Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6232–6236, October 1982
Biochemistry

In vitro assembly of the nonglycosylated membrane protein (M) of Sendai virus

(paramyxovirus/virus assembly/virus nucleocapsid/membrane protein interactions/optical diffraction)

MICHAEL H. HEGGENESS*, P. R. SMITH†, AND PURNELL W. CHOPPIN*

*The Rockefeller University, New York, New York 10021; and †New York University School of Medicine, New York, New York 10016

Contributed by Purnell W. Choppin, July 26, 1982

ABSTRACT **The nonglycosylated membrane protein (M) of Sendai virus was purified from virions and conditions were found under which the protein assembled *in vitro* into three types of ordered structures: narrow tubes, wide tubes, and sheets. These structures were examined by high resolution electron microscopy by using negative staining and metal shadowing techniques. The tubes and sheets are formed from strands 7.2 nm wide that are composed of annular subunits. The wide tubes appear to be formed by the rolling of a sheet into a cylinder in which the 7.2-nm strands are inclined with a pitch of 26–33° and have a left-handed orientation. In addition to the strong reflections corresponding to the 7.2-nm spacings generated by the strands, optical diffraction patterns also showed weak reflections that could be indexed on a lattice corresponding to real-space lattice constants of 7.6 nm and 5.3 nm, with an included angle of 71°. The dimensions and arrangements of these structures formed *in vitro* are strikingly similar to those of ordered arrays of particles found by others to be associated with the inner surface of the plasma membrane of infected cells. The results support the concept that ordered arrays of M protein, similar to those assembled *in vitro*, are involved in the assembly of the virus particle by budding from the cell membrane and that they provide specific recognition sites for the viral nucleocapsid at the cytoplasmic surface of the plasma membrane.**

The nonglycosylated membrane protein (M) of paramyxoviruses (M_r ≈34,000–41,000 depending on the virus strain) is a major protein of the virion that is associated with the inner surface of the lipid bilayer of the viral membrane (reviewed in ref. 1). Based on the location of the protein and on phenotypic mixing experiments that indicated that envelopment of the viral nucleocapsid depended on the presence of the appropriate M protein (2), it was postulated a decade ago that the M protein plays an important role in virus assembly, serving as the recognition site at the plasma membrane of the infected cell between the viral glycoproteins in the membrane and the nucleocapsid in the cytoplasm (1–3). The following findings have supported such a role: the association of the M protein with the nucleocapsid under certain conditions of fractionation of the virus or when the proteins are mixed *in vitro* (4, 5); the finding of M protein and the nucleocapsid protein subunit (NP) on the inner surface of the plasma membrane of infected cells or of erythrocytes with which Sendai virus had fused (6, 7); the crosslinking of M and NP by treatment of virions with crosslinking reagents (8); and the failure of assembly of virions in the absence of a functioning M protein, either in cells infected with a temperature-sensitive mutant of Sendai virus with a lesion thought to be in the M protein (9) or in brain cells in which the M protein is not expressed (10, 11). The M protein may also be involved in the regulation of viral RNA transcriptase activity (12).

Several reports have described the isolation, purification, and partial characterization of M proteins of the paramyxoviruses, Sendai and SV5 (13–16). Hewitt and Nermut (14, 15) found that under certain conditions, the M protein of Sendai virus formed narrow tubular structures composed of what appeared to be M dimers. Bächi and Büechi (17, 18), using freeze-fracture techniques and staining with ferritin- or fluorescein-labeled antibodies, found ordered arrays of particles associated with the inner surface of the plasma membrane of Sendai-infected cells. These particles were virus specific, and it was postulated that they could represent arrays of M protein.

In the present studies, the isolated purified M protein of Sendai virus has been found to assemble into narrow tubes similar to those reported previously (14), but two other forms of ordered structures, consisting of wider tubes and sheets, also have been found. The interrelationships of these structures and their implications for the assembly of the virion at the cell membrane are discussed.

MATERIALS AND METHODS

Virus. Sendai virus was grown in the allantoic sac of 11-day-old embryonated chicken eggs and purified as described (19). Purified virus was suspended in 10 mM sodium phosphate buffer at pH 7.2 (phosphate buffer) and stored at −80°C.

Purification of M Protein. M protein was purified from virus by discontinuous gradients (13) by using a procedure similar to that described for the purification of Sendai virus nucleocapsid (20). Two milliliters of Sendai virus, ≈2 mg of protein per ml, was lysed by the addition of 0.6 ml of 10% Triton X-100 in phosphate buffer and 0.2 ml of 10% sodium deoxycholate in water. The viral lysate was immediately layered onto a discontinuous gradient that consisted of 1.0 ml of 30% CsCl/1.5 ml of 20% CsCl/0.8 ml of 10% sucrose/0.6 ml of 5% sucrose with 1% Triton X-100; all of the above solutions were made up in phosphate buffer. Gradients were centrifuged for 90 min at 35,000 rpm in a Spinco SW56 rotor, and the M protein was recovered from the sucrose/CsCl interface. The M protein, which had been solubilized at the cesium layer, was dialyzed overnight in the cold against phosphate buffer, causing its precipitation (21). Electrophoresis was carried out in 10% polyacrylamide gels (22). Preparations of purified virions were included as polypeptide markers.

Electron Microscopy and Optical Diffraction. Precipitated M protein was gently resuspended in phosphate buffer and applied to grids coated with thin carbon support films. Specimens were first fixed by floating the grids on drops of freshly prepared 3% formaldehyde in phosphate buffer for a minimum of 10 min and then were washed on drops of distilled water. Spec-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: M, virus nonglycosylated membrane protein; NP, protein subunit of virus nucleocapsid; phosphate buffer, 10 mM sodium phosphate buffer at pH 7.2.

imens were either stained with 1% uranyl acetate or freeze-dried and platinum/carbon shadowed following the procedure of Smith (23). Micrographs were recorded on Kodak electron image plates in a Philips EM 301 electron microscope at a nominal magnification of ×70,000. Areas showing ordered structures were masked off from neighboring areas and optically diffracted by using standard procedures (24).

RESULTS

General Observations. The preparation of M protein by the discontinuous gradient method reproducibly yielded preparations of pure M protein, as shown in Fig. 1. An occasional preparation contained evidence of the viral NP and P proteins, but only in trace amounts. When examined in the electron microscope, most of the precipitated M protein was seen to be aggregated in masses; however, three different kinds of ordered structures could be seen in the regions adjacent to these masses. The most common of these structures were narrow tubes, similar to those described by Hewitt and Nermut (14). In addition, two new kinds of ordered structures were observed, which appeared as wide tubes and single-layered sheets. The sheets were observed to be less common than the narrow tubes, and the wide tubes were relatively rare. Both structures could be easily distinguished from the narrow tubes.

Narrow M Protein Tubes. M protein tubes (Fig. 2*a*) were found in clusters adjacent to larger masses of precipitated M. The average width of isolated narrow tubes was 15 nm, but the width can be quite variable depending on the staining conditions (14). The tubes can be up to 600 nm long, although one end usually remains embedded in a mass of precipitate, which appears in some micrographs to consist in part of large bundles of tubes. Isolated narrow tubes found in thin layers of stain (Fig. 2*a*) usually showed little structure, in contrast to nucleocapsids (Fig. 2*b*) from which they can be easily distinguished. However, careful screening revealed a small number of tubes that show a pattern of striations. When negatively stained tubes were optically diffracted (Fig. 3*a*), the spacing of the striations was measured to be 7.2 nm, and the pattern is clearly different from that of the nucleocapsid (Fig. 3*b*) which can be used for calibration purposes. The diameter of the helical nucleocapsid is 20 nm and the spacing of the turns of the helix is 5.6 nm, with a pitch of 8°.

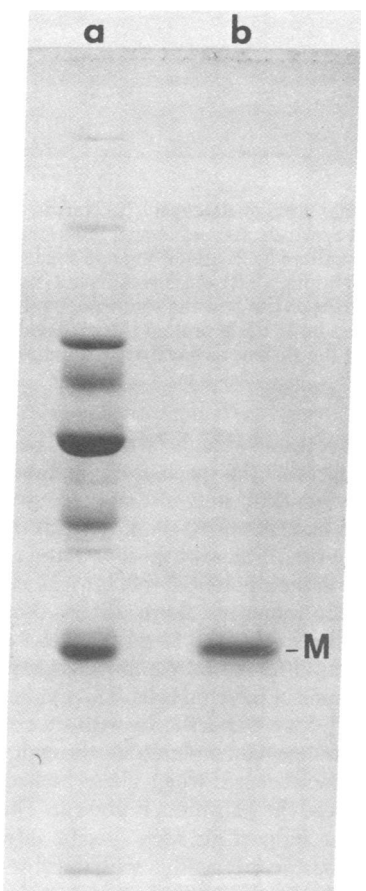

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified Sendai virions (lane a) and purified M protein isolated from virions (lane b).

Freeze-drying and shadowing revealed left-handed striations on the upper surface of the tubes with a pitch of 40–45° and a spacing of 6.1 nm. The measured width of these tubes allows the number of helically wound strands to be estimated (from the width and the pitch and spacing of the striations) to be between four and five. A more accurate estimate is not possible due to the variations in apparent strand spacing and tube diameter.

Wide Tubes and Sheets. Wide tubes and sheets (Fig. 2*c*) were found on the grids adjacent to, but usually not attached to, clumps of precipitated M protein. The wide tubes have variable widths between 56 and 43 nm and lengths that may exceed 1,000 nm. The tubes appear to be built from strands 7.2 nm wide, located side-by-side, and often containing a weak central stain line. The strands are inclined with a pitch of 26–33°, and the number of strands that form the wall of the tubes can be counted to be between five and eight, depending on their width. The strands are composed of annular units (indicated by arrowheads in Fig. 2*c*) that correspond to similar structures seen previously (14, 15) in negatively stained preparations of M protein and thought to represent dimers. Optical diffraction patterns of wide tubes (Fig. 3*c*) show a superposition of two sets of diffraction spots from the two sides of the tube, each corresponding to the 7.2-nm spacing. The right-handed striation pattern appears to be the strongest of the two, which would indicate that the tubes are left-handed, because the side of the tube closest to the support film is usually the most heavily contrasted.

Sheets were found to be more numerous then wide tubes but could often be found only as very small fragments on the grid. Usually they were found as rectangular structures whose width and length rarely exceeded 45 and 400 nm, respectively. As was the case with the wide tubes, the sheets appear to be built from bands 7.2 nm in width that are packed side-by-side. However, the sheets are often disordered with the band spacing varying between 6.5 and 8.0 nm and with adjacent bands not following exactly parallel paths.

The majority of sheets tend to be rectangular in outline with the bands running diagonally, making an angle of 25–35° to the narrowest dimension of the rectangle. Therefore, they give the impression of being the remnants of wide tubes that have attached to the grid and then disintegrated, leaving behind only those pieces stabilized by their contact with the support film.

Optical diffraction patterns from sheets (Fig. 3*d*) show the strong reflections corresponding to the spacings of 7.2 nm generated by the bands. Four additional weak reflections were also observed in rare cases, which could be indexed on a lattice corresponding to real-space lattice constants of 7.6 and 5.3 nm, with an included angle of 71°. These reflections were too weak to permit a filtration to be performed, which would have allowed the two-dimensional lattice to be observed.

Attempts were made to visualize surface structure on the sheets by freeze-drying of preparations that had been determined by negative staining to contain a significant proportion of sheets; however, no regular structures were observed by this method.

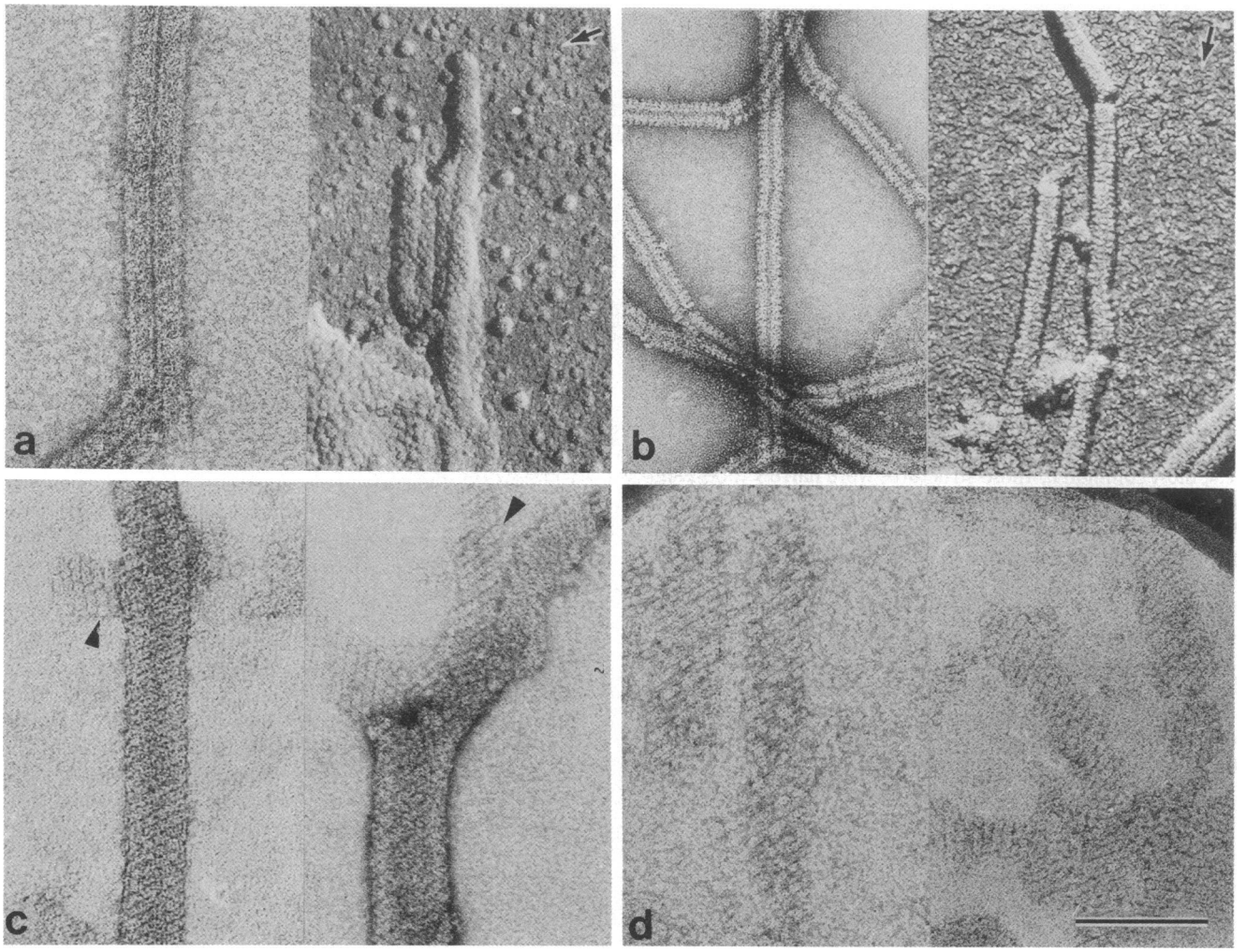

FIG. 2. Electron micrographs of structures formed by Sendai virus M protein and of Sendai virus nucleocapsid. (*a*) Narrow tubes of M protein shown after negative staining (left) and freeze-drying and metal shadowing (right). In the negatively stained image, faint striations can be seen that come from the side of the tube closest to the support film. The free side of the tube is visualized by freeze-drying and shadowing, which shows the helical striations on the tubes to be left-handed. (*b*) Purified nucleocapsids after negative staining (left) and freeze-drying (right). (*c*) Wide tubes of M protein after negative staining. The right panel shows a tube that has begun to disintegrate on the grid and split into the sheet structure from which it is constructed. Arrowheads indicate the annuli of M protein from which the tubes are built. (*d*) M protein sheets, negatively stained. The freeze-dried and metal-shadowed images in *a* and *b* show the presence of metal as light and the shadow as dark: arrows indicate the direction of metal deposit. The bar indicates 100 nm.

This negative result could be due to structural disordering of the specimen caused by the freeze-drying process, but another possibility is that the sheets may attach to the support film so as to leave an untextured side exposed.

DISCUSSION

Three different types of ordered structures have been found to be formed by the *in vitro* aggregation of the M protein of Sendai virus: narrow tubes, wide tubes, and sheets. The relationship between the wide tubes and sheets appears clear. As shown in the right panel of Fig. 2*c*, the wide tubes are formed by a sheet rolled into a cylinder. The sheets are built from strands of annuli that have the same appearance as those seen by Hewitt and Nermut (14, 15) in preparations of purified, disassembled M protein subunits and thought to be M dimers. Such annular units can be seen in the small fragment of a sheet beside the wide tube in Fig. 2*c*. However, in many preparations of sheets individual annuli cannot be detected, and the strands appear to possess a central stain line. Narrow tubes also have been found in association with sheets and strands of annuli. These findings, coupled with the fact that the spacings on the tubes and the sheets are essentially identical, suggest that these structures are built from the same basic elements—i.e., strands of annuli.

Much of the significance of these ordered assemblies of the M protein lies in their potential role in virus assembly. As summarized in the Introduction, there is a considerable body of evidence that indicates that the M protein plays a key role in the assembly by budding of the paramyxovirus particle from the plasma membrane of infected cells. This evidence suggests that the M protein interacts specifically with sites on the viral membrane glycoproteins which penetrate through the lipid bilayer and with the nucleocapsid which aligns beneath those areas of membrane where the M protein is present. The present results provide further support for such specific interactions. In addition, when considered together with the observations of Bächi and Büechi (17, 18), they provide persuasive evidence regarding the nature of the organization of the M protein at the inner surface of the plasma membrane of infected cells. These authors, using freeze-fracture techniques (17) and a procedure that

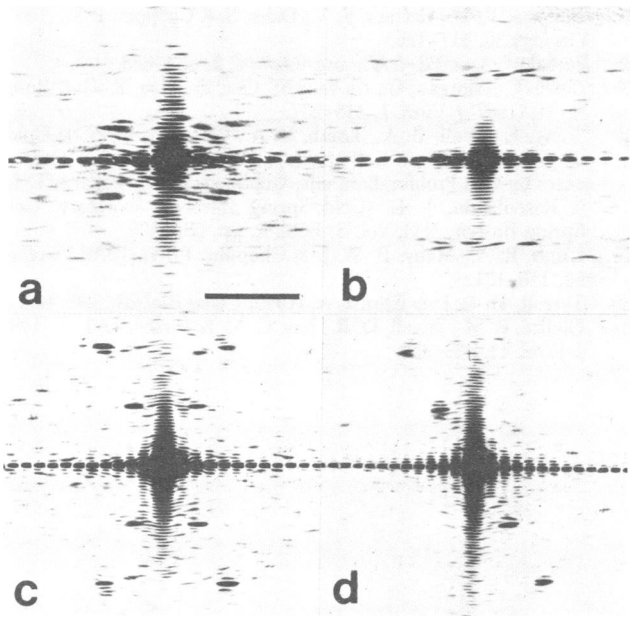

FIG. 3. Optical diffraction patterns of selected areas of typical negatively stained images of (*a*) narrow tubes, (*b*) nucleocapsids, (*c*) wide tubes, and (*d*) sheets. The bar indicates 0.5 nm.

permitted the visualization of the cytoplasmic surface of the membrane by immunoelectron microscopy and immunofluorescence microscopy with antiviral antibodies (18), detected several virus-specific structures associated with the inner surface of the cell membrane. These structures included the viral nucleocapsid and also ridge-like elevations with striations on them that were thought (18) to resemble the tubes of M protein seen by Hewitt and Nermut (14). These ridges were shown to be virus specific by staining with antibody against whole virus, but the specific presence of M was not demonstrated. The spacing of the striations on these ridges was 7 nm, which is remarkably similar to the 7.2-nm spacing found here on the tubes of purified M protein, suggesting that they do indeed contain M protein.

The most striking finding on the cytoplasmic surface of infected cells was patches of particles in crystalline array with a periodicity of 7.5 nm (17). These also were interpreted to be related to M protein, because of their location and because they were found in areas where the nucleocapsid was adherent to the membrane. The striking similarity between the 7.2-nm spacing of the sheets of purified M protein found in the present study and the arrays of particles at the inner surface of the plasma membrane of the infected cell suggests strongly that the ordered structures on the membrane are composed of M protein. The results further suggest that the sheets of M protein assembled *in vitro* in this study represent a structural organization that occurs *in vivo* at the plasma membrane, and this is probably the arrangement that is recognized by the nucleocapsid.

The ridges seen on the membrane (18) could represent partial rolling into a cylindrical structure of the sheets of M, similar to the formation of the wide tubes found *in vitro* in the present experiments. In this regard, it should be noted that the spacing of the M sheets (7.2 nm) is about 25% larger than the axial repeat of the tightly wound nucleocapsid helix (5.6 nm). However, it is pertinent to note that for the nucleocapsid, which has a unit length of 1,000 nm, to be enveloped within virions with an average diameter of ≈150 nm, the nucleocapsid must be flexible and must undergo some loosening of the helical winding, at least in certain regions. Further, it has been shown that the paramyxovirus nucleocapsid can reversibly coil and uncoil with changes in salt concentration (20, 22). Thus, if the helical nucleocapsid were in a loosely coiled configuration during the budding process, it could fit along one axis of a membrane-associated sheet of M protein. Such an interaction of nucleocapsid with a regular array of M protein could explain the striking arrays of regularly spaced, membrane-associated nucleocapsids seen in paramyxovirus-infected cells (25–27).

Thus, the available evidence suggests that the assembly of the paramyxovirus particle involves the specific interaction of the nucleocapsid with an ordered array of M protein associated with the inner surface of the plasma membrane which also interacts with the ends of the viral membrane glycoproteins extending through the lipid bilayer. It is not yet clear what events follow this interaction, although it is possible that the interaction of the nucleocapsid with the M protein initiates the budding process. It has been observed that the arrival of the nucleocapsid beneath the membrane is accompanied by the appearance of erect viral glycoprotein spikes on the area of membrane overlying the nucleocapsid (1, 25). The glycoproteins can be detected in the membrane by ferritin-labeled antibodies before the arrival of the nucleocapsid, but they are not seen without such a label. The arrival of the nucleocapsid apparently triggers a rearrangement that results in the appearance of erect spikes. We suggest that the events that are set in motion by the arrival of the nucleocapsid are mediated through cooperative interactions between it, an ordered array of M protein, and the glycoproteins, but the mechanisms remain to be elucidated. Other proteins also could be involved in the virus assembly process. The cellular protein actin has also been found in paramyxovirus particles (28, 29) and found to bind the M and NP proteins (30, 31). Whether this is a fortuitous association of an abundant cellular protein with viral proteins or whether it indicates the participation of cytoskeletal elements in the virus assembly process remains to be determined. Further studies on the specific interactions of these various proteins *in vitro* as well as *in vivo* should provide more information on the precise mechanisms of virus assembly budding from the cell membrane.

We thank Theresia Sonyi, Martha Hatchett, and Alexandra Moody for expert technical assistance. This work was supported by research grants from the National Institutes of Health (AI 05600 to P.W.C. and GM 26723 and RR 05399 to P.R.S.) and the National Science Foundation (PCM80-13464 to P.W.C.) M.H.H. was a U.S. Public Health Service Postdoctoral Fellow (AI 05965).

1. Choppin, P. W. & Compans, R. W. (1975) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. R. (Plenum, New York), Vol. 4, pp. 95–178.
2. McSharry, J. J., Compans, R. W. & Choppin, P. W. (1971) *J. Virol.* **8**, 722–729.
3. Choppin, P. W., Compans, R. W., Scheid, A., McSharry, J. J. & Lazarowitz, S. G. (1972) in *Membrane Research*, ed. Fox, C. F. (Academic, New York), pp. 163–179.
4. Shimizu, K. & Ishida, N. (1975) *Virology* **67**, 427–437.
5. Yoshida, T., Nagai, Y., Yoshii, S., Maeno, K., Matsumoto, T. & Hoshino, M. (1976) *Virology* **71**, 143–161.
6. Lyles, D. S. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5621–5625.
7. Bowen, H. A. & Lyles, D. S. (1981) *J. Virol.* **37**, 1079–1082.
8. Markwell, M. A. K. & Fox, C. F. (1980) *J. Virol.* **33**, 152–166.
9. Yoshida, T., Nagai, Y., Maeno, K., Iinuma, M., Hamaguchi, M., Matsumoto, T., Nagayoshi, S. & Hoshino, M. (1979) *Virology* **92**, 139–154.
10. Hall, W. W., Lamb, R. A. & Choppin, P. W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2047–2051.
11. Choppin, P. W. (1981) *Ann. Neurol.* **9**, 17–20.
12. Marx, P. A., Portner, A. & Kingsbury, D. W. (1974) *J. Virol.* **13**, 107–112.
13. McSharry, J. J., Compans, R. W., Lackland, H. & Choppin, P. W. (1975) *Virology* **67**, 365–374.

14. Hewitt, J. A. & Nermut, M. V. (1977) *J. Gen. Virol.* **34**, 127–136.
15. Hewitt, J. A. (1977) *FEBS Lett.* **81**, 395–398.
16. Semba, T., Hosaka, Y. & Sakiyama, F. (1977) *Biken J.* **20**, 77–80.
17. Bächi, T. (1980) *Virology* **106**, 41–49.
18. Büechi, M. & Bächi, T. (1982) *Virology* **120**, 349–359.
19. Hsu, M.-C., Scheid, A. & Choppin, P. W. (1979) *Virology* **95**, 476–491.
20. Heggeness, M. H., Scheid, A. & Choppin, P. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2631–2635.
21. Scheid, A. & Choppin, P. W. (1973) *J. Virol.* **11**, 263–271.
22. Heggeness, M. H., Scheid, A. & Choppin, P. W. (1981) *Virology* **114**, 555–562.
23. Smith, P. R. (1980) *J. Ultrastruct. Res.* **72**, 380–384.
24. Aebi, U., Smith, P. R., Dubochet, J., Henry, C. & Kellenberger, E. (1973) *J. Supramol. Struct.* **1**, 498–522.
25. Compans, R. W., Holmes, K. V., Dales, S. & Choppin, P. W. (1966) *Virology* **30**, 411–426.
26. Berkaloff, A. (1963) *J. Microsc. (Oxford)* **2**, 633–638.
27. Howe, C., Morgan, C., de Vaux St. Cyr, C., Hsu, K. C. & Rose, H. M. (1967) *J. Virol.* **1**, 215–237.
28. Wang, E., Wolf, B. A., Lamb, R. A., Choppin, P. W. & Goldberg, A. R. (1976) in *Cell Motility*, Cold Spring Harbor Conferences on Cell Proliferation, eds. Goldman, R. D., Pollard, T. D. & Rosenbaum, J. L. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 3, Book A, pp. 589–599.
29. Lamb, R. A., Mahy, B. W. J. & Choppin, P. W. (1976) *Virology* **69**, 116–131.
30. Tyrrell, D. L. J. & Ehrnst, A. (1979) *J. Cell Biol.* **81**, 396–402.
31. Giuffre, R. M., Tovell, D. R., Kay, C. M. & Tyrrell, D. L. J. (1982) *J. Virol.* **42**, 963–968.