INTRODUCTION

Virological literature tends, almost universally, to consider either the structural events leading to release of RNA from a virus particle into the cytoplasmic matrix (the so-called early events), or the subsequent functional expression of that RNA by the cellular protein synthetic machinery. Seldom are both aspects viewed together. This convenient, arbitrary division might not exist in vivo and we should perhaps consider these two processes occurring in concert, bearing in mind the likely fate of the released RNA.

As the title suggests, the principal aim of this review is to introduce a new perspective on the location and mechanism of the uncoating of positive-sense RNA viruses. Recent results from my laboratory (Wilson, 1984a, b) and work by several colleagues (Brisco et al., 1985; D. V. Sangar, personal communication) have alerted us to the possibility that nucleocapsid disassembly might occur in vivo as a consequence of translational events (i.e. 'co-translational disassembly').

Eukaryotic (80S) ribosomes contained in conventional cell-free translation systems (e.g. rabbit reticulocyte lysate or wheat germ extract) appear to be able to uncoat rod-shaped viruses, such as tobacco mosaic virus (TMV) (Wilson, 1984a, b), and also to bring about the release of template RNA from suitably swollen or pretreated isometric virus particles (Brisco et al., 1985; D. V. Sangar, personal communication). In the latter case, particles of foot-and-mouth disease virus (FMDV) and several spherical plant viruses (e.g. southern bean mosaic virus; brome mosaic virus, BMV) have been shown to programme virus-specific protein synthesis in vitro. In all cases, the template RNA would appear to remain predominantly encapsidated, and hence RNase-resistant, prior to the addition of the translation extract.

Unfortunately, one area where the gulf between plant and animal virology is particularly marked concerns the early events of virus infection: events which precede detectable levels of virus-specific gene expression or genome replication. Included in this category are: attachment of virus particles to the cell surface; entry into the cytoplasm, either directly or indirectly; and uncoating of the viral genome. In this review I shall concentrate mainly on recent information concerning the last of these 'events' and attempt to convince the reader that, at least for positive (messenger)-sense RNA viruses, nucleocapsid disassembly and early viral gene expression may perforce be connected more intimately than the current literature might suggest. To date, the identification and characterization of virus attachment, entry and uncoating sites and/or mechanisms has concentrated mainly on a structural/cytological approach and has been extensively reviewed elsewhere (Lonberg-Holm & Philipson, 1974; Kohn, 1979; De Zoeten, 1981; Dimmock, 1982; Bukrinskaya, 1982; White et al., 1983; Shaw, 1985).

In contrast to viruses with genomes of ssDNA, dsDNA, dsRNA or minus-sense ssRNA, viruses containing positive (messenger)-sense ssRNA have the possible advantage of being able to initiate virus-specific protein synthesis in an infected cell as soon as the nucleocapsid or nucleoprotein core structure has disassembled sufficiently to release all or part of the genetic material. The precise temporal relationship between viral RNA uncoating (or release) and early translation events remains almost entirely unknown in most cases. Workers experienced in
handling ssRNA *in vitro* are aware of its extreme sensitivity to ubiquitous ribonucleases. One might argue, intuitively, that completely naked, ssRNA could not remain intact and functional even for relatively short periods inside cells. Complete or partial disassembly of the protective viral coat protein shell (capsid), required for RNA release, must therefore occur very shortly before, if not simultaneously with, recruitment of the RNA into virus-specific polyribosomes or alternative ribonucleoprotein complexes (e.g. 'informosomes' (Spirin, 1969), or 'vRNP particles' (Dorokhov *et al.*, 1983; 1984a, b)), to ensure continued protection against nuclease attack.

*Are low pH compartments (endosomes) involved in uncoating animal virus nucleocapsids to release RNA?*

**Alphaviruses**

Cellular attachment and uptake of several enveloped animal viruses has been studied extensively (see reviews above; also Helenius *et al.*, 1980; Helenius & Marsh, 1982). Uptake of Semliki Forest virus (SFV), for example, follows the general receptor-mediated endocytic pathway involving coated pits and coated vesicles. However, the conventional route to the lysosomes, previously thought to be the site for proteolytic degradation of many viral nucleocapsids, is apparently interrupted when the prelysosomal vesicles (endosomes) become mildly acidic (pH ~ 6). At low pH, the viral membrane fuses with the cholesterol-containing, limiting membrane of the endosome (Kielian & Helenius, 1984), thereby releasing the nucleocapsid into the cytosol (Marsh *et al.*, 1983). Exactly how the 42S SFV RNA is released from the nucleocapsid after these events is still not clear. Early, non-structural proteins are eventually expressed from this RNA species via a polyprotein precursor (Lehtovaara *et al.*, 1980). All subsequent discussions in relevant papers mention only the presence of uncoated SFV RNA in the cytosol, and the onset of RNA replication (Marsh *et al.*, 1983). Clearly, the exact location and mechanism of nucleocapsid disassembly remains to be resolved, even for such an extensively studied alphavirus as SFV.

The nucleocapsids of alphaviruses appear to be composed of 32 capsomeres arranged in a T = 3 icosahedral surface lattice (Enzmann & Weiland, 1979). Therefore, they resemble the 'simple' isometric viruses, some of which have been found to release their RNA *in vitro* in the presence of 80S ribosomes and other components of the translational machinery (Brisco *et al.*, 1985; D. V. Sangar, personal communication). It would be extremely interesting to study the translational activity of isolated alphavirus nucleocapsids *in vitro*.

In this context, it may prove more than coincidental that alphavirus nucleocapsid (core) proteins (from SFV and Sindbis virus) associate with 60S ribosomal subunits both *in vitro* (Ulmanen *et al.*, 1979; Wengler *et al.*, 1984) and *in vivo* (Wengler & Wengler, 1984). It is suggested that the disassembly of so-called parental (incoming) alphavirus cores and reassembly of progeny cores is regulated by a saturable level of receptors on a finite number of 60S ribosomal subunits. One further possible consequence of these events would be alteration of the biological specificity/activity of the protein synthetic machinery in alphavirus-infected cells (Kääriäinen & Ranki, 1984). Some support for this hypothesis has appeared recently (Van Steeg *et al.*, 1984). Studies using Sindbis virus (Wengler & Wengler, 1984; Cassell *et al.*, 1984) also lend support to the view that intact alphavirus cores pass from the acidic (endosomal) vesicles into the cytosol prior to RNA release and early gene expression, since chloroquine (see below) inhibited the transfer of core protein to ribosomal subunits.

**Picornaviruses**

Various aspects of the uncoating of picornavirus particles have been examined and reviewed over the years (for reviews, see Rueckert, 1976; Crowell & Landau, 1983). Recent studies with poliovirus type 1 (Madshus *et al.*, 1984a, b) suggest that artificial acidification of the external medium facilitates entry of the viral nucleocapsid (or possibly the viral RNA) into the cytosol of infected cells. Once again, under normal conditions, entry into the cytosol is thought to occur from acidic, intracellular vesicles (endosomes) utilizing the pH gradient to establish the infection. Thus, lysosomotropic reagents (ammonium chloride, chloroquine) which raise the
endosomal pH, compounds which dissipate proton gradients across membranes (monensin, amines or protonophores), metabolic inhibitors which deplete cells of ATP, or compounds which inhibit the acidification process (N,N'-dicyclohexylcarbodiimide, tributylin), inhibited the entry of virus into the cytosol, but not the initial binding of virus. However, it should be stressed again that the data (Madshus et al., 1984a, b) do not discriminate between the release of RNA from nucleocapsids occurring either within the acidic vesicles (i.e. before transfer across the membrane) or during membrane transfer, or following transfer of whole virus particles from the vesicles into the cytosol. Additional complications arise because low pH also alters the virus structure. Poliovirus particles are hydrophilic at neutral pH, but expose hydrophobic regions at low pH (Madshus et al., 1984b). In all conditions that induce infection, virus particles are altered irreversibly to a more slowly sedimenting, swollen form, the altered or 'A-particles'. Membrane phospholipids may also be involved in the irreversible conversion to A-particles, by interacting with the newly exposed hydrophobic domains of the virus. Conversion to A-particles is a necessary, but not sufficient, condition to allow entry of the virus genome into the cytoplasm. Purified A-particles are not infectious, do not rebind to cell surface receptors and have lost VP4, one of the four structural proteins of the capsid (see below).

A protein kinase activity which has been found associated with purified poliovirions (Schärli & Koch, 1984) phosphorylates all the structural proteins, apparently leading to destabilization of the virus particles and the exposure of new antigenic sites. In general, phosphorylation reactions require reducing conditions; however, reducing agents or virus-stabilizing agents (cysteine or arildone) inhibit the poliovirus-associated kinase activity and affect the pattern of capsid protein phosphorylation. This suggests that capsid destabilization or an opening of the structure might be a prerequisite for phosphorylation. Particles of SFV and Sindbis virus also have protein kinase activity (Tan & Sokol, 1974); however, these resemble conventional kinases in that their activity is enhanced by reducing agents. Purified FMDV particles contain a protein kinase (Grubman et al., 1981) which is active only in preparations of disrupted particles, suggesting that it is an integral part of the viral capsid. Do these enzymes function in vivo to lock the nucleocapsid structure in its swollen conformation (A-particles)? Alternatively, are they involved directly in the shut-off of host cell functions (Kääriäinen & Ranki, 1984), e.g. by phosphorylation of eukaryotic initiation factor 3 (eIF-3) or cap-binding protein (CBP)? Recent data (Etchison et al., 1984) suggest that CBP is selectively inactivated in poliovirus-infected HeLa cells, and that this has a secondary effect on eIF-3. Since poliovirus RNA is not capped, some translational advantage no doubt accrues.

One further complication during the early events of infection by animal picornaviruses is the sequential loss of capsid proteins, particularly VP4 and VP2. This occurs either following attachment of the virus particles to the surfaces of susceptible cells (from which A-particles can be released) or after treatment with isolated membrane fractions in vitro under mildly alkaline conditions at low ionic strength. Contrast this with the isotonic, acidic pH conditions recently shown to be involved in poliovirus particle alteration in vivo, in endosomes (Madshus et al., 1984a, b). Do A-particles therefore represent abortive, partially uncoated intermediates? It may be significant that VP4 and VP2 arise from VP0 by a proteolytic cleavage event late in the virus assembly pathway. Is this a priming reaction, a 'trigger', to prepare virions for subsequent infection and uncoating in vivo?

One further clue to the probable site of release of picornavirus RNA can be found in studies of echovirus 12, by Eggers and co-workers (Eggers, 1977; Eggers et al., 1979). Attachment and uptake of virions was shown to be unaffected by rhodanine, a selective inhibitor of virus uncoating; radiolabelled virus particles were found randomly distributed throughout the cytoplasm and no selective association with any particular organelle could be seen. After reversal of the rhodanine block, uncoating occurred within about 5 min. These workers concluded that disassembly of the nucleocapsid occurs within the cytoplasmic matrix, perhaps following some relatively minor structural alterations on the cell surface or, as recent data would suggest, during entry from the endosomes (Madshus et al., 1984a, b).

Overall, one is left with the impression that most viral nucleocapsids containing plus-sense ssRNA probably enter the cytoplasm more or less intact, before RNA is released and early
protein synthesis begins. Straightforward in vitro experiments with artificially swollen isometric virions (Brisco et al., 1985), or pH 8-washed rod-shaped particles (Wilson, 1984a, b), may therefore have some relevance to early cytoplasmic events in vivo.

**Uncoating and expression of ssRNA viral genomes in plant cells**

Over 75% of all known plant viruses have ssRNA genomes of the positive sense. Apart from plant rhabdoviruses (minus-sense RNA), tomato spotted wilt virus (a possible member of the family Bunyaviridae: Matthews, 1982; Milne & Francki, 1984) and carrot mottle virus (Matthews, 1982), no plant viruses have outer envelopes of the type associated with many animal viruses. There are no known cell surface receptors for plant viruses, to aid specific attachment. Indeed, host specificity would appear to result from late events in infection, e.g. replication, cell-to-cell transport, etc. (Atabekov, 1975; Gaard & De Zoeten, 1979). The mechanism of virus entry into protoplasts or cells is still unclear. Pinocytosis (Cocking & Pojnar, 1969) and/or local, transient wounding of the plasmalemma (Kassanis et al., 1977; Burgess et al., 1973a, b) have been implicated. Recent data support the view that virus uptake into protoplasts is a passive process, primarily dependent on physical (electrostatic) interactions between virus and protoplast (Watts & King, 1984; Watts et al., 1981; for review, see Hull & Maule, 1985). Either of these putative routes would presumably result in more or less intact virions being found within the cytosol. That either route represents what actually happens when isolated protoplasts or plant tissues are inoculated is open to question (Shaw, 1985). Other alternatives exist (Zhuravlev et al., 1980).

The site and mechanism of uncoating of the viral RNA is also shrouded in controversy and confusion. In 1963, Caspar suggested that, since hydrophobic intersubunit bonds were involved in the assembly of the TMV nucleocapsid, a hydrophobic environment might lead to the uncoating of the viral RNA in vivo. Some possible support for this view has been provided by experiments with isolated membrane phospholipids (Kiho et al., 1979; Kiho & Abe, 1980), although poly-L-ornithine also appeared to be necessary.

Alternatively, plant cell walls, with their waxy cuticles, have been proposed as likely sites for nucleocapsid disassembly and RNA release (Gaard & De Zoeten, 1979; De Zoeten, 1981), a mechanism no doubt made attractive by the finding of end-on attachment of rod-shaped plant viruses to plant cell walls (Gerola et al., 1969). However, such an uncoating site would leave the naked RNA with a perilous journey to make before it reached the cytosol.

Many plant viruses contain binding sites for divalent cations (especially Ca\(^{2+}\)-binding sites) which may be involved in the regulation of nucleocapsid assembly-disassembly events (Durham et al., 1977, 1984; Durham, 1978). While Ca\(^{2+}\) ion gradients alone are thought unlikely to account for the release of RNA from rod-shaped or spherical virus capsids, Durham (1978) proposed that, in concert with the hydrophobic environment of a membrane (plasmalemma or intracellular?), nucleocapsid disassembly could take place. Such an amalgamation of destabilizing forces might be closer to the true situation in vivo. However, in the light of recent work on the co-translational disassembly, or release, of RNA from more or less intact nucleocapsids, one might speculate that complete uncoating and release of RNA, simply as a consequence of the prevailing physicochemical properties of the environmental conditions, would be too non-specific and risky from the virus' point of view. Some biological safety device, to ensure complete early gene expression, would seem desirable.

The stabilities of plant viral nucleocapsids vary widely. For example, turnip yellow mosaic virus requires pH 11-5 and above to release its RNA in vitro (Kurtz-Fritsch & Hirth, 1972; Keeling et al., 1979; Keeling & Matthews, 1982), whereas RNA is released from belladonna mottle virus at pH 7-0 and above (Virudachalam et al., 1983a, b). In both cases more or less intact (but swollen) protein shells are left behind, as the RNA exits, probably via a 'molecular trapdoor' (Keeling & Matthews, 1982) formed by a removable cluster of protein subunits (possibly one capsomere). The polar disassembly of several helical nucleocapsids in vitro has also been reviewed recently (Wilson & Shaw, 1985).

Overall, the conditions used to release most viral RNAs in vitro can have little relevance in vivo; however, they serve to illustrate the gulf between the relatively harsh chemical
destabilizing agents and the comparatively mild physiological conditions under which nucleocapsids must uncoat in vivo. Some cumulative effects of, or cooperative interactions with, subcellular components would seem to be involved. The example of Sindbis virus cores (Wengler & Wengler, 1984) is particularly attractive and encouraging here.

**Is there a unifying hypothesis?**

From the discussion above it would appear that many animal and plant viral nucleocapsids arrive more or less intact within the cytoplasm of infected cells, following a variety of entry routes. Some structural change in the nucleocapsid (e.g. swelling or loss of some protein subunits) probably occurs during entry as a consequence of encounters with hydrophobic (phospholipid) environments, protein receptors, Ca²⁺ ion gradients or subcellular compartments of low pH. There is no evidence for compartments of high pH. At this stage the encapsidated RNA would remain at least partially protected from ribonuclease attack, although some exposure of 5' leader signal sequences on the RNA would be expected to facilitate subsequent steps. It is interesting that at least the 5' cap of TMV RNA has recently been shown to be exposed at the concave end of the viral nucleoprotein helix, even at neutral pH (Wu et al., 1984). The topological location and biological function of the covalently attached 5' genome-linked proteins (VPgs) of many viruses may also prove relevant here.

It is proposed that when available components of the host cell translation machinery (e.g. CBP, initiation factors or 40S ribosomal subunits) encounter exposed viral translation initiation sequences, early protein synthesis would begin. Ultimately, ribosome translocation would lead to the sequential disassembly of rod-shaped, helical nucleocapsids or the extraction of RNA from isometric particles, perhaps with the concomitant collapse of the outer protein shell (e.g. BMV; Kurtz-Fritsch & Hirth, 1972). The obvious attraction of this hypothesis is that complete, early viral gene expression would occur only when and where competent translational machinery was available in the host cell. Until then, the protective influence of the viral capsid would continue to a greater or lesser extent.

In this context it is notable that plus-sense ssRNA viruses that employ translational strategies other than polyprotein processing (Davies & Hull, 1982) often have the genes which encode RNA-dependent RNA polymerase (replicase) located close to their 5' termini. These putative early gene sequences would be the first to be expressed during co-translational nucleocapsid disassembly or RNA extraction. The question then arises, at least for rod-shaped particles: does the newly synthesized replicase protein migrate to the 3' end of the virus to begin minus-strand synthesis and further uncoating (i.e. 3' → 5' co-replicational disassembly)?

At the outset, I stated that the aim of this review was to consider nucleocapsid disassembly and early translation of viral RNA as intimately linked events. The behaviour of many animal and plant RNA viruses could be seen to support this hypothesis. So far, few workers have conducted an obvious test of the major prediction from this hypothesis, i.e. that complete RNA release and/or nucleocapsid disassembly fails to occur in the presence of inhibitors of protein synthesis. We must also improve our understanding of the mechanism(s) whereby RNA contained in RNase-resistant nucleocapsids programmes virus-specific protein synthesis efficiently in vitro.

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**REFERENCES**


