction of the inoculum DNA can be demonstrated in vivo, the suggestion has been made that the enzyme acts at some stage during penetration outside or after internalization within the nucleus (25).

Not all of the virion-associated activities are necessarily virion specified or related to replication. Presence of host-derived adenosine triphosphatase (ATPase) has already been mentioned. Recently observed occurrence of a DNA polymerase activity in a poxvirus (196) and a small DNA agent, H-1 (175), could be the result of a nonspecific adsorption to the virion surface or the host cell activity, as suggested by the work of McAuslan (196). In the case of oncornaviruses, whose assembly occurs by budding from the cell surface, the acquisition of host enzymatic activity may be related to the incorporation of quanta of cytoplasmic material when the envelopes are formed around the nucleoid, as suggested by Temin and co-workers (137).

**VECTORIAL MOVEMENT OF THE INOCULUM TO SITES OF GENOME REPLICATION**

There is a great diversity of intracellular routes by which animal viruses are moved to the ultimate sites of their genomic function. Agents having a nuclear phase are carried rapidly toward the nucleus. With some, transfer occurs within phagocytic vacuoles, as is evident with polyoma, SV₄⁺ (12, 21, 96, 132), and avian oncorna agents (49). It is presumed that intact virus or subviral components gain access into the nucleoplasm after a fusion between membranes of the vacuoles carrying the virus and the envelopes enclosing the nucleus (96, 132).

The most detailed and interesting observations pertain to the transfer of genomes from the adenoviruses and an insect granulosis agent. During penetration, after being divested of enclosing membranes, where such exist, nucleocapsids of both agents are moved through the cytoplasmic matrix and make contact with the nuclear pore complex (35–37, 44, 193). A biochemical analysis of adenovirus 5 infection reveals that the bulk of inoculum DNA is transferred into the nucleus, whereas the protein is left outside (35). Although another investigation, based on radiochemical assays of cell fractions, suggests that the capsid proteins may also be moved into the nucleus (122), in this case evidence for the purity of the nuclear fractions was not presented. The observed rapid introduction of DNA into the nucleus (35) is consistent with the observed time-related conversion at the perinuclear zone of dense nucleocapsids into lucent virion shells (35, 36, 141, 193) (Fig. 18–20 and 21). In the case of the granulosis agent, elongated cylindrical nu-
cleocapsids attach themselves characteristically at their ends, precisely at the nuclear pores (Fig. 18–20), through which the dense component is presumably transferred. Although an analogous release of dense core material from adenovirus 7 has been observed (141), the authors of this study are of the opinion that there is not any predilection by the virus to lodge at the nu-
clear pores and that there is little evidence to incitate that the nuclear pores are the main portals of entry into the nucleus of adenovirus 7 DNA. By contrast, it was demonstrated (37) that a stable complex is formed between adenovirus 5 and the nuclear pores, which is not dissociated even after disruption of the nuclear envelope membrane by means of a detergent. Discharge of the core material through the nuclear pores could be an energetic process, perhaps related to an ATPase activity that has been localized biochemically in nuclear envelopes and cytochemically at the nuclear pores (37). The mechanism for vectorial transfer of adenovirus from the surface to the nuclear pores is currently under further investigation in our laboratory. Preliminary observations indicate that movement of virions may be related to the disposition of the microtubules. It is worthwhile to mention that nucleocapsids of herpes simplex virus, another nuclear DNA agent, also have been observed to lodge in the vicinity of the nuclear pores (see Fig. 15, 16, and 21 of reference 136), although no special significance appears to have been attached to this phenomenon. As to the release of herpes simplex virus DNA, it has been reported that the cores are released from the inoculum virions slowly, rather than becoming ejected at any instant. However, other studies indicate that with several of the herpes-type agents, including herpes simplex, equine abortion, and Epstein-Barr viruses, the inoculum DNA is deposited in its macromolecular form inside nuclei a short time after inoculation (80, 95), where it may remain conserved for many hours (98). Of the cytoplasmic DNA agents, the poxviruses have been studied extensively. The coated cores, after penetration out of vacuoles, become lodged in the cytoplasm where discharge (i.e., uncoating) of their DNA occurs. Prior to uncoating, some early mRNA sequences are synthesized rapidly via the endogenous polymerase and transferred out of the core (102, 104). Infection with high multiplicities of virus results in the deposition of inoculum DNA at sites of synthesis (45). If clumps of virus are introduced, there is a tendency for the cores to discharge their DNA cooperatively. The inoculum genomes probably commence to function soon after uncoating in the vicinity of their release and remain conserved within “factories” for many hours (45). Incidentally, such foci of synthesis are nucleoprotein complexes that, upon isolation, remain functional in transcription into vaccinia-specified mRNA (42, 43).

Unfortunately much less information is available about the intracellular movement of cytoplasmic RNA agents. With reoviruses it has been shown that the double-stranded RNA, most probably still in some state of coating and associated with a polymerizing activity, becomes attached to the microtubules, where transcription may commence (48, 181, 182). Autoradiographic studies (unpublished data) indicate that prior to replication some inoculum RNA binds to microtubular paracrystals, whose formation is induced by the alkaloid vinblastine, again emphasizing the specificity of association with microtubular proteins observed previously (48, 182). The state of the hypothesized subviral RNA-protein unit complexing with the mitotic spindle remains to be elucidated.

The released RNA of picornavirus agents may bind specifically to smooth endoplasmic reticulum membranes. Such membranes are sites for the activity of virus-specified RNA-dependent RNA polymerase (27). Later they may be converted into translation complexes by the addition of ribosomes to form polysomes. Regarding the paramyxovirus and rhabdovirus agents, these may commence their functions in transcription as nucleoprotein helices (24, 91). Autoradiography of cell-virus complexes, in which the inoculum is labeled in its RNA and protein, indicates that particles of VSV are transferred to the vicinity of the Golgi or per-Golgi region, where uncoating may occur and the protein coat label is concentrated but not degraded. By contrast, RNA replication may be initiated throughout the cytoplasm (207).

**UNCOATING**

In its strictest sense, “uncoating” is the event of release of the genome from a coated state in the virion prior to replication. This definition is satisfactory for describing the sequence with poxviruses in which accessibility of DNA to hydrolysis by DNAse is synonymous with uncoating (46, 99), although the DNA in its coated state can be transcribed. Unfortunately, “uncoating” has been applied more widely to describe any situation where modification of protective coats renders genomes of inoculum particles exposed to nucleases. This broader definition has led to difficulties of interpretation arising from comparisons between biochemical and morphological results, particularly in the case of the picornavirus and adenovirus, whose genomes can be accessible to hydrolysis without a physical separation from the capsid.

With the poxviruses, exemplified by vaccinia virus, uncoating in its specific sense occurs within 1 h after inoculation, when the DNA
passes out of the core structure into the cytoplasmic matrix through defined breaks in the proteinaceous coat (45, 46, 99). Release of DNA requires ongoing transcription and translation, as demonstrated by blocking experiments with appropriate inhibitors (46, 99). The uncoating process is most probably regulated by an autonomous transcriptase within the virion core (103, 104, 144), but it nevertheless depends upon the usual translation machinery of the host cell. It is unlikely that any functions of the nucleus are involved, because all events leading to DNA replication can occur in the cytoplasm of enucleated cells (166).

By comparison with the poxviruses, a different cytoplasmic DNA agent, FV, is rapidly uncoated in BHK cells without the mediation of ongoing protein synthesis (187). Nor does the uncoating of any of the nuclear DNA agents require such synthesis (12, 80, 115, 156, 194). With the adenoviruses, separation of the DNA core from the capsid can be monitored directly by electron microscopy, whereas the time of susceptibility of the genome to pancreatic DNAse is correlated with appearance of quasi-intact virions in the cytoplasm. At the time of becoming internalized, the inoculum particles may lose some of their capsid-related structures, most probably the fiber plus base penton units, thereby opening them to the action of DNAse (36, 115, 122, 194). The final extrusion of the DNA-rich core from the capsid occurs at the perinuclear zone (35–37, 41, 141), specifically at the nuclear pores (see Fig. 22; reference 37). Other DNA agents with a nuclear phase also become rapidly sensitive to DNAse. It was shown with SV40, carrying a double-isotope label in the DNA and protein, that both virion DNA and protein enter the nucleus within 30 min after initiating penetration into permissive simian cells, and this correlates in time with electron microscopy evidence showing the presence of intranuclear particles (10, 96). Intranuclear inoculum is initially refractory to DNAse, but after about 90 min is progressively more susceptible (12). Uncoating appears to be a stepwise process as judged by a gradual conversion of the virions to subviral structures of higher density. The free DNA, which ultimately appears in the nucleus, exists predominantly in its circular form, but in time becomes converted into linear molecules.

With herpes simplex virus, doubly-labeled inoculum was similarly employed to study uncoating. Occurrence of a time-related transfer into the nucleus of only the DNA and retention of the bulk of the protein in the cytoplasm could be demonstrated (80, 95). Intracytoplasmic separation of DNA cores is described in an electron microscopy study of the same agent (136). Loss of the dense cores from two morphological types of nucleocapsids, termed dense and light, is recognized, and the “light capsids” are said to be preferentially uncoated near the cell membrane, whereas the “light capsids” are deemed to be more stable and uncoated in the vicinity of the nucleus. More evidence is needed in support of the notion, suggested by the authors (136), that the two kinds of capsids have a duality of functions.

Reoviruses may be unique in their mode of uncoating. Although with many agents examined some inoculum is transported into the lysosomes, with the reoviruses transfer is very efficient, rapid, and specific. The outer coat of the virions is hydrolyzed within lysosomes, leaving the core intact (48, 182). Subviral inoculum particles recoverable from host cells after penetration are similar in structure, polypeptide constitution, and biological activity to cores artificially produced in vitro by hydrolysis with chymotrypsin (33, 181). Naturally derived cores obtained from the infected cells can transcribe faithfully the entire double-stranded RNA genomes, whereas in vivo only certain segments of RNA are copied (205). The loss of a portion of the outer capsid is concurrent with solubilization of about 40% of the inoculum virion protein (182), a process which doesn’t require ongoing protein synthesis. Proteolytic activity with lysosomes continues after these virus-containing organelles are isolated from infected cells and incubated in vitro.

With the picorna agents several investigations, particularly the studies of B. Mandel, have shown that separation of RNA from capsids is an intracellular event that is temperature dependent and that does not require protein synthesis (108, 127, 131). However, uncoating, defined in broader terms as susceptibility to RNAse, may result from an alteration of the capsid structure at the cell surface (31, 82) and occurs equally well with whole, nucleated cells, human erythrocytes, and isolated plasma membranes (31, 82, 157).

**ACTION OF ANTIBODY AND SPECIFIC INHIBITORS**

Neutralization of infectivity by antibody is the most specific mechanism evoked in response to viral infection in the body. The behavior of a neutralized virus can be examined in vitro in two ways. The virus either can be attached to the host cells, and then become neutralized by
specific antiserum, or virus particles in suspension can be mixed with neutralizing antibody with the complexes, thereafter, exposed to host cells. By using the first procedure, the virus may either (i) remain attached at the surface indefinitely; (ii) become eluted; or (iii) become internalized by viropexis much like the active virus particles. All three situations probably occur but in highly varying proportions, depending upon the animal agent and host cell. Previously adsorbed herpes simplex virus, whose envelopes are then coated with specific antibody, fit category (i) (140). Similar events occur when specific antibody against the neuraminidase or hemagglutinin of influenza virus is attached to the particles, which then are adsorbed to the host cells but which fail to penetrate (R. R. Dourmashkin, personal communication). More commonly observed effects of neutralization are either failure to absorb or an accelerated elution from host cells of previously adsorbed virions. This is evident with reovirus (unpublished data), Sendai (89), polio (130), NDV (174), and avian infectious bronchitis viruses (189). In passing, it should be mentioned that the situation in vivo appears to be analogous with certain target tissues and cells, but nevertheless the neutralized virions are eliminated from the circulation by actively phagocytic leukocytes, particularly the macrophages acting as scavengers of neutralized virus (18, 74). In some cases, as with poliovirus, elution from the surface may actually be reduced if 7S antibody is added at low concentrations after attachment to HeLa cells (130). The antiserum in this situation has no apparent affect on penetration, whereas the release of RNA into the HeLa cell fails to occur in the normal manner, and the genome is destroyed (130).

When elution fails to occur, the virion-antibody complexes may become phagocytized by the same process that internalizes the infectious particles. However, the subsequent steps of penetration are usually blocked, so that neutralized virions become hydrolyzed, presumably in lysosomes to which they are transported directly. This is evident with vaccinia virus (49, 180), NDV (22, 183), avian infectious bronchitis (189), and many other virus types. In one study, an infectivity-enhancing action by immunoglobulin has been reported with rabbit pox and Murray Valley encephalitis virus. The enhancement was observed transiently soon after attachment of the antibody. Upon prolonged interaction, the usual neutralization set in (76). The enhancement phenomenon may be related to some early intracellular event(s) as yet unspecified. As might be anticipated, neutralizing antibody suppresses fusion by agents carrying the cell fusion factor, including Visna (75) and Sendai viruses (150), presumably because the intimate contact with the cell surface required for expression of the fusion principle is blocked by antibody.

A so-called immune paralysis resulting from attachment of antecellular antibody has been invoked as a mechanism for suppression of infection by some picornaviruses. When such sera are adsorbed to the host cell surfaces they block phagocytosis of certain test materials such as colloidal gold and staphylococci and may act in a similar way with an echovirus by agglutinating adjacent parts of the cell membrane to establish a state of immune paralysis (29).

Most antiviral chemotherapeutic compounds are known to act at steps subsequent to uncoating. In this resume, I shall only be concerned with inhibition occurring prior to release of the genomes. The primary step amenable to inhibition occurs at the cell surface. Because phagocytosis is well known to be an energy-dependent process, it is not surprising that vaccinia inoculum attached to cells treated with fluoride, which affects one of the pathways of the energy generating reactions, prevents internalization of the virus (50). Aerobic oxidation also appears to be obligatory for syncytigenesis by Sendai virus, for in the presence of 2-4-dinitrophenol, or under anaerobic conditions, cell fusion is suppressed (147).

The inhibitory action of adamantamine and related compounds is directed against some early steps in penetration of certain enveloped viruses, although it is not clear at present which of these is affected. Since the original investigation of the action of adamantamine on the arrest of infection by certain strains of influenza virus (81), corroborating evidence has revealed that the drug is not virucidal and doesn’t affect attachment (63, 68, 105, 121; R. R. Dourmashkin, personal communication), but it can suppress infectivity of Rous sarcoma, murine sarcoma (170, 203), lymphocytic choriomeningitis (206), and other enveloped agents. The action of adamantamine is in some respect mimicked by the ammonium ion (158), although the site of action of NH₄⁺ may be different (158). Evidence has been obtained that, because of treatment with adamantamine, there is a delay in penetration (penetration in this system being measured by the amount of residual neutralizable virus or hemagglutinin at the surface) (63, 68, 81, 203, 206). Results from the studies on fowl plaque virus by Kato and Eggers (105) indicate, in-
stead, that penetration to the point of antibody insensitivity is slowed only minimally but that uncoating, measured as the loss of photosensitivity of neutral red particles, is affected by the drug after penetration. The mechanism of action of the adamantamines deserves further attention in the future, for these compounds are heretofore unique chemotherapeutic agents capable of blocking infection at the earliest step prior to manifestation of cytotoxicity or expression of any genome functions. Another specific substance has been reported to affect penetration. In the case of the picornavirus agent, echo-

virus 12, rhodanine (2-thio-4-oxothiazolidine) prevents the uncoating, defined here as loss of photosensitivity of neutral red virus, at some step beyond antiserum accessibility (59). In this connection it was shown that rhodanine also interferes with an in vitro release of the RNA from echovirus 12 particles.

A specific block of uncoating can be instituted in the case of the poxviruses, presumably because this step is regulated by transcription from the virus core. Application of RNA and protein inhibitors at the time of penetration, or by irradiating the virions with UV light, blocks uncoating (46, 99). However, if host cell RNA metabolism is first blocked by pretreatment with actinomycin D, then after carefully washing out any unbound antibiotic, infection is started; the uncoating is not suppressed (126), much like the situation with enucleated cells (166). Interferon and interferon inducers affecting virion-specified synthesis suppress uncoating of vaccinia virus (125), but only partially and, in these circumstances, later synthesis of virus DNA and RNA may also be affected by interferon (13, 118). Inhibitors acting rapidly on protein synthesis, such as streptovitacin A and cycloheximide, are very effective in arresting irreversibly the uncoating process (46, 142). Introduced at the critical time, these inhibitors may prevent synthesis of some specific viral protein required for translation, because the mRNA is produced from the core, but fails to associate into polyribosome complexes (142). Less definitive information exists about the role of protein synthesis in the release of genomes from other agents. Uncoating of NDV is viewed as a two-stage process of which the second phase, related to the freeing of the RNA from its helical nucleoprotein, is contingent upon prevailing protein synthesis (57).

Biochemical evidence indicates that penetration of herpes simplex virus DNA into the nucleus is unaffected by inhibition of protein synthesis (80). This is at variance with an electron microscopy study, the significance of which remains to be elucidated, indicating that pretreatment of cells with actinomycin D or puromycin, or after extensive irradiation of inoculum with UV light, delays intracellular release of the DNA-rich cores (136).

**SUBVIRAL COMPONENTS**

Since the original demonstration showing that RNA of picornaviruses is by itself infectious (60, 112), many other instances, too numerous to cite individually, of infectious nucleic acids from animal viruses have been published. The reports about nucleic acids, which by themselves have been shown to carry infectivity, include RNA of other picornaviruses in both its single-stranded and replicative form, RNA from arboviruses, DNA from papova agents and others. Recently evidence was presented for the infectivity of DNA from an adenovirus, although the possibility remains that some residual protein bound to the DNA played a role in the infection (145). A purported demonstration of infectious DNA from the poxviruses has not been adequately substantiated (99). The mechanism of penetration of free nucleic acids remains obscure. In the case of some tailed bacteriophages, the inoculum DNA passes via the shaft through the cell wall and is extruded on the outer surface of the plasma membrane. Because the "needle" may not penetrate directly into the cell cytoplasm (184), there must exist another mechanism for internalizing the DNA.

Enhancement of infectivity by addition of polycations such as DEAE-dextran may either enhance the adsorption of the nucleic acid, stimulate its internalization, or provide protection against surface or intracellular nucleases (112). It remains to be shown whether the free nucleic acid penetrates directly through the plasma membrane or is introduced through the phagocytic mechanism.

Partial dismemberment of many virus types doesn't necessarily abolish completely their infectivity, but only reduces it to a varying degree. In the case of reoviruses, the outer capsid can be removed under controlled conditions, either naturally in the lysosomes or artificially in vitro by chymotrypsin. In either case, the specific infectivity of the resulting cores is lowered considerably (186), with the exception of one report citing enhancement of infectivity (188). The infectious cores retain three out of six polypeptides of whole virions, but all 10 double-stranded RNA segments and the polymerase (10, 181), endowing them with the essential requirements for initiating intracellular replication.
The nucleocapsids of herpes-type viruses can also attach, penetrate, and initiate infectivity (1), although their specific infectivity is much lower than that of enveloped particles. The so-called skeletons, or nucleoprotein helices of VSV, likewise are infectious (30). In one instance it is claimed that subviral particles of poxviruses possess some residual infectivity (195). In the case of Sindbis, an arborvirus, removal of the envelope eliminates capacity of the nucleoprotein cores to infect by abolishing their attachment, whereas the RNA by itself remains infectious (19).

THE ROLE OF LYSOSOMES IN THE EARLY PROCESS OF INFECTION

The authors of many publications infer the presence of inoculum virions in the dense membrane-bounded granules and identify such organelles as lysosomes (12, 53, 65, 96), although biochemical and cytochemical identification of morphologically similar granules as bona fide lysosomes has been infrequent (34, 36, 180, 182). It has been supposed also, most probably erroneously, that the consequence of phagocytosis is a transfer of the inoculum into lysosomes (78, 141). Lysosomes do not appear to be connected with viropexis, because there is no cytochemical or other evidence to indicate that acid hydrolases occur within phagocytic vacuoles. Concerning many of the virus types examined, whenundenatured virions suspensions are mixed with host cells, some particles proceed directly to their respective sites of uncoating, whereas the remaining fraction is transferred into lysosomes. The proportion of virus that is shunted into the lytic organelles varies a great deal with the virus types involved and even between serotypes within a single group, such as the adenoviruses (36). The inoculum virions rerouted into lysosomes are those that have failed to proceed along the normal pathway of penetration and are thereby destined for destruction. The single exception to this generality is observed with reovirus particles which are carried rapidly and efficiently into the lysosomes within minutes after inoculation. In lysosomes, they are converted into cores and thereby activated for transcription after a specific cleavage of a capsid polypeptide (33, 181, 182). Inside these organelles the proteinaceous coats become hydrolyzed, whereas the RNA genomes survive. Direct evidence for digestion of reovirus coats is derived from experiments involving in vitro incubation of preparations of isolated lysosomes containing labeled particles (181) and from polyaclrilamide gel analysis (33, 181). A dependence on protein catabolism to activate the polymerase of the virus in vitro (10, 33, 181) further substantiates the enzymatic character of the uncoating process with this group of agents. The prolonged digestion of reovirus protein within lysosomes inside host cells indicates that in this case uncoating is asynchronous. The double-stranded RNA genome of reovirus is totally refractory to degradation within L cells and is conserved over a 12-h period or longer (48, 182). Presumably the lysosomes of host cells are not endowed with a nuclease capable of degrading the double-stranded RNA species. Labilizers and stabilizers of lysosomes have been tested to determine their effects on infectivity. In the case of mouse hepatitis virus, evidence was presented that treatment with chloroquine, under appropriate conditions, decreases the virus yield, and this was interpreted to indicate an effect on the uncoating process. Application of chloroquine was ineffectual in arresting reovirus type 3 infection (S. C. Silverstein and S. Dales, unpublished data).

Transfer into lysosomes may occur with inoculum that has been rendered noninfectious. Upon thermal inactivation of vaccinia virus at 56 C or neutralization by antiserum, escape of cores by lysis of virus and vacuole membranes is abolished, and the engulfed particles are instead transported to lysosomes where they are concentrated and gradually digested (50). With vaccinia, NDV, and other agents, a fraction of membrane-attached and antiserum-neutralized inoculum can undergo viropexis and enter lysosomes (50, 183). When macrophages and leukocytes become the target cells, whether in vivo or in vitro, addition of antibody to the system enhances phagocytosis and the subsequent digestion of the virus as demonstrated with influenza (18, 74), ectromelia (134), and vaccinia viruses (180). Regarding the role of macrophages and leukocytes as the host defences against viral infections, the evidence remains inconclusive, because it is not clear whether in all cases phagocytosis arrests further dissemination. Likewise, in vivo studies are incomplete and too few in number to allow generalization about the role of lysosomes in dealing with inactivated particles. The current data provide a most tenable hypothesis which predicts that: modification at the interacting surfaces of virus inoculum resulting in suppression of infectivity, but not viropexis, alters the normal route of penetration and channels the virions into lysosomes where they are destroyed.
HOST- AND VIRUS-DEPENDENT MODIFICATIONS AND RESTRICTIONS

The specificity of receptors on the erythrocytes and plasma or internal membranes of primate cells for picorna viruses such as poliovirus and echovirus has already been mentioned in the section on Attachment. Human and simian adenoviruses also bind to specific receptors on their respective host cells (64, 122, 158). In the case of simian adenovirus SV 15, attachment and penetration are observed with both rhesus monkey kidney cells, the permissive host, and BHK-21 cells, the host in which an abortive infection occurs, although much less virus is adsorbed to the nonpermissive host. In another system the paramyxovirus SV 1, adsorbs to and replicates in both primary monkey kidney and BHK-21 cells, but the inoculum is syncytigenic for only the latter host (41). Another paramyxovirus, Sendai, when propagated in chicken eggs is highly syncytigenic. However, after passage through L cells, its capacity for hemolysis, fusion, and infection is markedly reduced (83). The inactivation is apparently related to a proteolytic masking substance removable by trypsinization, whereby all of the above activities of L-cell-grown Sendai can be restored to the level observed with egg-propagated virus.

With the poxviruses there is no evidence for any restriction on attachment to nonpermissive cells. After viropexis, the inoculum in all cases is efficiently converted into cores (167, 180). However, host-controlled restriction of viral functions may be expressed at other stages subsequent to core formation. In the case of rabbit pox mutant RPu6-2, the virus was inoculated onto L929, HeLa, chicken embryo fibroblast, and PK2a porcine kidney cells. Only the chicken embryo fibroblast cells were totally permissive, whereas the PK2a line was totally restrictive. In the other two cells some virion-specific antigens did appear (176).

In the case of conditional lethal virus mutants, some selected strain mutants of rabbit pox are unable to express their genome functions as related to DNA replication in PK2a cells (62). It would be useful to possess a strain mutation in the endogenous RNA polymerase to determine whether uncoating is blocked, because transcriptions from the core would be suppressed. Unfortunately, such mutants have not as yet been identified. With another agent, the plaque-type mutant of SV 15 multiplying in CV-1 cells is restricted at some early step following adsorption during either penetration or uncoating. The restriction presumably resides in the coat, for extracted DNA is normally infectious (152).

CONCLUDING REMARKS

The foregoing resume attempts to inform the reader about both the general and detailed observations concerning the destiny of animal viruses soon after they have become associated with their hosts. It is now clear that envelopes, coats, and enzymatic activities of the interacting particles, as well as their genomes, play a fundamental role in the orderly penetration and uncoating of the parental nucleic acid. The normal cellular activities, including membrane-to-membrane associations, internalization of material in bulk, vectorial movement of vacuoles and particulates, and nucleocytoplasmic transfer are also intimately involved in preparation of the inoculum for expression of its genomic functions. Events in the penetration sequence are shown schematically in Fig. 23, where representative virus types are diagrammatically represented within individual panels. This diagram reflects in large measure the author’s own judgment and evaluation of the available information. Despite the great variety of pathways traversed and mechanisms involved with uncoating, certain distinctive patterns emerge. The simple, cytoplasmic RNA viruses release their genomes promptly after becoming internalized. The genomes of more complex DNA and RNA cytoplasmic agents are freed in the form of either naked nucleic acid or as a nucleoprotein complex, once a rupture of the envelope or fusion of the envelope and plasma (or vacuole) membrane has occurred. In the case of the reoviruses, activation of the genome may be a uniquely intralysosomal event. Nuclear agents may be delivered to the periphery of the nucleus within phagocytic vacuoles, as appears to be the case with papova, oncorna-, and influenza viruses, or they may be moved through the cytoplasmic matrix directly to the nuclear pores, as observed with adenov-, granulosis, and perhaps, herpes-viruses. Many details about the biochemical events, as related to the morphological ones described, remain to be unravelled. One expects to know, for example, how virions are guided vectorially to the nucleus, why they become lodged at the nuclear pores, and for what reason they are uncoated there. One also continues to be intrigued by the apparent differences in the uncoating of myxo- and oncorna-viruses on the one hand and the structurally similar rhabdo- and paramyxoviruses on the other. These and other detailed
questions about the penetration sequence must await future investigations.

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