Detection of Viral-like Particles by Electron Microscopy of Negatively Stained Extracts from Grapevines.

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Viral-like particles were detected by electron microscopy of negatively stained extracts from young leaves and roots of grapevines infected with fanleaf virus and purported to be infected with the graft-transmissible agents of leafroll, corky bark, fleck, and stem grooving. Preparations were extracted in 0.01 M phosphate buffer (pH 7.0) containing 2.5% nicotine and negatively stained with 2% ammonium molydate. Membrane associated spherical particles were detected in extracts from grapevines that tested positive for fanleaf virus by ELISA and bioassay. Similar membrane associated particles were detected in herbaceous plants inoculated with grapevine extracts. Rigid rod tobacco mosaic virus-like particles were detected in extracts from some grapevines but they were not disease associated. Flexuous rod viral-like particles about 11 x 800 nm with cross-banding helical substructure similar to closteroviruses were detected in extracts from leafroll infected grapevines.

In 1964, M. A. Lauffer wrote "The most fundamental question in modern virology, therefore, is the identification of the biological role of characteristic particles". After 20 years, the statement is still relevant, especially when considering diseases for which the agents are only graft-transmissible. Woody deciduous fruit crops, especially those vegetatively progagated on rootstocks other than their own, have a propensity to accumulate such agents. Grapevines are no exception for in 1970 according to Hewitt 19 different viral diseases or viral disease complexes had been recognized and that the diseases involved 17 different viruses. Ten years later, Bovey, *et al.*, (1980) listed 33 viruses or virus diseases of the grapevine. Of these, the agent or agents of 19 are mechanically transmissible with specific virus particles recognized as the causal agents. The remaining 14 diseases are considered viral because symptoms are induced by a graft-transmissible agent or agents and other pathogens such as viroids, prokaryotes (fastidious bacteria, wall-less forms) fungi and nematodes have not been associated with the symptoms. Many of the diseases are probably world wide in distribution and several are of great economic importance. These diseases, whose agent or agents are only graft-transmissible, are difficult to work with because the method of transmission is laborious, time and space consuming, may require years for symptom production, and uneven distribution of the agent(s) in the test material may give false negatives. The agents have not yet been mechanically transmitted to herbaceous hosts, they have no known vector (other than man) and a specific viral-like particle(s) has not been detected in tissue or in extracts from grapevines exhibiting symptoms of the following diseases: corky bark, stem grooving (legno riccio), flat trunk, yellow speckle, fleck, vein mosaic, vein necrosis, asteroid mosaic, infectious necrosis, infectious chlorosis, shoot necrosis and chlorotic leaf curl. Bovey, *et al.*, (1980) included grapevine leafroll in this group of diseases with unknown vector and unknown specific particle. The disease, grapevine leafroll, because of its world-wide distribution in wine and table grapevines, its high incidence and effect on quality and production has attracted much attention. Based on graft-transmission experiments the grapevine leafroll disease has been considered viral (Goheen, *et al.*, 1958) and attempts to mechanically transmit or detect and associate particles with the disease have produced conflicting results.

Symptoms, because of the effects of environmental and other factors such as nutritional disorders, herbicidal, insecticidal, fungicidal sprays, and genetical diversity of the host, are not always reliable for diagnosis of viral infections. Graft-transmission as a means for detection and diagnosis may also not be reliable for the scion or stock material may already contain a symptomless virus and the addition of a second agent may cause a reduction or a synergistic effect on symptoms. The presence of one or more graft-transmissible agents may also interfere with the movement of the agent in question. Likewise, uneven distribution of the agent in the test material or failure of the agent to move across the graft union may falsely indicate that the indexed material is "virus-free". Detection of viruses by biological assay, the most sensitive method (Gibbs & Harrison, 1976), detects only those viruses that are mechanically transmissible and that induce symptoms in the test plants used. The serological technique of enzyme-linked immunosorbent assay (ELISA) has become the preferred method, by some research laboratories, for detecting viruses. Unfortunately, all serological procedures detect only the virus (protein) to which the antiserum was produced. The detection of viral-like particles, especially those with anisometric particles (rod morphology), by electron microscopy of plant extracts is a relatively easy, quick and reliable indexing procedure. Electron microscopy, although not specific for virus identification, will give the operator an indication that the plant extracts contain viral-like particles and possibly the group (e.g. tobamo, rhabdo, tobra, etc.) to which it has affinities.

For detecting viruses, bioassay has been reported to be the most sensitive procedure and plant viruses with a single component species can be detected if the preparation contains approximately $10^5 - 10^6$ virus particles per ml. If the virus requires two active components for replication then approximately 10^7 particles per ml are needed and 10^9 particles per ml are required if the virus needs 3 or 4 components for replication. Serological and chemical methods of detection require approximately 10^9 particles per ml and physical tests need even more, 10^{10} particles per ml for detection (Gibbs & Harrison, 1976). Van Regenmortel (1982) reported that the sensitivity of the serological tests ELISA and immunoelectron microscopy was from 1 to 10 ng of antigen (virus) per ml.

By comparison, the electron microscope has been reported (Gibbs & Harrison, 1976) to detect virus-like particles in preparation at concentrations of 10^8 particles per ml. The procedure requires little plantmaterial, is fast, easy, reliable and is the topic of this communication.

MATERIALS AND METHODS

The research reported here was accomplished while the senior author was on sabbatical leave, July-December 1982, at the Plant Protection Research Institute, Stellenbosch, consequently various sources of grapevines that had exhibited symptoms associated with virus infection were used. Grapevines of the virus indicators LN33 (Courderc 1613 X Thompson seedless) and Mission had been previously graft inoculated by F. A. Máre with tissue from grapevines exhibiting symptoms of leafroll, leafroll plus stem grooving (legno riccio), leafroll plus fleck, leafroll plus stem grooving plus fleck, and leafroll plus corky bark plus stem grooving. Cane (dormant wood) cuttings from these grafted plants and from field grown grapevines that exhibited various symptoms, including those of the fanleaf complex, were forced in the laboratory for shoot and root formation.

Extracts were obtained from young leaves and or young roots by grinding tissue, approximately 1:5 (w/v) with extraction buffer with a mortar and pestle. The extraction buffer consisted of 0.01M sodium phosphate buffer (pH 7.0) containing 2.5% nicotine, final pH 9.8 (Cadman *et al.*, 1960). One drop of extract, crude juice plus extraction buffer was placed on a carbon-coated collodion-covered electron microscope grid for about one minute. The drop was flushed off with a drop of extraction buffer and depending on the turbidity of the sample, the grid was rinsed with 2-5 drops of extraction buffer applied dropwise to the grid surface. Residual liquid was removed from the grid by touching the side of the grid to absorbent paper. The preparation was stained dropwise with 2-4 drops of 2% aqueous ammonium molybdate pH 5.0 and residual stain was removed as above. The specimen was air-dried and examined in a Philips model 201-C electron microscope at magnifications of 20,000 – 45,000 at an acceleration voltage of 60,000.

Extracts were made from 212 grapevines and 312 preparations were examined in the electron microscope. Extracts of some samples were tested in the greenhouse on herbceous plants for the presence of mechanically transmissible viruses and also indexed serologically by Ms. Petro Kooijman and Ms. Roleen Human for the presence of grapevine fanleaf virus by ELISA (enzyme-linked immunosorbent assay).

RESULTS AND DISCUSSION

Crude juice extracts from young grapevine leaves and roots had pH values of 2.9 and 4.5, respectively. When 1 ml of grapevine leaf or root extract was mixed with 5 ml of 0.01M phosphate buffer pH 7.0 containing 2.5% nicotine the final pH of the mixtures were 8.2 and 8.9, respectively. Electron microscopy of leaf or root extracts, negatively stained with 2% ammonium molybdate, from 212 grapevines revealed that 73 of the preparations contained viral-like particles of spherical or rod morphology. This study was designed to detect by electron microscopy viral-like particles in extracts from grapevines and not necessarily associate a specific particle to a specific disease. The particles are referred to, at this time, as viral-like because infectivity has not been associated with a particle of specific morphology. It is always possible in any heterogenous mixture of particles, as seen in electron microscopy of crude preparations, that a minor component may be the infectius particle. It is also possible that any "viral disease", for which the agent(s) is only graft-transmissible, may be caused by one or more viruses. Thus, until a "specific particle" (the form) is directly related to a "disease" (the function) it is prudent to refer to all such particles as "viral-like" even though the particles have similar or identical morphologies to specific particles, spheres, rigid or flexuous rods, that are already associated with other virus diseases of plants.

Electron microscopy of tissue-dip preparations from roots and shoots of some grapevines showed the presence of rigid rod viral-like particles 16 x 300 nm that exhibited a central electron dense core or channel (Fig. 1). These particles are similar if not identical to those of tobacco mosaic virus (TMV) and were termed TMV-like (Zaitlin & Israel, 1975). TMV has been reported from grapevines in the U.S.A., West Germany, Italy, Bulgaria, Yugoslavia and U.S.S.R. but its presence in grapevines has not been associated with a specific disease nor has it caused an apparent economic loss (Bovey, *et al.*, 1980).

The soil-borne nepoviruses (acronym for *ne*matode transmitted virus with *po*lyhedral particles) which includes the grapevines fanleaf virus complex (fanleaf, yellow mosaic, veinbanding) is perhaps the most readily recognized grapevine virus disease because the symptoms associated with the diseases are obvious and the diseases are world wide in distribution and inflict great economic loss. The virus, grapevine fanleaf (GFV), is readily transmitted mechanically in crude juice plus additives from infected grapevines to herbaceous plants that serve as virus indicators (Cadman, *et al.*, 1960). GFV has been characterized, purified and specific antiserum is available for indexing by serological tests. Unfortunately the ease with which GFV can be detected in grapevines may be a disadvantage for grapevines that test positive by bioassay and/or serology for GFV may not be tested for other viruses. Since bioassay only detects viruses that are mechanically transmissible and infect, with symptoms, the test plants used and serological tests only detect proteins to which the antiserum was prepared all other viruses go undetected.

The detection by electron microscopy of viruses with anisometric (rods, rigid or flexous) particles in plant extracts is a relatively easy, fast and reliable indexing procedure (Corbett, 1974). On the othe hand the detection of viruses with spherical or isometric (icosahedral) parti-

cles is difficult unless the virus occurs in high concentrations or the particles are associated with membranes that enhance their detection. Electron microscopy of metal-shadowed or negatively stained crude plant extracts may only detect and not identify viruses but the procedure of serological specific electron microscopy can detect and identify viruses with a degree of sensitivity equal to that of other procedures (Van Regenmortel, 1982).

Electron microscopy of extracts from young leaves of laboratory forced field produced dormant canes of a Chenel grapevine showed the presence of membrane associated 30 nm spherical viral-like particles (Fig. 2) that compares with viruses of the nepo group, specifically GFV (Hewitt, *et al.*, 1970). Similar membrane associated spherical viral-like particles have been visualized by electron microscopy of GFV infected *Chenopodium quinoa* leaf tissue (Reynolds & Corbett, 1980). Leaf extracts from a Chenel grapevine tested positively against GFV antiserum in ELISA tests and when mechanically inoculated to various herbaceous plants induced systemic symptoms in *Chenopodium quinoa, C. murale, Gomphrena globosa, Nicotiana clevelandii* and *Phaseolus vulgaris* 'Pinto'. Electron microscopy of extracts negatively stained in 2% ammonium molybdate from systemically infected leaves of Pinto bean showed the presence of 29 nm spherical or icosahedral particles (Fig. 3). Two predominant types of particles, with regards to staining properties are present, electron lucent, considered RNA containg intact particles and electron dense, RNA deficient or ghost particles. Membrane associated particles were also present in extracts from systemically infected leaves of *N. clevelandii* inoculated with extracts from *Vitis cinerea* grapevine that tested positive for GFV by ELISA (Fig. 4).

Spherical viral-like particles, approximately 28 nm in diameter, with prominent structural units, or capsomers, were detected in extracts from roots of the LN33 indicator that had been chip-budded with tissue from a Riesling Renano grapevine (Fig. 5). The latter had been tested by F. A. Maré to index positively for leafroll and fleck diseases. Extracts from the vine indexed negative for GFV by ELISA and bioassay on herbaceous hosts. Although the particles could be those of a nepovirus they also appear similar in morphology to particles associated with viruses of the bromovirus, cucumovirus or comovirus group.

Before discussing the flexuous rod viral-like particles it should be noted that whenever crude plant extracts from roots or shoots are examined in the electron microscope it is always possible to detect normal cellular filamentous particles such as microtubules, protofilaments, microfilaments, aggregated phloem protein and various types of bacterial flagella. These structures, or fragments of them, are in the size range of filamentous viruses and could easily be confused or mistaken for virus particles. The most commonly occurring flagellar-like particles are of two types, those that are arched approximately 15 nm in diameter and about 900 nm long (Fig. 6) and those that are about the same width but very long (10-15μm) and often have a typical sine wave morphology (Fig. 7). Extracts from roots of the cultivar Donhepski-Muscat contained rod particles that were approximately 13 x 860 nm (Fig. 8). The particles did not show definite surface subunit arrangements and could represent a normal cellular component or an unknown virus.

Electron microscopy of leaf or root extracts from a Chenel grapevine showed the presence of flexuous rod particles that exhibited a coated surface (Fig. 9). The particles were about 13 – 15 nm wide by 970 nm long and the surface appeared to be absorbed with extraneous material as if decorated. These particles were detected in extracts from several different grapevines and were not correlated or associated with any specific normal cellular component or disease caused by a graft-transmissible agent. They may be an artifact of the preparation, extraction and/or staining procedures.

The flexuous rod particles (Fig. 10) that created the most interest had definite cross-bandings similar to those of the closterovirus group (Bar-Joseph, *et al.*, 1979). Viruses of this group are often divided, on the basis of particle length, into two groups – those longer than 1000 nm and those shorter than 1000 nm. Particles of both groups are about 11 – 12 nm wide and exhibit a helical substructure with a pitch of 3.4 to 3.75 nm. The flexuous viral-like particle in Fig. 10 is from a Colomino grapevine that had been determined by F. A. Maré with grapevine indicators to be positive for the leafroll disease. The electron micrograph is at a magnification of 180,000 and the particle is about 11 x 780 nm with a helical pitch of about 3.5 nm (Fig. 11). Similar particles were detected in extracts from vines of the cultivars Baco blanc (Fig. 12), Colomino (Fig. 13), Ruby Cabernet (Fig. 14), Muscat Patras (Fig. 15) and also in extracts from the indicators Mission, LN33 and Baco blanc (Fig. 16) that had been chip-budded with tissue from grapevines tested (indexed) for leafroll.

Closterovirus-like particles were also detected in extracts from roots of grapevines that had been derived from shoot apices (less than 1 nm) of grapevines that had been heat treated for 14 weeks at diurnal temperatures of 37 ° C for 16 light hours and 34 °C for 8 dark hours indicating that the particles are present in apical tissues and relatively heat stable.

Electron microscopy can detect viral-like particles in extracts from roots and young leaves of grapevines that are symptomless and those that exhibit symptoms of fanleaf and leafroll. The direct association of these particles, especially the closterovirus type, to the leafroll disease requires the performance of Koch's postulates, since the agent(s) of the disease has been reported only to be graft-transmissible (Goheen, *et al.*, 1958), and any number of agents may be involved in the production of specific symptoms of disease.

Similar clostervirus-like particles have been associated with grapevine leafroll in Japan (Namba, *et al.*, 1979), Italy (Faoro, *et al.*, 1981), and also with grapevine stem-pitting disease in Italy (Conti, *et al.*, 1980). Whereas, other workers have failed to associate a specific viral-like particle with grapevines that exhibit symptoms of leafroll (Von der Brelie & Nienhaus, 1982, Castellano, *et al.*, 1983). Viral-like particles of a potyvirus (Tanne, *et al.*, 1974; Tanne, *et al.*, 1977) and a tobamovirus (Ochs, 1960; Brandes, 1961) have also been associated with grapevines exhibiting symptoms of leafroll. At present confusion exists regarding the etiology of several diseases of the grapevine because the agents are only graft-transmissible and it has not been possible to separate the agent or agents responsible. The ability to detect viral-like particles in grapevine extracts by electron microscopy should enable or assist in the purification and characterization of a specific particle and/or particles which may eventually be directly associated with a specific disease.

FIGURES 1 – 16. All figures are electron micrographs of particles in crude plant extracts negatively stained with 2% ammonium molybdate.
FIGURE 1. Tobacco mosaic viral-like particles in extracts from leaves of a Chenel grapevine, magnification 150,000X.
FIGURE 2. Membrane associate viral-like particles in extracts from leaves of a Chenel grapevine, magnification 225,000X.
FIGURE 3. Spherical viral-like particles in extracts from leaves of Pinto bean (*Phaseolus vulgaris*), magnification 225,000X. The bean plant had been inoculated with extracts from a Chenel grapevine.
FIGURE 4. Membrane associated viral-like particles in extracts from leaves of *Nicotiana clevelandii*, magnification 225,000X. The *N. clevelandii* plant had been inoculated with extracts from a *Vitis cinerea* vine that tested serologically positive for fanleaf virus.
FIGURE 5. Spherical viral-like particles in extracts from leaves of a Riesling Renano grapevine which indexed positively for leafroll and fleck, diseases, magnification 225,000X.
FIGURE 6. Bacterial flagellum, magnification 100,000X.
FIGURE 7. Bacterial flagellum, magnification 126,000X. Flagella are often present in crude plant extracts.
FIGURE 8. Rod-shaped viral-like particle in extracts from roots of a Donhepski-Muscat grapevine, magnification 150,000X.
FIGURE 9. Flexuous rod viral-like particle in extracts from roots a Chenel grapevine, magnification 225,000X.
FIGURE 10. Flexuous rod viral-like particle, with cross-banding periodicity, in extracts from roots of a Colomino grapevine, magnification 180,000X. Particle is similar to those of the closterovirus group.
FIGURE 11. Portion of particle in Fig. 10 at a magnification of 525,000X.
FIGURE 12. Closterovirus-like particle in extracts from roots of a Baco blanc grapevine, magnification 180,000X.
FIGURE 13. Closterovirus-like particle in extracts from roots of a Colomino grapevine after 14 weeks heat treatment, magnification 180,000X.
FIGURE 14. Closterovirus-like particle in extracts from roots of a Ruby Cabernet grapevine that exhibited symptoms of leafroll magnification 270,000X.
FIGURE 15. Closterovirus-like particle in extracts from roots of a LN33 grapevine that had been chip-budded with tissue from Muscat Patras for leafroll, magnification 270,000X.
FIGURE 16. Closterovirus-like particle in extracts from roots of a Baco blanc grapevine that had been chip-budded with tissue from Colomino (Fig. 13), magnification 180,000X.

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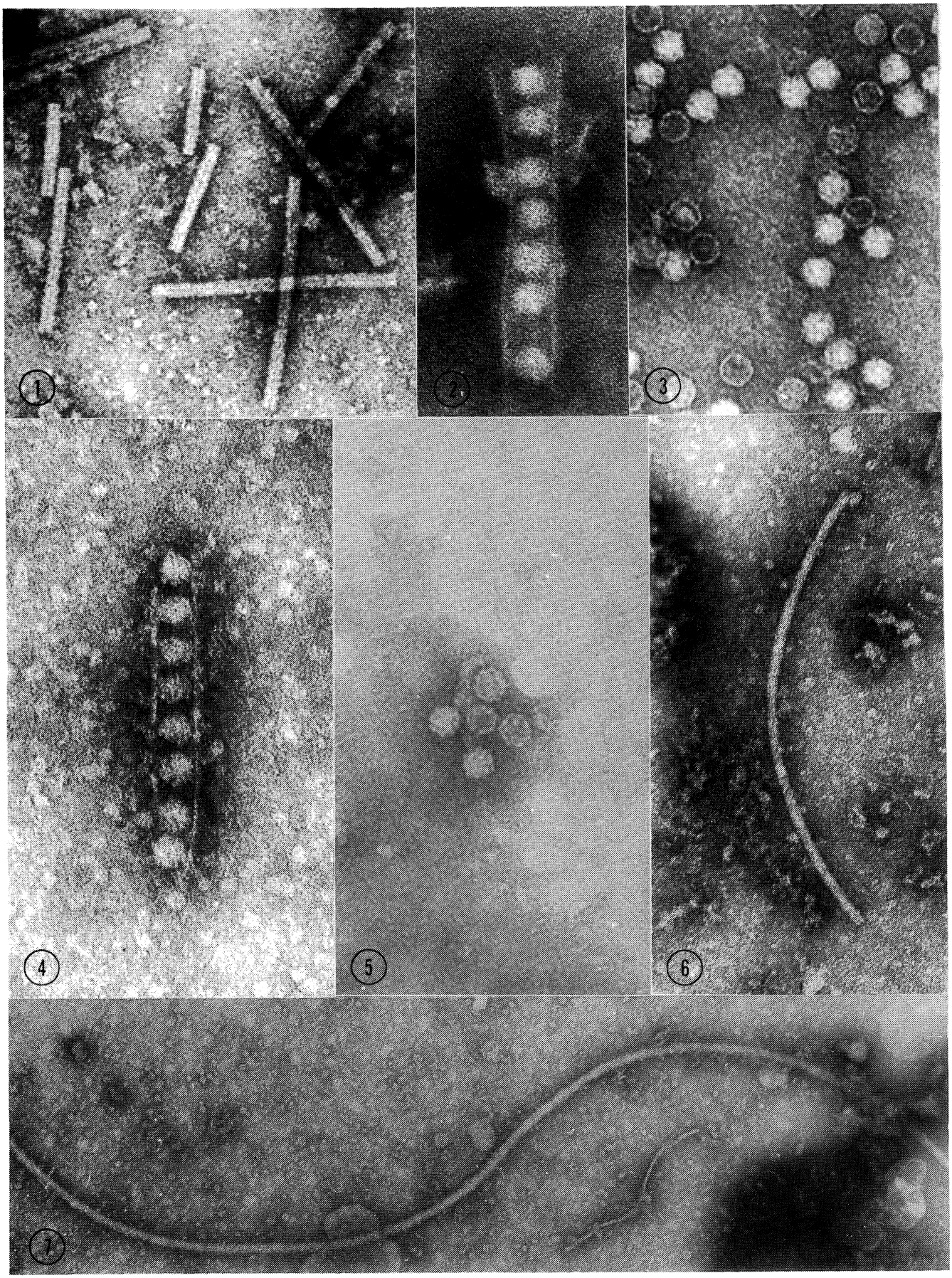
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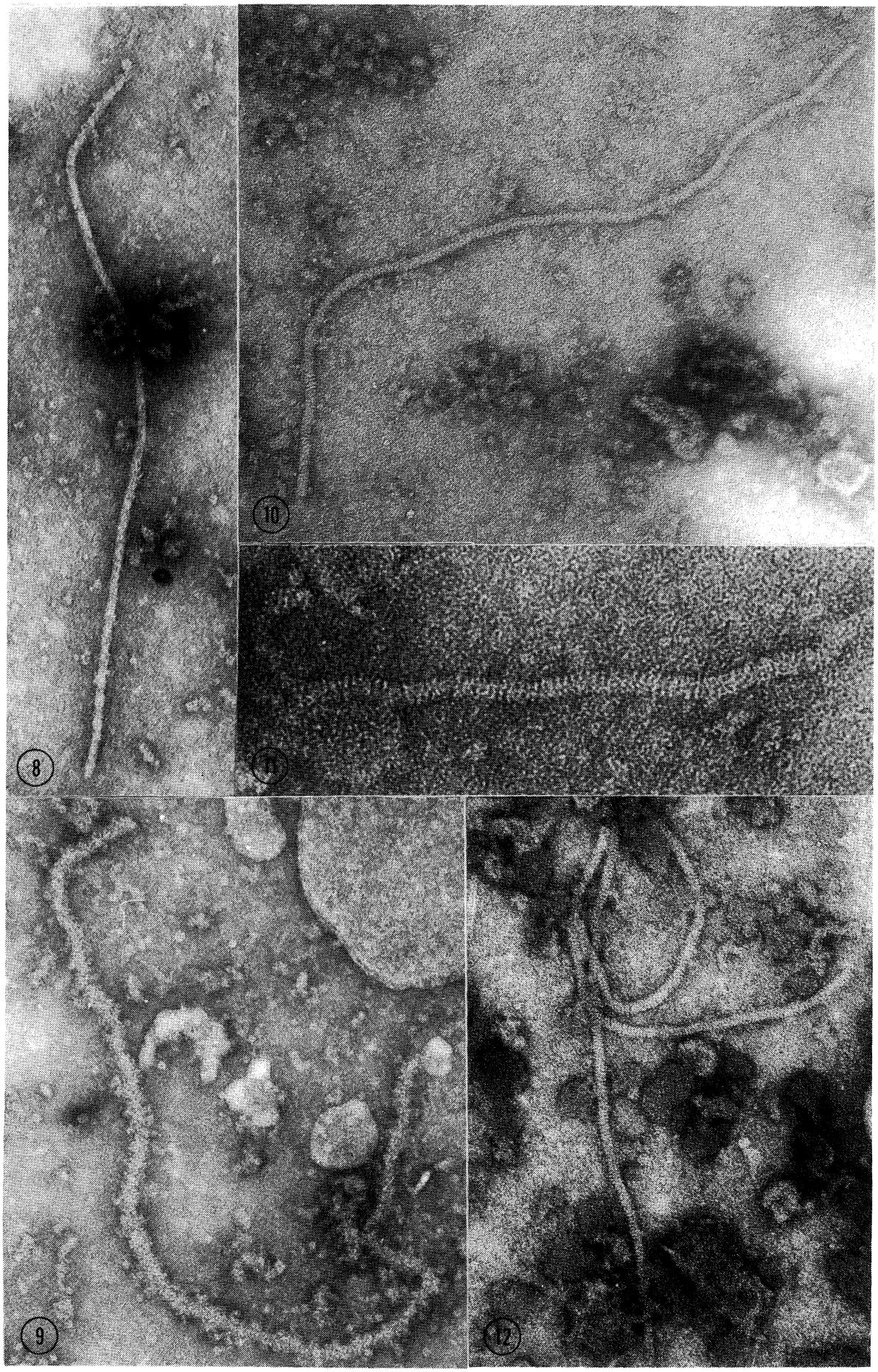
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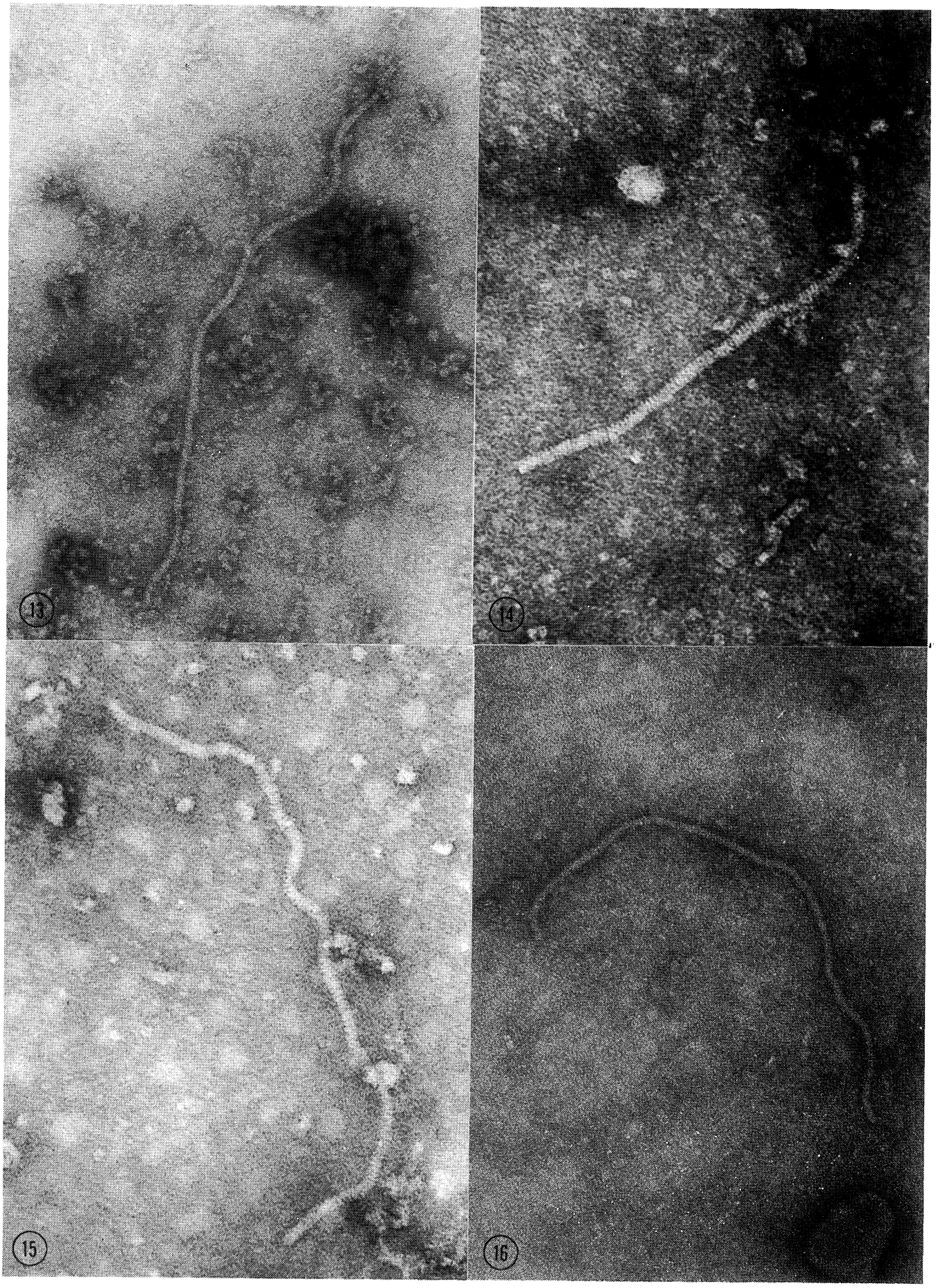
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