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Electron Microscopy: Current Applications to Plant Virology

Ever since tobacco mosaic virus (TMV) was first visualized by electron microscopy (EM) in 1939, EM has been a valuable tool in plant virology. Transmission electron microscopy (TEM), in particular, is extremely useful for virus identification and for basic and applied research with plant viruses. Familiar applications of TEM are in viewing virions and their in situ location in ultrathin sections of plant or insect tissues. In purified or crude preparations, TEM is used to: 1) detect the presence of virions, 2) give information on morphology and size, provided magnification calibration standards are used, and 3) monitor the purity and relative amount of virus during purification. Scanning electron microscopy (SEM), while lacking the resolution capabilities of TEM, offers simpler and less tedious specimen preparation than TEM, provides three-dimensional information, and permits examination of large samples.

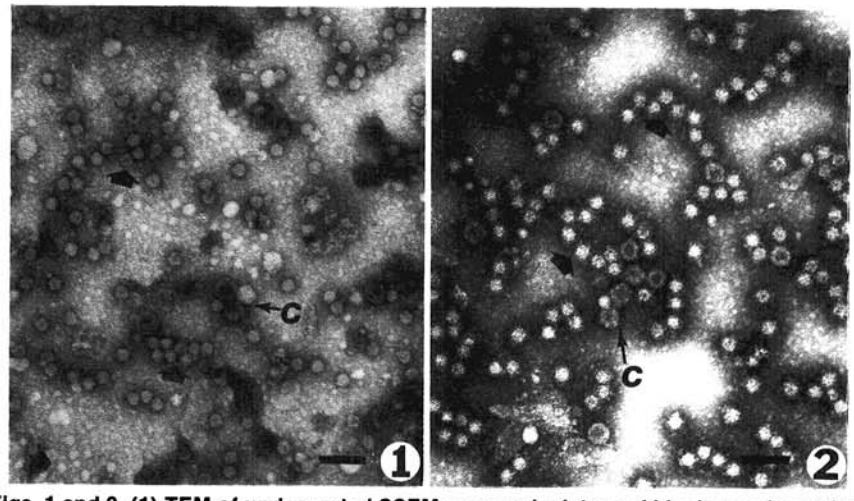
Several recent and powerful developments in TEM and SEM applications have increased the rigor and specificity of EM as a tool in studying the identification, characterization, and vectoring of plant viruses.

Immunologic Adaptation of TEM

The detection of plant viruses in crude preparations has come a long way since 1957 when Brandes (3) first reported a leaf-dip method of visualizing the gross morphology of a plant virus in sap that diffused from a cut leaf into a drop of buffer. Ball (1) improved the leaf-dip method by adding, to the crude sample in buffer, virus-specific antiserum along with potassium phosphotungstate and vanadatomolybdate to stain the virus before applying the mixture to a collodion-coated, carbon-backed grid. In 1973, Derrick (7) reported a method of directly detecting plant viruses in crude sap in a quantitative manner. He called this method "serologically specific electron microscopy" (SSEM), now synonymous with "immunosorbent electron microscopy" (ISEM). Derrick's method consisted of making TEM grids serologically specific for a given virus. This was done by floating TEM grids coated with carbon-backed Parlodion films on a drop of diluted antiserum to the virus to be detected. This trapped the virions onto the grid and also allowed for washing the grid to get rid of some of the nonspecific plant salts, crystals, and other compounds that could substantially interfere with viewing. Trapped virions were shadowed with platinum-palladium to enhance their viewing. Up to 40 to 50 times more virions were visible in the TEM as a result of this method. Derrick reported a linear relationship between the log of the number of virus particles attached to the serologically specific grids and the dilution of tobacco sap infected with TMV. In 1974, Ball (2) published a more detailed leaf-dip serology method for TEM using Derrick's modifications.

Milne and Luisoni in 1977 (19) published a technique that significantly improved the ISEM method. Their technique introduced "decoration," the addition of more virus-specific serum to the virions already trapped on the serum-coated grid. The method employs negative stain (after the decoration step), with uranyl acetate rather than phosphotungstic acid, which is destructive to some viruses. The main advantage to the decoration modification is that one can see in the TEM the halo of virus-specific globulin surrounding the trapped virus particles (Figs. 1 and 2). This takes much of the guesswork out of identifying spherical virions, especially those that may otherwise be very difficult to discern from various types of particulate matter present in crude preparations.

The advantages and disadvantages of ISEM are difficult to describe without also mentioning enzyme-linked immunosorbent assay (ELISA) and radioimmunosorbent assay (RISA), since all three methods can be used as ultrasensitive virus assays. The advent of ELISA has made possible accurate and direct detection of small amounts of plant viruses in crude preparations without the use of the electron microscope. Recently, Clark (6)



Figs. 1 and 2. (1) TEM of undecorated SSEM prepared mixture of blueberry shoestring virus (BBSSV) (large arrows) and cauliflower mosaic virus (CaMV) (C). (2) TEM of decorated SSEM prepared mixture of BBSSV (large arrows) and CaMV (C) using antibody prepared against BBSSV. Note "halo" of antibody molecules around BBSSV but not around CaMV. Scale bars = 0.1 μm.

compared the merits of ELISA and it variations with those of RISA and ISEM. Each method has its strengths and weaknesses.

We have compared the three immunosorbent methods for detection of blueberry shoestring virus (BBSSV) in its aphid vector, *Illinoia pepperi* MacG., using purified virions (11). ELISA detected a minimum level of 5.0 ng, RISA detected 0.6 ng, and ISEM (with decoration) detected <0.15 ng. When known quantities of purified BBSSV were added to homogenates of nonviruliferous *I. pepperi*, however, ELISA still detected BBSSV at a minimum level of 5.0 ng and RISA detected 0.6 ng but ISEM detected only 20 ng. The more than 100-fold reduction in sensitivity of ISEM resulted from the interfering particulate matter in the aphid homogenate. Many round and amorphous entities in the TEM field caused the ISEM technique to be unreliable at virus concentrations of <20 ng when blind tests were run. In addition, ELISA and RISA are less time-consuming than ISEM for testing a large number of samples. Therefore, we selected RISA as the assay of choice for reliable detection of BBSSV in individual aphids. We now use RISA rather than ELISA or ISEM for field epidemiologic studies involving the aphid vector.

Other researchers have found ISEM to be more useful than ELISA. For example, ISEM (without decoration) was compared with ELISA for the detection of pea seedborne mosaic virus (PSbMV) in seed lots containing various percentages of infected seed (12). ISEM was superior in this case because it could detect PSbMV in seed lots containing 1–5% infected seed, while ELISA could not. The advantage for ISEM was probably due to being able to see the virions on the grid and to count them, whereas with the ELISA technique, the background from healthy seed (A_{405nm}) was rather high, resulting in poorer discrimination between healthy and diseased samples, especially where the amount of virus in the seeds was very low.

ISEM (without decoration) was also shown to be very useful for direct detection of six nepoviruses in their nematode vectors (23). Virus in the vectors could be detected much more reliably by ISEM than by "slash tests" in which nematodes that have had an acquisition access period on diseased herbaceous plants are ground in buffer and rub-inoculated to herbaceous indicator plants to determine if they contain virus. The ISEM method is very effective for checking the status of both test plants and nematodes for the presence of virus, especially when in low titer. ISEM has also been used to successfully detect potato leafroll virus (PLRV), a spherical virus of low titer in the aphid vectors *Myzus persicae* Sulz. and *Macrosiphum euphorbiae* Thos. (24). When decoration of the trapped particles was done, the virus was detected readily in single aphids.

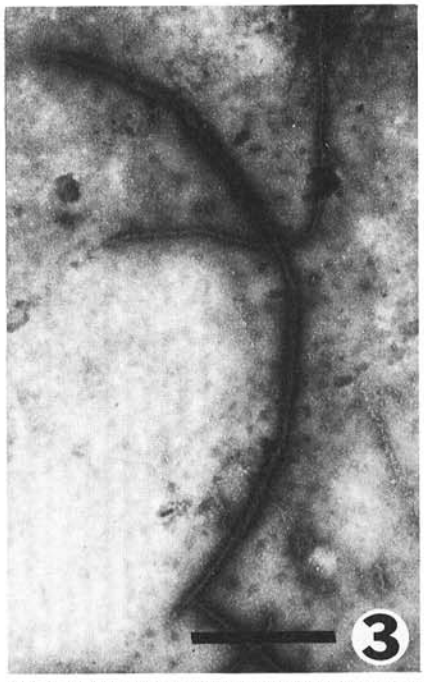


Fig. 3. TEM of decorated SSEM preparation of wheat spindle streak mosaic virus. SSEM was used to monitor virus purification. Scale bar = 1 μm. (Courtesy Karen Haufler)

Wheat spindle streak mosaic virus (WSSMV) causes a serious disease of wheat in Michigan, the eastern United States, and Canada, as well as in other countries. Until only recently, symptomatology and/or detection of pinwheel inclusions with the TEM in ultrathin sections was the only way to diagnose the disease. Japanese workers (26) have succeeded in purifying this virus in small quantities and making a somewhat weak but usable antiserum. Researchers in cooperation with our laboratories were able to adopt an ISEM assay with the Japanese serum for WSSMV, using decoration (14) (Fig. 3). This assay has made it possible to monitor further purification steps to markedly improve the yield of WSSMV and also to make more antiserum of higher titer. The fact that ISEM requires very little antiserum makes it a method of choice for virus detection when the supply of serum is limited. Also, WSSMV is a long flexuous rod and therefore easier to locate in a negative stain preparation than small spherical viruses and easier to distinguish from any contaminating material. ISEM can now be used for routine diagnosis of WSSMV in breeding lines as an aid in determining relative susceptibility, resistance, or tolerance of wheat. ISEM will also be used to facilitate vector relationship studies with the probable vector, *Polymyxa graminis* Led.

A little known but very useful spin-off of the ISEM technique is its use in determining serological relationships (17). ISEM can be an ancillary test to the agar gel double-diffusion technique to confirm or disprove distant serological reactions that may occur between strains of a virus, as, for instance, when testing a new or unknown plant virus A against antisera to several strains of a known virus B. If unknown virus A is really related to one of the strains of known virus B, then many more particles of virus A will be trapped and decorated on the grids containing antiserum to B than there would be trapped on the control grids.

ISEM is a powerful tool for routine diagnosis of small numbers of samples as well as for research purposes. However, the proper controls must be used, such as an antiserum to an unrelated virus or normal serum. Proper dilutions of antiserum should be empirically tested for the optimum dilution of virus that will maximize the binding of virus and antibody on the grids. ISEM can be a rapid, cost-effective means of detecting viruses provided the needed antisera are available and one has ready access to an electron microscope.

Nucleic Acid Characterization

The TEM can also be used to gain a considerable amount of information relative to the morphology and fine structure of nucleic acids from plant viruses, viroids, and "virusoids." Nucleic acid extraction followed by treatment with RNase and/or DNase, sucrose density centrifugation and buoyant density centrifugation in cesium chloride (optionally including ethidium bromide), and electrophoresis in polyacrylamide gels can provide information about the type of nucleic acid (RNA or DNA) and also aid in obtaining highly purified nucleic acid by a combination of these techniques. Thermal melting kinetics of the nucleic acid will also give further information as to its strandedness. After some or all of these physicochemical property manipulations are completed and information is gained about the general properties of the nucleic acid at hand, the TEM can be used to gain further information about its fine structure.

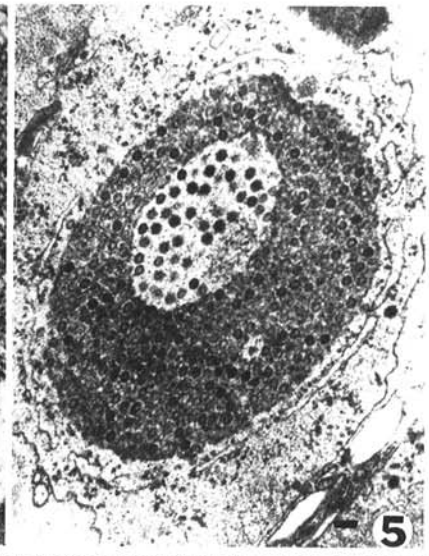
For TEM examination, the nucleic acid is spread on a monolayer consisting of cytochrome c (the "Kleinschmidt method") or on a monolayer of a nonprotein material called BAC (benzyldimethylalkyl-ammonium chloride) (8). The BAC method can give better resolution of small structural features, but it can also cause tangling of nucleic acid molecules and may give poor contrast to single-stranded nucleic acids in the TEM. After the nucleic acid is spread on the monolayer, it is stained, usually with uranyl acetate. The specimen may be shadow-casted with heavy metal for further contrast. A known reference marker of single- or double-stranded DNA or RNA should be included for a size and shape reference.

A number of significant breakthroughs

in plant virology were made possible by the use of the protein monolayer TEM method of visualizing virus or viruslike nucleic acids. In the case of viroids, the studies of Sanger et al (25) and McClements and Kaesberg (18) have elucidated two types of structure for potato spindle tuber viroid (PSTV). Both research groups used TEM to visualize completely denatured viroid molecules. They demonstrated the presence of linear strands twice the length of native undenatured molecules and, conversely, ringlike (circular) molecules with a circumference about twice the length of native viroids. The circular forms were seen to be covalently closed. These forms occur because of the extensive areas of intramolecular complementarity in the form of collapsed circles and hairpin structure, appearing as double-stranded molecules in the TEM.

Cauliflower mosaic virus (CaMV), a double-stranded DNA virus (dsDNA) was recently shown (5,16) to contain two forms of dsDNA. By various centrifugal and electrophoretic methods and by the protein monolayer method of viewing the DNA, CaMV DNA was shown to be composed of linear and circular forms. Furthermore, the circular forms were both twisted and not twisted, and infectivity was associated only with the twisted forms from centrifuged sucrose density gradients (5).

A relatively new group of plant viruses, the geminiviruses, are small quasi-isometric particles that occur in pairs. The individual particles are 15–20 nm in diameter. Two geminiviruses, cassava latent and maize streak, were recently shown, by physicochemical methods and by viewing the DNA spread on a protein monolayer, to be single-stranded DNA viruses. Two forms were seen, circular and linear (13); it was hypothesized that



Figs. 4 and 5. (4) TEM of ultrathin section through crystalline array of maize mottle virus in sieve tube of maize. Scale bar = 0.1 μm. (5) TEM of ultrathin section through inclusion body of red ringspot virus in blueberry. Scale bar = 1 μm. (Courtesy K. S. Kim)

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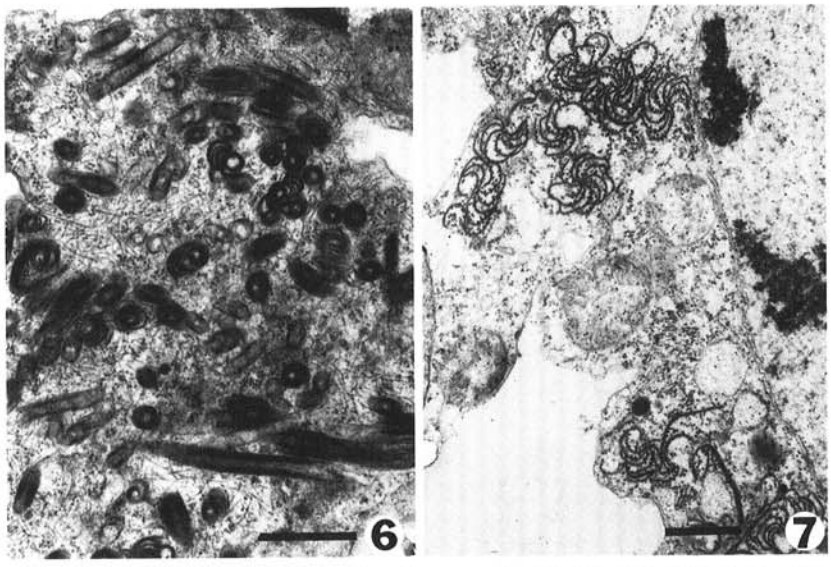
the linear forms resulted from nicked circular forms.

Finally, a new type of virus, termed a "virusoid," has been described with the aid of protein monolayer TEM techniques. Virusoids are encapsidated spherical virions that contain regular single-stranded RNA of molecular weight approximately 1.5×10^6 daltons plus a number of much smaller circular single-stranded RNAs that are viroidlike; velvet tobacco mottle virus (VTMoV) is an example (22). It is interesting that the nucleotide composition of the small RNA is unrelated to that of the larger RNA.

In all the cases we have described, new information has been obtained about the type, form, and species of nucleic acids associated with these virus and viruslike entities. The information could not have been obtained without the TEM and protein monolayer method used in conjunction with other laboratory methodology.



Figs. 6 and 7. (6) TEM of ultrathin section through wheat leaf infected with wheat streak mosaic virus. Note scrolls and laminated aggregate inclusions. Scale bar = 1 μm. (7) TEM of ultrathin section through wheat spindle streak mosaic virus. Note pinwheel inclusion. Scale bar = 0.5 μm. (Courtesy Karen Haufler)

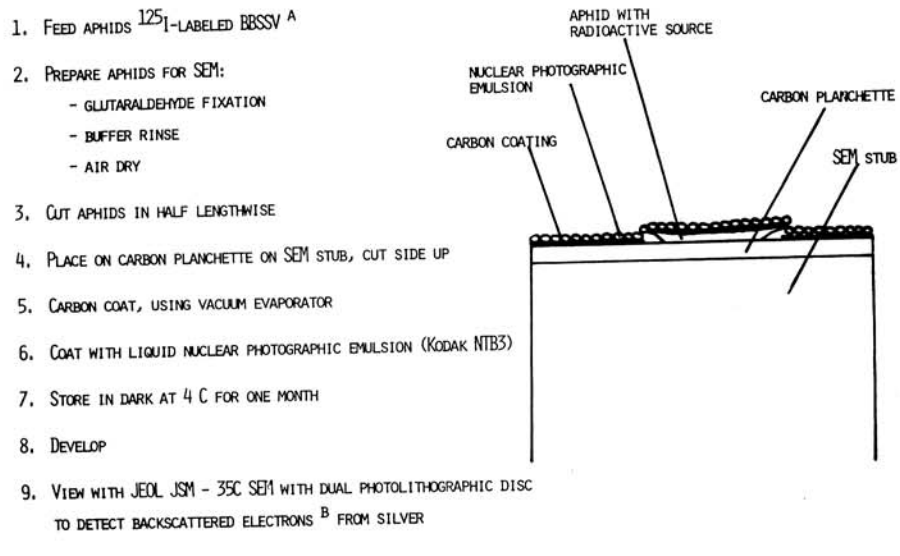


Fig. 8. Preparation procedure for scanning electron microscopy autoradiography. (Courtesy Maureen Petersen)

Host-Virus Interactions

The TEM has also been used to examine the host-virus interactions within the host cells. It is often useful to examine diseased host tissues to ascertain whether the virus particles are in crystalline arrays (Fig. 4), associated with specific organelles, or producing protein inclusion bodies (Fig. 5). Crystalline arrays or the mere presence of virions is particularly important when purifying the virus is difficult or impossible.

Visualization of the specific morphology of various protein inclusions, especially in the potato virus Y (PVY) group of viruses, would not have been possible without the use of ultrathin sections and TEM, although they are partially resolved with the light microscope (4). Wheat streak mosaic virus (WSMV) and WSSMV are examples. Although these viruses cause similar symptoms at times, they each produce characteristic protein inclusion bodies (Figs. 6 and 7). Visualizing the specific inclusions, then, can aid in accurate diagnosis.

Analytical Applications of EM

Analytical EM is the use of TEM and/or SEM beyond their normal applications in morphological and anatomic studies. Autoradiography, specific staining, specific antibody-ferritin or specific antibody-colloidal gold labeling, and X-ray analysis are examples of analytical EM. We have used the SEM as an analytical tool to study virus-vector relationships. Specifically, autoradiography was adapted at the SEM level to corroborate light microscopic autoradiography (LM-AR) (21).

SEM autoradiography (SEM-AR) (Fig. 8) is a rather new and potentially powerful analytical method with useful applications to plant virology. SEM-AR was used in conjunction with LM-AR to trace the movement of BBSSV virus in its aphid vector, *I. pepperi* (20). The virus was iodinated with ^{125}I, which served as a radiation emitter, to allow autoradiographic detection of the virus in aphids that had acquired it. A backscatter detector on the SEM was used to detect the silver produced by the autoradiographic process (9).

Backscattered electron (BSE) energies are related to the atomic number of the element that produced them. In general, as the atomic number (Z) of the element increases, the backscatter coefficient is measured as Z^2. Therefore, areas of the sample producing bright spots on the SEM screen are indicative of high atomic number elements. For our use, the silver produced by the autoradiographic process was easily located by the backscatter detector and was assumed to be spatially related to the presence of ^{125}I-labeled BBSSV. With an SEM equipped with a BSE detector, Petersen (20,21) was able to follow the movement of BBSSV

through the aphids as the virus acquisition access period varied.

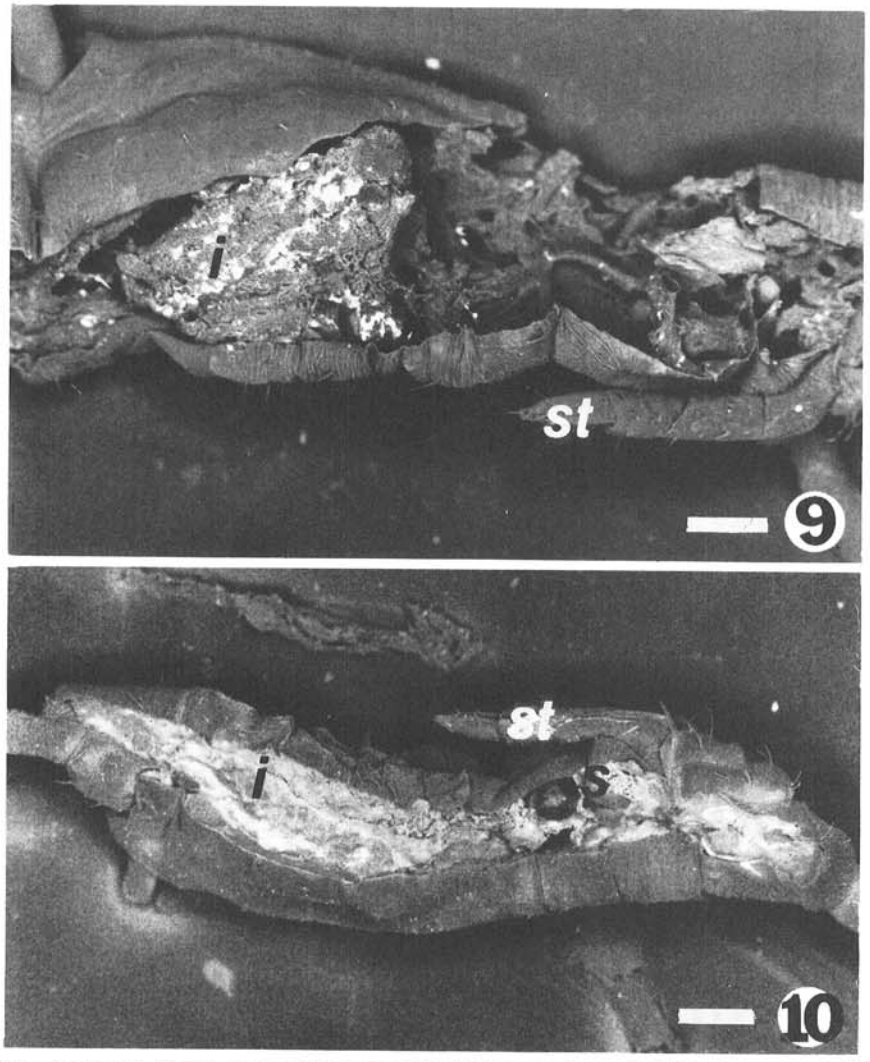
Silver was found on the stomach after an acquisition period of 6 hours; on the intestines after 12, 24 (Fig. 9), 36, and 48 (Fig. 10) hours; and throughout the intestines to the anus after 72 hours. The silver appeared bright, indicating its high atomic number (Z = 47), against the dark, mostly carbon (Z = 12) composition of the aphid. The presence of silver was confirmed with a Tracor Northern energy-dispersive X-ray analyzer. This allowed for the specific identification of silver via collection and sorting of X rays produced by bombarding the sample with the electron beam. The amount of silver appeared to increase with the increase in the acquisition period (Fig. 11).

The TEM can also be used in an "analytical" mode even without the use of auxiliary equipment. Analytical data, eg, data beyond morphological information, can be generated provided the end product either is electron-dense, as in the case of labeled antibody, or is somehow visible in the transmitted electron mode of a TEM, as in the case of decoration of virions in the ISEM.

Ferritin- and colloidal gold-labeled antibodies are the most common probes used for localization at the TEM level. Both ferritin and colloidal gold can be directly conjugated to specific antibodies made to virus coat protein or they can be used in a two-step procedure by conjugating to a secondary antibody, such as goat antirabbit antibody.

Gildow (10) used ferritin-labeled antibodies to follow the transport of luteoviruses through the salivary glands of *M. persicae*. Ferritin molecules associated with viruslike particles are an indication that the particles are actually the specific virions to which the antibody was made. This method of labeling is particularly useful when examining insects, since many insects contain viruslike particles and/or other nonplant-pathogenic viruses.

Colloidal gold is widely accepted as a label for use in light and fluorescent microscopy as well as in SEM and TEM (15). As with ferritin, colloidal gold can be directly conjugated to a specific viral antibody or to a secondary antibody. Labeling of ultrathin sections of TEM with colloidal-gold conjugated antibody is generally accomplished with less



Figs. 9 and 10. SEMs of longitudinal sections through the aphid *Illinoia pepperi*. (9) Backscatter electron image showing silver (bright area) from the autoradiographic procedure on the intestines (i) of aphid given a 24-hour acquisition access period on ^{125}I-labeled blueberry shoestring virus (BBSSV). St = stylet. Scale bar = 100 μm. (Courtesy Maureen Petersen). (10) Backscatter electron image showing silver (bright area) from the autoradiographic procedure throughout the intestines (i) with some on the stomach (s) of aphid given a 48-hour acquisition access period on ^{125}I-labeled BBSSV. St = stylet. Scale bar = 100 μm. (Courtesy Maureen Petersen)

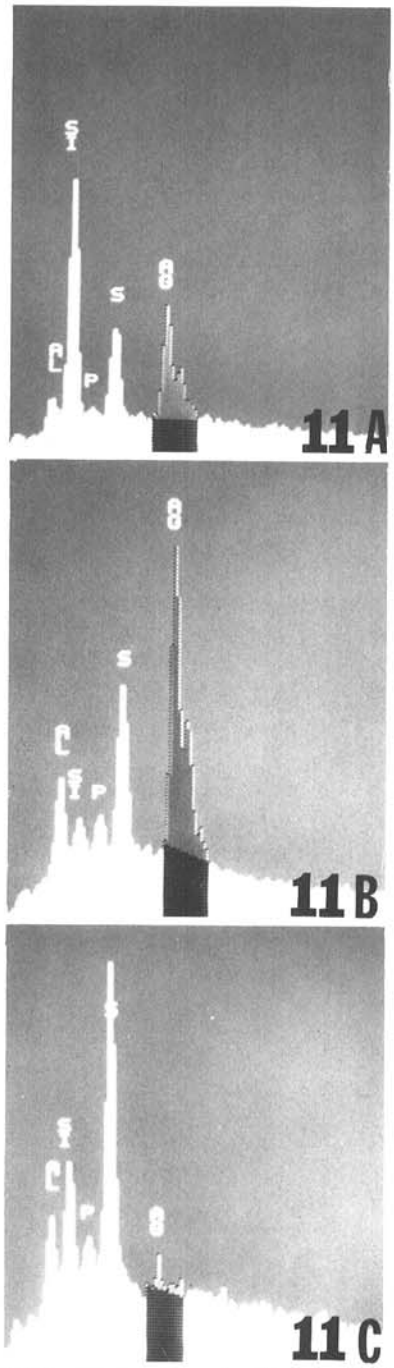


Fig. 11. Energy-dispersive X-ray analysis spectra from aphids allowed (A) 24-hour and (B) 48-hour acquisition access period on ^{125}I-labeled blueberry shoestring virus; (C) no ^{125}I-labeled virus. AG = silver peak. (Courtesy Maureen Petersen)

nonspecific binding than when ferritin is used. In addition, gold particles can be made in varying sizes to permit use at the SEM level or to allow for differentiation of two different antibodies on the same section. We are beginning to apply the colloidal gold-antibody labeling technique to a variety of projects in virology, including vector relations and other studies of host-pathogen interactions. Methodology for applying gold-labeled antibody to ultrathin sections with high specificity and low background is currently being developed.

Conclusions and Outlook

Research using the TEM and the SEM has made invaluable contributions to the science of plant virology. The TEM has provided, and will continue to provide, unique data on virus morphology and titer, subcellular effects of diseases on hosts, host-virus-vector relationships, and disease diagnosis. When combined with immunologic techniques, the TEM and the SEM become powerful tools for further probing host-virus-vector interactions, monitoring virus purification, and examining viral strain relationships. The resolution attainable by each microscope provides visualization of subcellular details not available by other means. Finally, when combined with additional analytical equipment such as backscatter electron detection and energy dispersive X-ray analysis for use in autoradiography or confirmation of other techniques, the SEM, in particular, can provide three-dimensional, ultrastructural information that is truly unique. The TEM and, more recently, the SEM have been used in many phases of plant virology. The application and usefulness of each continues to increase as more and more methods are adapted for use at the ultrastructural level and, perhaps more significantly, as our demand for high-resolution subcellular and analytical information increases.

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