The Structure of Particles of Tobacco Ringspot Nepovirus: Evidence from Electron Microscopy

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(Accepted 13 May 1988)

SUMMARY

Particles of tobacco ringspot nepovirus from purified preparations were trapped on grids by immunosorbent electron microscopy and then either negatively stained, or freeze-dried and shadowed with uranium, for structural studies. Particle dimensions differed considerably with different stains and methods of preparation, but no obvious substructure was apparent. In contrast, particles which were freeze-dried and then shadowed exhibited either fivefold or threefold symmetry and had a structure resembling that of models made up of 60 units in clusters of five arranged in a $T = 1$ lattice. Both chemical and morphological evidence are compatible with this structure.

INTRODUCTION

The structure of particles of several plant viruses has been determined by examination of electron micrographs of negatively stained preparations (Horne, 1961; Finch & Klug, 1966). One of the main problems with negative staining, however, is that depending on the depth of stain surrounding the particle, information derived from opposite sides of the particle may be superimposed, and image analysis by computer is then needed to help interpret the images (Finch et al., 1970; Adolph & Butler, 1974; Crowther et al., 1974; Horne, 1979).

One way of avoiding this problem is to use a method which provides information on the three-dimensional structure of only one side of a particle; high resolution shadowing (Roberts & Mayo, 1980) of particles which have been freeze-dried for better structural preservation (Roberts & Duncan, 1981) is such a method. Modern shadowing techniques can give a resolution of approx. 2 nm, similar to that achieved with negative stains, and freeze-drying can prevent collapse of virus particles on the support film, so retaining structural features which may be damaged during staining or subsequent air-drying.

This paper describes the use of these techniques to study the structure of the particles of tobacco ringspot virus (TRSV), the type member of the nepovirus group, no other member of which has had its particle structure elucidated convincingly.

TRSV has particles about 27 nm in diameter which sediment as three components. All three components contain the same protein which is the only major constituent of the slower sedimenting component (T). Middle component (M) particles contain, in addition, one molecule of the smaller genome segment RNA-2 ($M_r 1.34 \times 10^6$), whereas particles of the fastest sedimenting component (B) contain either one molecule of the larger genome segment, RNA-1 ($M_r 2.73 \times 10^6$), or two molecules of RNA-2 (Stace-Smith, 1985). The particle protein has an $M_r$ of approx. 57 000 (Mayo et al., 1971) although there is evidence to suggest that each subunit is a stable tetramer of a protein of $M_r$ approx. 13 000 (Chu & Francki, 1979). The data presented here indicate that the particles of TRSV have $T = 1$ symmetry and may be constructed of 60 subunits, a finding which conflicts with previous reports (Chambers et al., 1965; Chu & Francki, 1979) in which a $T = 4$ structure was postulated.

METHODS

Virus purification and particle assessment. TRSV (New Jersey blueberry ringspot isolate) was propagated in Nicotiana clevelandii and its particles were purified as described by Mayo et al. (1982). In some tests, impure
whether, after minimal treatment, the virus was suitable for high resolution electron microscopy. To assess the suitability of the virus particles for structural studies, fresh (<3 days old) preparations were diluted in newly prepared 0.1 M-ammonium acetate or 0.1 M-ammonium formate (pH 7.2 to 7.4, unadjusted), and examined by electron microscopy using different negative stains. In subsequent tests these media, which volatilize readily during freeze-drying, gave markedly better results than distilled water or phosphate buffer.

Attachment of virus particles to grids. To prepare grids with suitable numbers of evenly dispersed virus particles, the particles were trapped by antibody, as in immunosorbet electron microscopy (ISEM) (Roberts & Harrison, 1979; Roberts, 1986b). Copper/rhodium grids (400 mesh) (Graticules, Tonbridge, U.K.) coated with a thin carbon-stabilized film of pyroxylin were used for all experiments. These grids were first floated for 15 to 30 min on 10 µl drops of TRSV antiserum which had been diluted $10^{-4}$ in 0.1 M-ammonium acetate, pH 7.2 and were then thoroughly washed in wells containing 2 to 3 ml ammonium acetate solution before being floated on 10 µl drops of the virus samples diluted in 0.1 M-ammonium acetate or 0.1 M-ammonium formate. When a suitable distribution of particles was obtained, usually after 2 to 10 min, all remaining grids were removed from the virus drops and transferred to wells containing either 0.1 M-ammonium acetate or 0.1 M-ammonium formate, where they were washed for 10 to 15 min before negative staining or freeze-drying. Virus preparations which were originally diluted in ammonium acetate were subsequently treated with that solution; in other tests ammonium formate was used throughout.

Negative staining. Grids with virus particles which had been washed with ammonium acetate or ammonium formate were stained with 2% ammonium molybdate, pH 7.0, 2% methylamine tungstate (Faberge & Oliver, 1974), pH 7.0, or 1% aqueous uranyl acetate, pH 3.8. In every instance, a grid with attached virus particles was removed from the washing well and, without drying, washed with three to four drops of stain. The grid was immediately drained with filter paper and dried. Washing the grids with ammonium salts before staining allowed the same procedure to be used for all stains, and it resulted in retention of less non-viral material on the grids.

Freeze-drying. The method used was essentially that described by Roberts & Duncan (1981). Grids to be freeze-dried were removed from the wash wells, blotted and frozen as described by Roberts & Duncan (1981), and loaded into the freeze-drying module (Agar Aids, Stansted, U.K.) under liquid nitrogen. At least three grids were prepared from every sample and air-dried control grids were also included and shadowed under the same conditions. The module was used with the base adjusted to a mass of 240 g, and the top to 420 g. During the freeze-drying run, the vacuum in the coating unit was monitored using a Servoscribe 1S chart recorder attached to a Penning gauge and operating at a chart speed of 30 mm/h. The method to select the best time to separate the top from the base, for shadowing, and the operating vacuum is illustrated in Fig. 1.

Shadowing. High resolution shadowing with uranium was done using the method of Roberts & Mayo (1980). The shadowing angle in all runs was nominally 45°, however, because of sagging of the support film over the grid holes this allowed examination of virus particles which had been shadowed at angles ranging from 40° to 50°. Shadowing was done slowly, 5 to 7 min being the normal run, and during that time the vacuum was always <2 mPa (E in Fig. 5). The rate of evaporation and the amount of uranium deposited was estimated using a foil screen behind the evaporation source, a single spot being the minimum amount detected, representing a metal thickness of <2 nm (calculated according to Roberts & Mayo, 1980). After the grids had been shadowed, they were left under vacuum overnight to allow the freeze-drying module to reach room temperature. They were then removed and examined.

Electron microscopy. Grids were examined in a Philips 301G or a Jeol JEM 100S electron microscope operating at 40, 60 or 80 kV. All negatively stained samples were photographed at 80 kV and the micrographs printed in the normal way. Shadowed samples were usually photographed at 60 kV because this gave better structural information and more clearly defined shadow profiles, and all such micrographs were printed directly onto contrast reversal film (Kodagraph Transtar TPP5).

Catalase crystals (Wrigley, 1968) were used to calibrate the microscopes. Micrographs of suitable crystals were taken at the beginning, in the middle, and at the end of each series of micrographs (Roberts, 1986a,b), using the same magnification and objective lens current values. The mean value of the catalase calibrations was used for measurement of the virus particles, and experience of this calibration method over several years indicates an accuracy of $\pm 5\%$. It is worth noting that differences between the stated and calculated magnifications of both microscopes sometimes exceeded $\pm 5\%$ over short periods of time (hours), and emphasized the need for inclusion of calibration micrographs with those from the samples.

Examination of micrographs. Micrographs which had been taken at microscope magnifications of 20000 × to 400000 × and printed to give final magnifications of approx. 150000 × were suitable for general assessment of particle condition, distribution, shadowing quality etc., but were not suitable for detailed structural examination. To do this, micrograph negatives were examined directly in a microfiche reader (Bell & Howell, Model ABR-900), which offered a range of magnifications between 18 × and 43 × , and the inclusion of a reference grid allowed selected particles to be relocated as required. Also, by fitting a simple blade shutter between the lens and the viewing screen, and incorporating suitable filters (Roberts, 1982), the microfiche reader could be used as a
photographic enlarger capable of producing good quality prints of micrographs (see Fig. 2, 6 and 7). Furthermore, by defocusing the image, structural features on the virus particles could, in many instances, be enhanced (Fig. 6).

Particle size measurements. Micrographs of negatively stained or shadowed virus particles were projected at approx. 20 × onto sheets of paper and their diameters marked, or their circumferences traced (Roberts, 1986a). Both types of measurement were used initially and were in good agreement; later, however, only circumference measurements were used because problems with ovoid or angular particles were thereby eliminated. Traces of particles (minimum 100) were measured using an ID-TT-20 digitizer (Summagraphics, Fairfield, Conn., U.S.A.) linked to a 4051 graphics system (Tektronix, Beaverton, Ore., U.S.A.), and histograms of particle size distributions plotted using the calibrated magnification values.

Models. Several models of virus particles were constructed using information obtained from the electron micrographs. The model shown (Fig. 8a) comprises 60 corks, mounted together as twelve pentamer clusters in a T = 1 structure (see Fig. 8, D and 9, E of Horne & Wildy, 1961). This model was photographed in different orientations with light sources and backgrounds to mimic shadowing, and at different amounts of defocus to simulate the effects seen on micrographs when examined with the microfiche reader.

RESULTS

Negatively stained preparations

The appearance of virus particles differed with the stain used, and the best results were obtained with ammonium molybdate. In this stain particles were intact, consistent in size and appearance, many showed a regular angularity, and the stain clearly differentiated the top and bottom component particles (Fig. 2). Particles in methylamine tungstate were less angular and larger (swollen or collapsed), while staining with uranyl acetate produced a high proportion of irregularly shaped and damaged particles. Moreover, in uranyl acetate, the top and bottom component particles were not distinguishable.

The particles showed no obvious substructure in any of the stains tested, although a few particles in ammonium molybdate had some regular features suggestive of capsomere-like structures (Fig. 3). However, these features were not consistently found nor could they be interpreted as part of a regular structure.

Particle sizes

There were considerable differences in the size of particles prepared by freeze-drying or after staining in different negative stains (Fig. 4). Particles which were freeze-dried and shadowed
Fig. 2. TRSV particles stained with 2% ammonium molybdate, pH 7.0. Two kinds of particles are distinguished. These are presumed to be T and B component particles; many particles have an angular outline. Bar marker represents 100 nm.

Fig. 3. Montage of individual TRSV particles in ammonium molybdate stain after selection and photography in a microfiche reader. Some particles show features suggestive of capsomeres. Bar marker represents 25 nm.

had mean diameters of $24.5 \pm 0.95$ (from ammonium acetate solution) and $25 \pm 0.85$ nm (from ammonium formate solution). Diameters of particles in ammonium molybdate were $27 \pm 1.1$ nm, but when methylamine tungstate or uranyl acetate was used, the mean diameters were considerably larger at $32 \pm 1.1$ nm. The length and shape of the shadow profiles (Fig. 5a, b) gave
Fig. 4. Frequency of circumference measurements for 100 particles after different treatments. Mean ± standard deviation in brackets. (a) Ammonium formate, freeze-dried and shadowed (77 ± 2.92 nm); (b) ammonium acetate, freeze-dried and shadowed (79.5 ± 2.5 nm); (c) ammonium molybdate, pH 7.0 (84.6 ± 3.6 nm); (d) methylamine tungstate, pH 7.0 (99.7 ± 3.5 nm); (e) uranyl acetate, pH 3.8 (100.2 ± 3.2 nm).

Fig. 5. TRSV particles after ammonium acetate treatment and uranium shadowing. (a, b) Particles after freeze-drying showing good preservation and some structural features, (c) particles after air-drying, obscured by debris or collapsed and showing no substructure. Bar marker represents 100 nm.

no evidence that the freeze-dried particles had collapsed whereas the irregular shape of the particles that were negatively stained with methylamine tungstate or uranyl acetate indicated damage resulting in swelling and/or collapse of the particles on the support film during drying.

**Freeze-dried, shadowed preparations**

Freeze-drying of grids using the technique and device described by Roberts & Duncan (1981) and high resolution shadowing with uranium (Roberts & Mayo, 1980) was essential for
observing structural detail on the virus particles. Furthermore, the capsomeres were more distinct with freshly purified virus samples compared with older (>48 h) preparations. No differences were observed between such preparations after negative staining. Particles which had been air-dried and shadowed were irregular in shape and size, and did not show well defined shadow profiles (Fig. 5c). In contrast, freeze-dried preparations had angular particles whose shadow outlines clearly indicated that there had been no collapse or deformation on the grid (Fig. 5a, b).

The best results were obtained when the top of the freeze-drying module had a mass of 420 g; this gave a better ‘etching’ effect (Roberts & Duncan, 1981) than using the top with less mass, presumably by providing a greater temperature differential between the top and the base and by maintaining a lower temperature for a longer time. Careful monitoring of the vacuum during the freeze-drying run using a chart recorder coupled to the high vacuum gauge also indicated the best time for separation of the top and base. This was just before the main peak (C in Fig. 1) on the graph, and some 20 min after etching began (B in Fig. 1). After separation of the top from the base, the grids were left for a further 90 min before being shadowed. Shadowing with uranium had several advantages over other metals. It gave a resolution comparable to that obtained with negative staining, and evaporation could be performed very slowly to give a uniform and reproducible deposition of metal. Moreover, there was no marked decrease in vacuum during evaporation, a feature common to other shadowing techniques and perhaps harmful to the specimens.

At low magnifications (approx. 130000 ×) only a few structural features could be seen on some particles. Using the microfiche reader at higher magnifications of 600000 × to 900000 ×, many more particles showed morphological units or projections in a regular array (Fig. 6, 7). These structural features were enhanced by defocusing the projected images (Fig. 6a, b, c) and showed numerous pentagonally shaped particles with fivefold symmetry. Careful examination also showed an electron-transparent ‘hole’ in many of these projections suggesting that they are

Fig. 6. TRSV particles from ammonium formate medium after freeze-drying and shadowing. Prints were prepared directly from microfiche reader (see Methods) with increasing defocus from (a) to (c). Note that the appearance of the capsomeres is enhanced with increasing defocus. Arrow indicates direction of shadowing. Bar marker represents 50 nm.
hollow tubes or rings of smaller units (Fig. 7). These 'holes' were approx. 2 nm in diameter and the morphological unit was approx. 8 nm in diameter.

The vast majority of particles showing substructure had fivefold symmetry (Fig. 7a, b) but only a few of these were aligned about the fivefold axis (Fig. 7b). More rarely, particles were aligned about their threefold axis (Fig. 7c) but no unequivocal examples of twofold symmetry were seen. Nonetheless, these features corresponded to an icosahedral structure with T = 1 symmetry, and were compared with a model of such a structure (see Methods). When the model was photographed in different orientations and matched with several shadowed particles there was a considerable similarity (compare Fig. 7 and 8). This similarity is further evidence that the surface of TRSV particles has the icosahedral symmetry of a T = 1 lattice.

**DISCUSSION**

Careful preparation of the specimens on the grids before freeze-drying and shadowing was essential for showing structural details of the virus particles. ISEM allowed the virus particle numbers to be controlled, and the firm attachment of the particles to the support film permitted thorough washing. Although various washing procedures were examined, ammonium acetate and ammonium formate each consistently gave good results and their use was compatible with ISEM.

The widely different sizes of particles after different treatments was not expected. Although uranyl acetate is known to damage the particles of many plant viruses (Roberts, 1977, 1986a, b; Duncan & Robinson, 1981), the 20% increase in particle diameter over that for particles stained with ammonium molybdate, and the approx. 25% increase over freeze-dried, shadowed samples (allowing for a metal thickness of 2 nm) raises serious doubts about sizes of quasi-spherical virus particles measured in this stain. The similarity in measurements of ammonium molybdate-stained particles and of those which were freeze-dried and shadowed suggests that uranyl acetate and methylamine tungstate cause swelling, and not that freeze-drying causes shrinkage. This swelling, together with the superimposition of structural information from both sides of the particle in negative stain, may help to explain the lack of obvious substructural features after staining.

The reproducible evidence of capsomeres on particles of TRSV and some other spherical plant viruses (Roberts, 1984) indicates that they are not preparation artefacts. Moreover, they are unlikely to be shadowing artefacts caused by progressive aggregation of metal particles, because the deposited metal layer is thin (approx. 2 nm), and holes of about this diameter can be discerned in the centre of many capsomeres. Thicker metal layers would be more likely to cover such details and create a smooth profile thus obscuring structural features such as capsomeres. Also, the size and appearance of the capsomeres, some of which appear to be made up of five smaller units, are reproducible and unlike any areas of metal deposition on the support film which one might expect to find if they were artefacts.

The appearance of particles of TRSV after freeze-drying and shadowing conforms closely to micrographs of bacteriophage φX174 (Hall et al., 1959), and to models constructed of 60 subunits clustered in groups of five in a T = 1 structure, indicating this is the triangulation number of the virus particle. This contrasts with the interpretation of Chambers et al. (1965), who suggested a 42 capsomere structure, but is compatible with estimates of Mr 57 000 for the particle protein of TRSV together with a calculated weight for the protein shell of Mr 3.5 x 10^6 of TRSV particles (Mayo et al., 1971). Previous evidence for a T = 1 structure was challenged by Chu & Francki (1979) who obtained evidence for a polypeptide of Mr, 13000 in preparations of TRSV particles and suggested that they comprised 240 subunits in a T = 4 structure. However, none of the particles of TRSV examined in my study showed any of the special structural features of particles of Nudaurelia capensis β virus, the first shown to have T = 4 symmetry and which contain 240 protein subunits (Finch et al., 1974).

All the evidence presented here indicates therefore that TRSV particles are constructed from 60 subunits clustered in 12 pentamers to give a T = 1 structure and supports the conclusions of Mayo et al. (1971). Whether the individual protein subunits have an Mr of 57000 or are
Fig. 7. Montages of selected freeze-dried, shadowed, particles showing structural symmetry and tracings of their most prominent morphological features. The particles have been printed directly using the microfiche reader. (a) Particles showing obvious fivefold symmetry but not necessarily axially aligned, (b) particles showing the fivefold rotational axis, and (c) particles showing the threefold rotational axis. Arrow indicates direction of shadowing. Bar marker represents 50 nm.
themselves stable tetramers as suggested by Chu & Francki (1979) must await the results of further study.

I thank A. F. Murant for supplies of purified virus particles and S. Malecki for photography of the models and simulated images.

REFERENCES


*(Received 5 April 1988)*