Part II. Virus-host interactions.

Chairman: R. Bovey

THE STUDY OF PLANT VIRUSES IN HOST CELLS BY THIN SECTIONING FOR ELECTRON MICROSCOPY

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Introduction

Examination of thin sections with the electron microscope provides a direct means of studying virus-host relationships. Although much of the method's potential has not yet been realized, studies of thin sections have given a clear indication of where, in cells, many plant viruses accumulate and information has begun to emerge on the manner in which some viruses are assembled into stable nucleoprotein particles. As histological methods become more refined, much should be learned about these and other events in virus infection.

Because the thin-sectioning method is becoming increasingly useful to plant virologists in general, it may help to discuss some of the specific techniques which have been found most effective for studying plant viruses *in situ* and to review, briefly, the results of studies already published.

Methods

Data obtained from studying thin sections are limited largely by the manner of specimen preparation. In the general references dealing with the preparation of tissue sections for electron microscopy (1, 44, 65)* no attention is given to those methods which are most suitable for studying plant viruses *in situ*. Certain methods of killing, embedding, and staining virus-infected cells have been found to yield better results than others. Although no single procedure can be prescribed for all virus-tissue systems, there are some which have wide application and others which usually should be avoided.

Fixation. The selection of a fixative largely depends upon the type of tissue being studied and the purpose of the investigation. Pease (65) lists the fixatives used for biological electron microscopy as those containing osmium tetraoxide, or aldehydes, or solutions of permanganate salts.

Solutions of osmium tetraoxide, buffered slightly alkaline (64), are the most widely used fixatives for electron microscopy, and are particularly useful for animal tissues although less so for plants. Cells of higher plants which have been fixed with buffered osmium tetraoxide alone are usually poorly preserved and much of the fine structure is lost; its use in conjunction with other fixing agents seems to give more satisfactory results. Dalton's chrome osmium solution (17) was found to be superior to osmium tetraoxide alone in studies on TMV in tomato leaflets (74). The most useful application of osmium

* Numbers in parentheses refer to "Literature Cited" at end of this paper.

tetraoxide to date is for post-fixation following a primary aldehyde fixation.

Chrispeels and Vatter (12) reported successful preservation of ultrastructure in root cells of *Zea mays* using dilute solutions of glutaric acid dialdehyde (glutaraldehyde). Additional stabilization of ultrastructure and enhancement of contrast was obtained if the tissues were then post-fixed with osmium tetraoxide. A variation of this technique was used with striking results by Kolehmainen *et al.* for studies of TMV in tobacco leaves (48). Their method was to fix small pieces of leaves for 3 hours in 6.5% glutaraldehyde in 0.07 M phosphate buffer (pH 7.0 at 4°C). The tissues were then washed for 6 hours in the same buffer then post-fixed in buffered 2% OsO_4 at 4°C.

Glutaraldehyde has an additional advantage as a fixative in that it does not inactivate many of the enzymes and antigens present in the cell. Preliminary studies on the localization of TMV antigen in cells using ferritin-antibody conjugates have shown that intracellular TMV antigen retains its ability to combine specifically with homologous antiserum conjugates following glutaraldehyde fixation (75). Thus, this material is of value in cytochemical and immunological as well as structural studies of plant cells (12).

Permanganate fixatives were introduced to electron microscopy by Luft (50), who obtained good preparations of liver tissues using a 2% potassium permanganate solution. Subsequently, Whaley *et al.* (108) demonstrated the usefulness of potassium permanganate for studying membrane systems in plant cells. To virologists, treatment of tissues with permanganate has the severe disadvantage of failing to preserve some types of ribonucleoprotein particles. Luft noted that ribosomes were destroyed in liver tissues fixed in potassium permanganate (50), and this also seems to be true for ribosomes in plant cells. Similarly, isolated TMV particles are degraded by potassium permanganate in concentrations normally used for tissue fixation (73). Virus particles are seldom found in thin sections of cells infected with TMV and fixed in permanganate solutions (73, 107). Because many of the plant viruses are small, ribonucleoprotein particles (similar to TMV and, in some respects, to ribosomes) it seems probable that permanganate fixation will be of limited value for studying these viruses in cells although it may be valuable when studying intracellular formation of larger membrane-bound plant viruses (3). Kitajima observed particles 80-120 m μ in diameter, and believed to be Brazilian Tomato Spotted Wilt virus, in roots of host plants which had been fixed with permanganate (47). Also, permanganate fixation is useful for studying the effects of virus infection on cell organelles and membranes *per se* (70).

Embedding. The medium in which tissues are embedded for sectioning has a great influence on the appearance of cells when examined with the electron microscope. This is especially true for plant tissues.

The two most widely used media are methacrylates and epoxy resins. Methacrylate penetrates tissues easily in the monomeric state; when polymerized it cuts quite easily, and, because it partly volatilizes in the electron beam, a high degree of contrast is imparted to imbedded biological material (65). For plant tissue, these advantages are far outweighed by the fact that extensive damage to cell structure occurs during its polymerization. This damage may range from rupture of cell membranes and distortion of organelles to mass destruction of the entire tissue piece. These effects can be reduced to some extent by using partially polymerized methacrylate for infiltration (65), but far better results can be achieved simply by employing a different medium such as an epoxy resin.

Three of the more common epoxy resin embedding media are Araldite, Epon, and Maraglass (65). Any of these seem to be satisfactory for use with most plant tissues. Araldite 6005 (CIBA) is routinely used in our laboratory for a wide range of different plant materials. The following proportion of Araldite, hardener (dodecenyl succinic anhydride) and accelerator (n-benzyl dimethylamine) is used:

Araldite	1.43 volume
Hardener	1.00 volume
Accelerator	1.41 percent

This is mixed vigorously in large batches, placed under vacuum for several minutes to remove air bubbles, then frozen in lots of 10-20 ml until used. For embedding, pieces of fixed and ethanol-dehydrated tissue are placed directly in the resin mixture and incubated at 40°C for 1 hour with occasional stirring. The tissue is then transferred to recently thawed resin in capsules or forms for flat castings. These are immediately placed under vacuum at 70°C. After most of the bubbles have dissipated, nitrogen gas is introduced into the oven to atmospheric pressure and the samples are cured for 24 hours at the same temperature.

Staining. Because of the low electron-scattering capacity of viruses, the ability to resolve them in sections of infected cells depends to a high degree on their being stained with heavy atoms. When tissues are fixed in solutions containing osmium tetraoxide, osmium atoms are bound differentially to various substances in the cells and some degree of contrast is imparted to ultrastructure as viewed with the electron microscope. Although some plant viruses have been demonstrated in sections of cells which were merely fixed in this manner (5, 22, 49), in most studies some additional treatment with heavy atoms has been necessary.

One means of increasing contrast of embedded virus is by removal of the embedding medium. In this method, thin sections of methacrylate embedded tissue are mounted on collodion-coated grids and immersed in liquid benzene for several minutes. This treatment removes the methacrylate and, after drying, the sections may be shadowed with a heavy metal (72) or negatively stained with phosphotungstic acid (98). Although some useful information has been obtained from studies of sections prepared in this manner, the method greatly modifies cytological detail and is of little general use.

Wehrmeyer observed that the contrast of intracellular TMV could be enhanced if tissues were placed in a solution of uranyl acetate after fixation in osmium tetraoxide and prior to dehydration and embedding (101, 102, 103). Post-fixation staining with uranyl acetate or lead subacetate has also been used for tissues infected with several of the small filamentous viruses (35, 36); but with these viruses, as with TMV, the degree of staining was not spectacular. The larger, membrane-bound viruses such as the corn virus of Herold *et al.* (34) seem to be more effectively stained in this way.

Biological electron microscopy received a large boost when Gibbons and Bradfield discovered that staining could be achieved by floating intact sections on solutions of heavy metals (27). Subsequently it was found that the electron density of embedded TMV could be enhanced by floating sections on solutions of phosphotungstic acid (71). Using a variety of other heavy atom compounds, this method now provides a highly effective means of staining viruses *in situ*.

Paradoxically, permanganate salts, which degrade nonimbedded virus, are very effective as post-sectioning virus stains. We routinely stain in the following manner: the sections are mounted on collodion-coated grids, inverted and allowed to float on a 1% aqueous solution of strontium permanganate for 10 minutes. The grids are then rinsed by floating them on distilled water and blotted dry. Care is taken to avoid excess rinsing, as the sections are readily destained. A major disadvantage of permanganate staining is the difficulty in removing residue of the staining solution from the sections.

Comparatively residue-free preparations may be obtained by staining with 1% uranyl acetate for about 1 hour. This procedure imparted high contrast to intracellular TMV (48) and tobacco rattle virus (113). Urynal acetate has also been used in a double-staining procedure with solutions of lead compounds with excellent results (16). Milne obtained good staining of TMV using lead citrate (personal communication). In this case the tissues were also soaked in a uranyl solution before being imbedded and sectioned.

Although treatment of sections with solutions of heavy atoms is a highly effective means of staining embedded virus, the method has inherent faults of which the microscopist should be aware. For example, the apparent morphology of a structure which derives all of its ability to scatter electrons through the staining of sections can vary, depending on the completeness with which the staining material penetrates the section. We have found that the apparent width of TMV varies directly with the time the sections are exposed to solutions of strontium permanganate (up to 6 minutes). Furthermore, embedded TMV commonly appears narrower than it actually is if the surface of the section to be stained is allowed to dry between the time of sectioning and staining (78). Thus, incomplete staining could lead to inaccuracy in estimating the morphology of intracellular virus.

Another point is that all of the virus particles in a section may not be stained. Most evidence with TMV indicates that stain penetrates into sections primarily through sites where virus particles intersect the surface. Presumably, in a section of usual thickness (exhibiting a silver interference color) some virus particles do not intersect both surfaces of the section, and those which do not intersect the surface which is exposed to the staining solution are not stained. This belief is supported by the finding that the number of identifiable TMV particles per unit area increases when both surfaces of sections are stained rather than only one surface (78).

Identification of Virus in Cells

The problems of identifying virus structures in cell sections have been discussed by Williams (109). Once a cell has been killed, embedded, and sectioned there is no way in which infectivity (the primary defining property of a virus) can be ascribed to a particular class of particle seen therein. Identification of particles as virus is therefore based primarily on their morphological similarity to a known virus and their presence only in cells presumed to be infected.

With many plant viruses, identification on the basis of morphology can be made with a high level of certainty. The filamentous and rod-shaped viruses possess a gross morphology quite unlike most particles thus far observed in non-infected cells. Many have been found to have a coaxial core which further differentiates them. When particles with such a form are observed only in cells of infected plants, and their size corresponds to that of particles in purified virus preparations, it is quite probable that they are the causal virus. Similarly, the large insect-transmitted viruses are clearly distinguishable from normal cell components. With rice dwarf virus, for example, the particles seen in sections of infected leafhopper and plant tissue are so characteristic and resemble so closely those seen in sections of partially purified, infective extracts that there can be little doubt that the intracellular particles are rice dwarf virus (24).

Recognition and identification of the small isometric viruses is more difficult. Years ago Smith (92) pointed out the difficulty of identifying viruses such as turnip yellow mosaic virus in sections, because they so closely resemble normal cellular components (i.e., ribosomes). He stressed that the chances of recognizing them were considerably improved if the virus particles were aggregated in a crystalline array, as were particles of tomato bushy stunt virus in leaves of *Datura stramonium L.* (92). With improved preparative methods the prospects for studying these viruses in thin sections are much brighter. Gerola *et al.* recently published micrographs showing clusters of heavily stained particles in leaves of *Chenopodium amaranticolor* infected with Arabis mosaic virus (25). The particles closely resembled those present in purified preparations of AMV. Also, Shikata *et al.* demonstrated particles believed to be those of pea enation mosaic virus in sections of diseased pea plants and infective aphids (81). Both AMV and PEMV appeared slightly larger, were more regular in outline, and were much more heavily stained than cytoplasmic ribosomes, and were therefore easily distinguished from them. Identification of these particles (as with anisometric ones) is largely dependent on a prior knowledge of the virus' morphology.

In cases where characteristic virus-like particles are seen in diseased tissue, but where size and form of the infecting virus is not known, it is presumptuous to conclude that they are the causal agent of the disease. Abnormal structures other than virus are produced in plant cells following infection with certain viruses (48, 74). Unless the morphology of the virus is known it is not possible to say which of the abnormal structures observed in the sectioned cells are virus.

Once an intracellular particle has been tentatively identified as virus on the basis of its morphology, there are additional circumstances which may strengthen the identification. These include the consistent presence of the particles in cells of different host species infected by the virus, a progressive increase in the number of particles with time after inoculation, and their accumulation in cytological structures known to contain infective material. An example of the latter is the aggregation of rods seen in sections of TMV-infected cells to form crystalline inclusion bodies.

A potentially important means of specific cytological identification takes advantage of the antigenic properties of viruses. Singer (83) has adapted the fluorescent antibody technique of Coons *et al.* (13) for use in electron microscopy. The method involves conjugating antibody and ferritin molecules using a bifunctional coupling agent such as toluene 2,4-diisocyanate. Ferritin is a spherical molecule consisting of a protein shell surrounding an inner core of crystalline ferric hydroxide-phosphate which is 55 A° in diameter and easily visualized with the electron microscope (84). Ferritin-conjugated antibodies retain the ability to combine with homologous antigen and thereby provide a specific means of staining virus antigen.

Although this method has proved useful for identifying some animal viruses in tissues, there has been little success in similar attempts to label plant viruses *in situ*. The main difficulty is the inability of the large ferritin-antibody conjugate to diffuse into plant cells and reach sites occupied by viruses. This can be overcome somewhat by freeze-sectioning glutaraldehyde-fixed tissue before application of the conjugate (75). This has the effect of opening cells to allow entry of the ferritin-antibodies. Cells so treated can then be embedded in plastic for ultra-thin sectioning. Although it has been possible to specifically stain intracellular TMV-antigen in this way, the method is extremely time consuming, and preservation of cytological detail is poor. Singer and McLean have developed an alternate means of overcoming penetration difficulties whereby ultra-thin sections of tissue embedded in a polyampholytic methacrylate polymer are directly exposed to the conjugates (84). This is a highly promising approach but the technique needs further refinement before it will be suitable for routine use.

Relation of Plant Viruses to Their Host Cells

Since the first report on the visualization of a virus in a plant cell (4), nearly one hundred papers have been published on similar thin-sectioning studies with plant viruses. Although TMV has been investigated to the largest extent, at least 37 other plant viruses have been studied in this manner (Table 1). The following attempts to summarize the results of these studies, with emphasis on what they have demonstrated concerning the relation of plant viruses to their host cells.

Table 1. Plant viruses studied in infected tissues, using thin-sectioning methods for electron microscopy.

Virus	References
Small anisometric viruses (rods, filaments)	
Barley stripe mosaic virus	72,76
Bean common mosaic virus	a[*]
Bean yellow mosaic virus	20, a
Clover yellow mosaic virus	a
Cucumber mosaic virus 4	8, 40, 41, 91
Cucumber virus 2	88
Cymbidium mosaic virus	a
Lettuce mosaic virus	a
Narcissus yellow stripe virus	14, 15
Papaya ringspot virus	35
Passiflora virus	69
Petunia ringspot virus	67
Potato virus X	5, 7, 45, 91, a
Potato virus Y	22, a
Sugar beet western yellows virus	18
Sugar beet yellows virus	18
Sugar cane mosaic virus	36
Tobacco etch virus	58, 59, 68, a
Tobacco mosaic virus	4, 6, 7, 9, 10, 21, 26, 28, 29, 30, 31, 32, 37, 41, 48, 53, 54, 55, 56, 57, 60, 61, 62, 63, 71, 72, 74, 75, 77, 78, 84, 85, 86, 87, 89, 91, 95, 98, 100, 101, 102, 103, 104, 105, 106, 107, a
Tobacco rattle virus	113, a
Tulip mosaic virus	110
Turnip mosaic virus	33
Watermelon mosaic virus	a
Wheat mosaic virus	38

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[*] John R. Edwardson, personal communication (see throughout).

[**] Morphology of the causal virus not known.

Table 1 (continued).

Small isometric viruses	
Arabis mosaic virus	25
Pea enation mosaic virus	81
Prunus ringspot virus	70
Tobacco ringspot virus	6
Tomato bushy stunt virus	90, 92
Tomato ringspot virus	19
Turnip yellow mosaic virus	90
Large insect-borne viruses	
Albutilon chlorosis virus[*]	99
Corn mosaic virus	34
Lettuce necrotic yellows virus	11
Rice dwarf virus	23, 24, 79
Tomato spotted wilt virus	2, 42, 46, 47, 52
Wheat striate mosaic virus	49
Wound tumor virus	51, 79, 80, 82

Intracellular location of viruses. The sites where viruses are synthesized and assembled in cells have long been the objects of speculation and experimentation. To date, thin-sectioning studies have not helped to answer the question of where viral RNA and protein are synthesized. However, the places where viruses can be seen in cells could indicate where their components, having been synthesized, are assembled into stable, infective particles. As will be seen from the following, it isn't that simple.

When cells of plants systemically infected with TMV are examined, masses of TMV particles are often seen scattered throughout the central cell vacuole (4, 7, 72). The vacuole would seem the least likely place in the cell where virus particles might be assembled and, in fact, there is much evidence to indicate that they are not formed there. The presence of virus in the vacuole is apparently the result of a breakdown of inclusion bodies when the cells are killed in preparation for electron microscopy. The crystalline inclusions, long known from light-microscope studies (43), are now known to be composed largely of TMV particles (97) and to occur within the cytoplasmic matrix of the cell. During fixation with osmium tetraoxide or other agents they often dissolve, the tonoplast is ruptured, and virus pours into the vacuole (29, 72).

Another instance where apparent location of virus in the cell could lead to wrong conclusions about sites of virus formation has been brought out in a study of TMV infection in tomato leaflets (74). In response to TMV infection tomato chloroplasts become abnormally amoeboid. They constantly change shape, and develop long appendages which may be extended and contracted rapidly. In some instances these projections become cup-like and fold back upon the main body of the chloroplast. In the process, portions of the hyaloplasm become surrounded by these projections and are either partially or completely incorporated into the body of the plastid. Viewed in section through the electron microscope these cytoplasmic regions appear as small vacuoles within the chloroplast stroma. They often contain normal cytoplasmic organelles, such as

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[*] Morphology of the causal virus not known

mitochondria, and sometimes large masses of TMV particles. In such instances virus particles appear to be inside the chloroplasts, and it would be tempting to conclude that they were formed there. However, most indications are that their presence within the plastids is a result of their being engulfed by a process akin to phagocytosis.

These are forceful reminders that virus particles can arrive at sites within the cell without being formed there. They also point to the need for exercising extreme caution in interpreting the appearance of thin sections of chemically-fixed material.

In nearly all of the studies cited in table 1, particles which could reasonably be identified as virus were observed only in the hyaloplasm of infected cells (as defined by Robbins *et al.* [66], hyaloplasm is that part of the cytoplasm exclusive of mitochondria and plastids). Except for TMV in tomato (see above), identifiable virus particles have not been found in chloroplasts and only one virus, pea enation mosaic virus (81), has been found in host nuclei. None has been found in mitochondria.

Inclusion bodies have been found in nuclei of plants infected with tobacco etch virus (58, 68) and bean yellow mosaic virus (Englebrecht, personal communication) but it is questionable whether the inclusions contain virus. The fine structure of TEV intranuclear inclusions suggests they are not composed of particles of a size and shape which corresponds to that known for tobacco etch virus (58). Similarly, inclusion bodies have been found in chloroplasts of plants infected with sugar beet western yellows, sugar beet yellows and tomato ringspot viruses (18, 19). These all possess a crystalline structure as viewed with the electron microscope which is not easily identifiable as virus. Virus-like particles have been reported in chloroplasts of diseased tissue by Hohl (39) and Sun (99), though in neither case was the morphology of the causal agent known.

Of the viruses which have been found in the hyaloplasm, some have a close association with structures therein or with other organelles. In mesophyll cells of bean leaves infected with bean yellow mosaic virus, particles believed to be virus were usually observed in close proximity to the nucleus (20). In other studies, particles of barley stripe mosaic virus were often found attached by one of their ends to the outer surface of chloroplasts in mechanically inoculated leaves of barley, but were mostly scattered free in the hyaloplasm in leaves infected from seed (76). Most of the large viruses which have been studied, such as tomato spotted wilt and wound tumor viruses, were always seen in the hyaloplasm and were usually surrounded by membranes believed to be elements of endoplasmic reticulum (47, 82).

As mentioned, the presence of a particle within a certain region of the cell does not necessarily mean that it was assembled, much less synthesized, there. However, the consistent appearance of many different viruses only in the hyaloplasm seems to be very strong evidence that many of them probably are assembled there--otherwise virus particles would surely be seen more often in other regions (such as chloroplasts or nuclei) if they were also assembled there. Many viruses are probably assembled in the hyaloplasm, but it is possible that synthesis of viral components may occur in other regions of the cell. Indeed, with TMV there is considerable evidence that this is so (112). It is quite possible that some viruses may be both synthesized and assembled in nuclei (viz., pea enation mosaic virus) or chloroplasts.

Manner of particle assembly. From studies on extracts of tobacco hair cells Zech has proposed that during assembly of TMV into stable, ribonucleoprotein particles an intermediate structure is formed which is larger than mature TMV rods (111). According to Zech, flexible large strands appeared in cell extracts approximately 16 hours after the cells were infected; these strands contained threads which were sensitive to ribonuclease and appeared to contract with progressing infection to form stiff TMV particles. He proposed that the RNA portion of TMV was synthesized first and then underwent a stepwise coating with TMV protein which was usually complete 30 hours after infection. He suggested that these infection products were rich in water and that most of this was lost with time, resulting in a shrinking of intermolecular distances and formation of the stiff TMV particles.

Strands of comparable size and morphology to those shown in Zech's micrographs were found in thin sections of tomato leaflets during the period of exponential increase of TMV in inoculated leaves (74). These were 60-70 mμ in diameter and variable in length, sometimes nearly 2μ. They usually were arranged in orderly bundles having a pattern similar to that of TMV particles in crystalline inclusions. They occurred exclusively in the hyaloplasm, and were often closely associated with elements of endoplasmic reticulum and ribosomes. Similar structures have also been observed in sections of TMV-infected tomato by Smirnova (87) and more recently in tobacco leaves by Kolehmainen *et al.* (48) and Milne (personal communication). Kolehmainen *et al* also show micrographs of structures which could be interpreted as intermediate stages between the large, voluminous strands and mature TMV particles. Milne observed the large strands when the first recognizable TMV particles could be seen 15 hours after inoculation.

It seems probable that the large strands observed in cell extracts and sections are intermediate forms in the assembly of TMV. However, the infection of plant cells is not synchronous and it is therefore not possible to obtain a timed sequence of events in virus formation by studying sections of manually inoculated leaves. A more precise control over the sequence of events in infection must be achieved before it can be shown that the large filaments seen in sections actually precede the formation of TMV particles. This could be accomplished by studying sections of individually inoculated cells. Treatment of the strands with enzymes and ferritin conjugated anti-bodies *in situ* could help in establishing biochemical and antigenic relationships between the strands and TMV. Finally, it will be significant to learn whether analogous structures are formed during multiplication of other plant viruses. Similar structures have been found in cells infected with petunia ringspot (67), but in this case the morphology of the virus itself was not known.

Structure and formation of inclusion bodies. Iwanowski first reported that abnormal intracellular inclusions were formed in mosaic infected tobacco plants (43). Since then, numerous types of inclusions have been described in cells of plants infected by various viruses and their possible composition has greatly interested electron microscopists. The occurrence and structure of plant virus inclusions has been extensively reviewed by Smith (93). Only information bearing on the nature of inclusions which has been found from thin-sectioning studies since Smith's review will be discussed here.

Although the structure and viral content of the crystalline inclusions formed in cells infected with TMV is well known (96, 101), the manner of crystal formation has received little attention at the electron microscope level. Some information on this has been obtained from studies of TMV infected tomato leaves (74). In samples taken 48 hours after leaves were inoculated with a 38 μg/ml suspension of TMV, individual TMV particles were found scattered in the hyaloplasm of mesophyl cells. At the same time small aggregates of particles were observed in some of the cells. Transverse and longitudinal serial sections of these aggregates revealed that they were cylindrical, about 0.3μ long and 0.1 to 0.3μ in diameter, and composed of several to a hundred virus particles. These were interpreted as early stages in virus aggregation leading to the formation of crystalline inclusion bodies. Of particular interest was the large number of these aggregates distributed throughout the hyaloplasm of many cells. In samples taken 70 hours after inoculation, as many as seven separate aggregates were commonly observed in one thin section of a cell. From volume measurements and counts in over 60 sectioned cells, it was estimated that many cells contained from 50 to 100 individual aggregates (Shalla, unpublished data). In cells sampled at subsequent intervals, some of the aggregates appeared to increase in diameter by the deposition of TMV particles to their periphery. Some of these aggregates were 1-2μ in diameter and probably correspond to the grey plates described from phase microscopy of living cells (94). Eventually (200-550 hours after inoculation) many cells contained 1 or 2 massive aggregates of particles consisting of several layers of virus particles and corresponding to the characteristic crystalline inclusions seen with the light microscope. The fact that few large crystals eventually form in a given cell, while early in infection aggregation began at numerous sites suggests that only a few of the small aggregates grew into large ones. This is supported by the lack of any indication that the small ones fused to form larger ones, and by the fact that many small aggregates were still found in cells with large crystals as late as 550 hours after inoculation.

While the composition and structure of crystalline TMV inclusions is well-known, knowledge about the nature of amorphous X-bodies is still meager. There are conflicting reports concerning the appearance of X-bodies in thin sections of TMV-infected tissue. In one study they were reported to be composed mainly of TMV particles (7). In another, they appeared as amorphous spheres containing a granular substance and rarely virus (56), and in a third report they were depicted as highly membranous bodies in which mitochondria, small vacuoles, some TMV particles, and numerous filaments (suspected to be intermediate assembly products of the virus) were embedded (74). It is doubtful whether all of these structures actually correspond to the inclusions described as X-bodies from light microscope studies. In one case they could have been crystalline inclusions disrupted as a result of preparative procedures (7), and in another report (56) some of the structures described as X-bodies bore close resemblance to poorly preserved host nuclei. The body described as containing small vacuoles and mitochondria among other things (74) seems to be the best candidate, as these have long been recognized as components of X-bodies as seen with the light microscope. Also, this structure agrees closely with that of X-bodies observed with the electron microscope in cells infected with petunia ringspot virus (67). It is clear that critical studies are needed--studies in which light and electron microscopy are integrated--before the problem of X-body structure will be solved.

Several other types of inclusions have been described whose nature has not been determined. The crystals which occur in chloroplasts (18, 19) and nuclei (58) are examples. Also, a new family of structures has been described from studies with the electron microscope. They are variously referred to as "looped profiles", "dense bands","looped masses", and "rings" (33, 58, 110). Edwardson suggests that most of these belong to a single type of inclusion having thin plates radiating from an elongate axis (personal communication). Whether they appear as "pinwheels" or "dense bands" depends on whether they are sectioned longitudinally or transversely. These forms are common in the hyaloplasm of plants infected with many of the viruses having filamentous particles 730-750 mμ long. It is not known whether they contain virus, but there is evidence that similar inclusions from narcissus plants infected with yellow stripe virus are composed of filaments resembling yellow stripe virus (14).

Local lesions. Although many viruses may readily be observed in sections of systemically infected plants, the presence of virus particles in local lesion hosts has only recently been demonstrated convincingly by thin-sectioning methods (10). Early work on electron microscopy of local lesions induced by TMV in *Nicotiana glutinosa L.* and *Datura stramonium L.* failed to reveal the presence of typical 300 mμ long rods (72), even though extracts of lesions were highly infective. Subsequently, Hayashi and Matsui (31) reported visualization of structures, purported to be the rod-shaped particles of TMV, within local lesions on *N. glutinosa.* Weintraub and Ragetli (105), however, offered an alternative interpretation of the micrographs of Hayashi and Matsui. They suggested that the morphology and length of the rod-shaped structures coincided more closely with fragments of chloroplast lamellae frequently observed in degenerating cells. Consequently, they suggested that insufficient evidence had been presented to confirm the presence of TMV rods in a local lesion host. In rebuttal, Hayashi and Matsui (32) reasserted that the particles they observed were TMV and published another micrograph showing filamentous particles in the lumen of a degenerating cell.

Whether TMV has been seen in sections of *N. glutinosa* is still questionable, but there is little doubt as to its having been demonstrated in sections of lesions on *D. stramonium* (10). In *D. stramonium*, mono- and multilayered aggregates of TMV particles were found in the hyaloplasm of cells at the periphery of local lesions that were at least 5 days old. The aggregates were similar in structure to crystalline inclusion bodies commonly observed in cells of systemic hosts. Carroll (9) examined sections of over 2,000 cells in lesions and observed virus in only 6% of them. It is not surprising, therefore, that TMV evades detection in thin sections of *N. glutinosa*, as the concentration of virus in lesions of this host is far lower than in lesions of *D. stramonium* (72).

Weintraub and Ragetli (106) obtained a detailed account of cytological changes leading to necrosis in leaves of *N. glutinosa* inoculated with a high concentration of TMV. Of particular interest was an increase in the number of mitochondria to about twice that present in normal cells 24-28 hours after inoculation. These changes in numbers of mitochondria were correlated with changes in levels of respiration in intact tissues following inoculation. Apparent increases in the number of mitochondria has also been noted in cells at the margins of expanding TMV lesions on *N. glutinosa* (32) and *D. stramonium* (9).

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