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An Electron Microscope Study of Tomato Spotted Wilt Virus in Sections of Infected Cells and in Negative Stain Preparations

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SUMMARY

Mature particles and the early stages of growth of tomato spotted wilt virus (TSWV) were examined by electron microscopy in thin sections and by negative staining. Early evidence of infection included: (*a*) amorphous, darkly stained material in the cytoplasm; (*b*) spherical virus-like particles 100 nm. in diameter, with two concentric membranes; (*c*) configurations interpreted as membranes budding to form the particles in (*b*); (*d*) mature virus particles resembling the inner membranes plus cores of the particles in (*b*). Similar structures were seen in thin sections of developing local lesions. Local and systemic infections more than five days old contained only mature particles, and not structures (*a*), (*b*) and (*c*).

A possible sequence for virus maturation is suggested. The evidence does not support the suggestion that TSWV is a myxovirus.

INTRODUCTION

Work on the chemistry and structure of tomato spotted wilt virus (TSWV) has shown that it differs greatly from other plant viruses, save possibly carrot mottle virus (Murant *et al.* 1969), and that it may resemble myxoviruses (Best, 1966, 1968; Milne, 1967*a*, *b*; van Kammen, Henstra & Ie, 1966; Kitajima, 1965; Ie, 1964; Martin, 1964; Black, Brakke & Vatter, 1963). Particles of different sizes and shapes, spheres and dumb-bells, have been reported (Best, 1968; Ie, 1964). The present study of the mature TSWV particle and its development was made to clarify these points.

METHODS

Virus. TSWV strains from the following sources were examined: (*a*) a strain (here called YAR I) long maintained in the glasshouse and kindly supplied by Dr C. E. Yarwood from Berkeley, California; (*b*) a recent isolate from a naturally infected plant also supplied by Dr Yarwood; (*c*) a strain isolated and supplied by Dr M. Hollings from southern England; (*d*) a strain long kept in cultivation by sap-transmission at Wye College, Kent, England, provided by Dr T. A. Hill. Nearly all work was done with the YAR I virus, which was passed through three consecutive single-lesion isolations on *Nicotiana clevelandii* before use. All strains were maintained by inoculating tomato plants with infective sap at least once every 2 weeks.

Evidence for YAR I being spotted wilt virus is as follows. The thermal inactivation point is 46° and infectivity in tomato sap at room temperature is lost after 30 min.; longevity may be extended to about 24 hr by adding sodium sulphite, keeping the pH

above seven and the temperature below 4°. The symptoms and host range accord with those published (see Best, 1968) and the virus was transmitted by *Thrips* species (unpublished results of Dr H. C. Finch, San Luis Obispo Polytechnic College, California). The virus particles resemble those described for TSWV by van Kammen *et al.* (1966) and Kitajima (1965).

Plants. The virus strains were grown systemically in tomato plants, var. Kondine Red which were kept in a glasshouse or in growth rooms maintained at 20° with a 16 hr day and illumination of 3600 ft candles from warm-white fluorescent tubes. Early stages of virus growth were examined by sampling sap-inoculated leaves of tomatoes at 5 to 8 hr intervals, or by sectioning single expanding local lesions in leaves of *Nicotiana clevelandii*, *N. glutinosa*, *Vigna sinensis* and *Chenopodium amaranticolor*.

Inoculation and infectivity assay. Plants to be infected were dusted with carborundum and wiped on their upper leaf surfaces with a finger dipped in inoculum. This consisted of infected tomato leaf ground up in ice-cold 0·02 M-sodium sulphite to give a dilution (w/v) of 1 in 5. Tissue to be assayed for infectivity was ground up in ice-cold 0·02 M-sodium sulphite and adjusted to a dilution of 1 in 20 (w/v). This was immediately rubbed on to at least eight leaves of *N. clevelandii* that had been dusted with carborundum. Inoculations made at different times were only roughly comparable and close comparison of infectivities was not attempted.

Sectioning. Pieces of healthy and infected leaf were vacuum-infiltrated and fixed in 2·5% (w/v) glutaraldehyde in 0·1 M-phosphate buffer pH 6·8 for 1 hr at room temperature, blotted and transferred to 1 or 0·1% (w/v) osmium tetroxide in the same buffer at room temperature for 3 hr. The 1 and 0·1% osmium tetroxide gave similar results (see Harrison & Roberts, 1968). The fixed leaf pieces were washed in 50% (v/v) aqueous acetone and soaked in 70% (v/v) aqueous acetone saturated with uranyl acetate for 3 hr. After passage through 100% acetone, the material was embedded in Epon without using propylene oxide, sectioned on a Reichert Om U2 microtome with a diamond knife (Dehmer, Germany) and placed on uncoated 400-mesh grids. Staining for 1 min. in Reynolds' lead citrate was followed by examination in a Siemens Elmiskop 1A electron microscope. An anticontaminator was available during a small part of the work.

Negative staining. Virus particles were not consistently seen in negatively stained dip preparations or leaf crushes of infected material, but the following method always revealed them. A container was filled with 5% (w/v) glutaraldehyde made up in double-distilled water and, after cleaning by sweeping with a glass rod, the surface was sprinkled with talc. When about 4 $mm.^2$ of leaf was crushed and touched to the prepared surface, the cell contents spread out, displacing the talc to a diameter of some 4 cm., and a carbon-filmed grid was touched to the surface at the edge of the spread material. The grid was then washed with a few drops of negative stain. 2% (w/v) neutral sodium phosphotungstate, 2% (w/v) uranyl acetate, 2% (w/v) uranyl formate and 2% (w/v) ammonium molybdate, made up in water, were used as negative stains.

RESULTS

Virus strains

In all respects studied, the two strains from California and the one from Dr Hollings in southern England were similar. The strain from Wye agreed in *in vitro* properties and host range but virus particles were never seen in material infected with it, despite exhaustive search. The results that follow refer specifically to the YAR 1 strain.

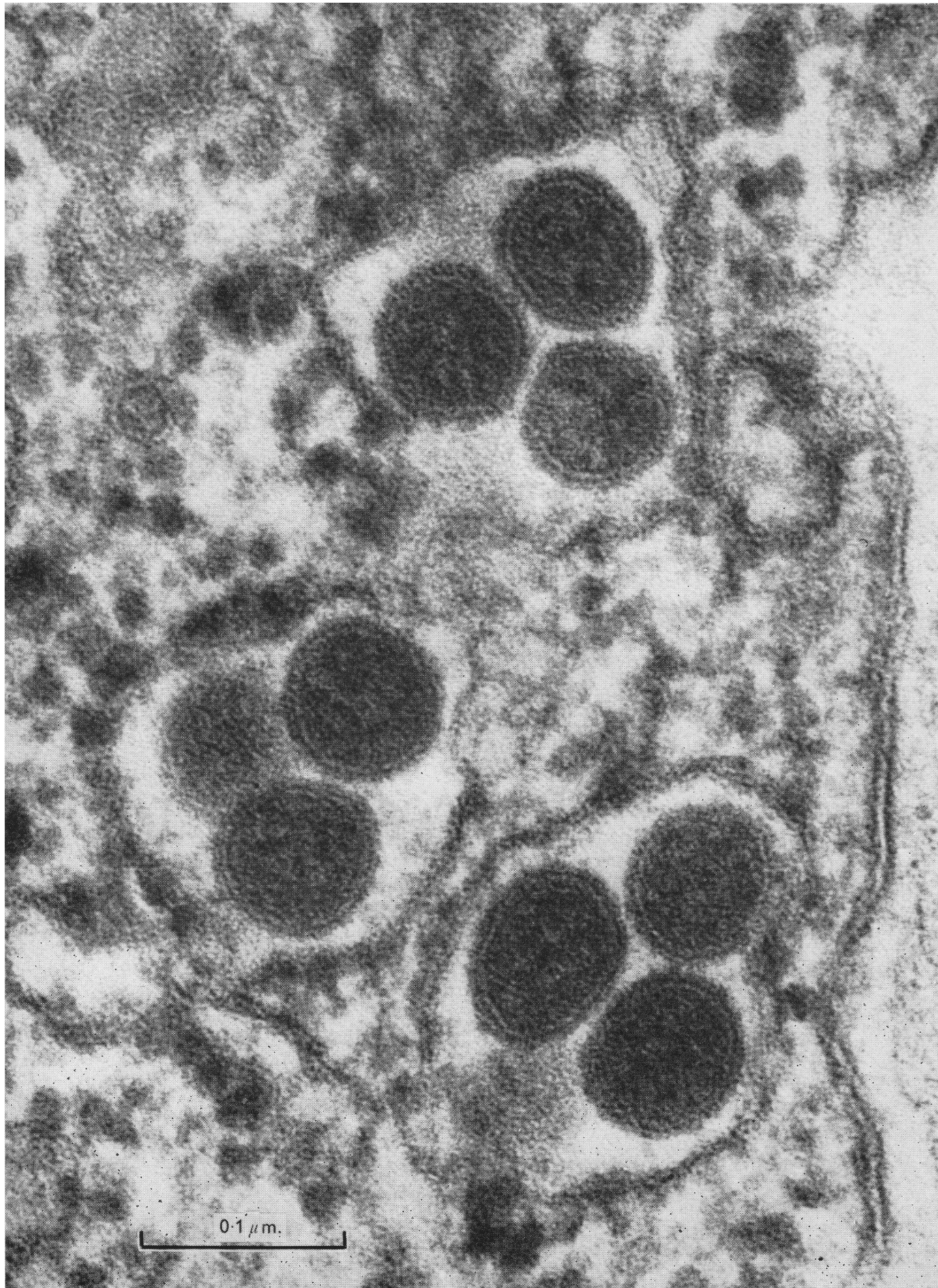

Fig. 1. Groups of mature virus particles in tomato mesophyll cytoplasm.

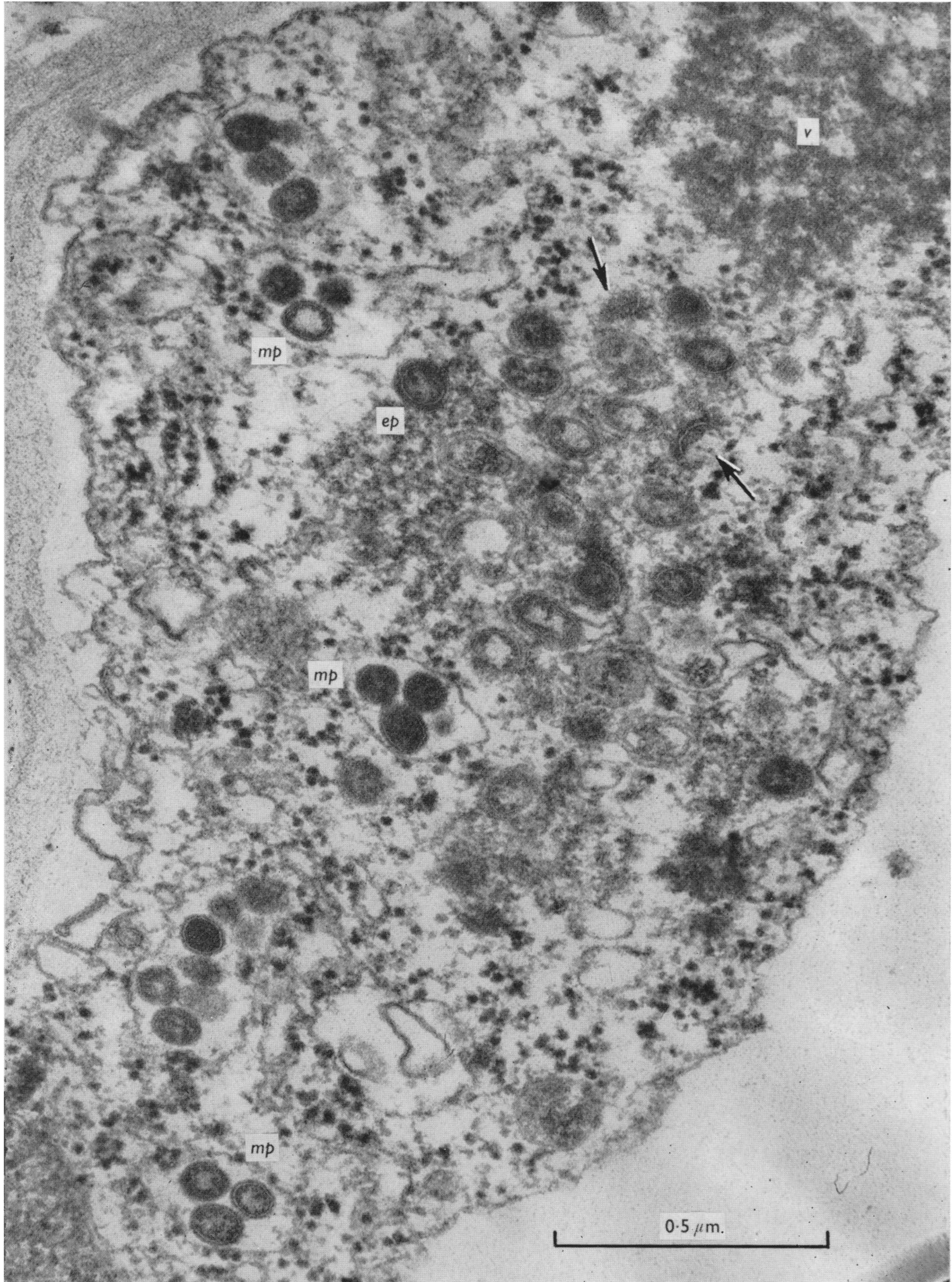

Fig. 2. Part of an infected tomato mesophyll cell. Viroplasm (*v*) is seen as amorphous material. Some mature virus particles (*mp*) and enveloped particles (*ep*) appear empty though others have dense cores. Arrows indicate incomplete enveloped particles.

Sectioned material

Inoculated leaves of tomato plants kept at 20° first became infective at about 50 hr after inoculation. Infectivity increased until about 90 hr and then remained at a high level until necrosis set in. Sectioning showed that nuclei and mitochondria in inoculated leaves remained normal in appearance after virus particles were produced. Chloroplasts were sometimes invaginated and contained large starch grains. From about 55 to 125 hr after inoculation, several structures, in addition to mature virus particles, were consistently seen in the cytoplasm. Later, only mature particles were seen.

The mature particle

Completed virus particles occurred in membrane-bound interconnecting cisternae that contained no other visible components (Fig. 1). The particles were nearly spherical, some hollow, usually 75 to 80 nm. in diameter, and varied slightly in shape and size (extreme diameters were 70 and 100 nm.). The mottled core of the particle was surrounded by a three-layered membrane whose middle layer was typically found at a diameter of 65 nm. Outside the membrane was a thin, densely staining coat. No smaller particles, dumb-bell-shaped or tailed particles were seen.

Viroplasm

This material occurred as densely staining amorphous masses in the cytoplasm (Fig. 2).

Enveloped particles

These nearly spherical particles, about 100 nm. in diameter (95 to 120 nm.) were always associated with viroplasm (Fig. 2, 3*a*, 4*a*, *c*). The central core of the particle was surrounded by a membrane at a mean diameter of 65 nm. and outside this was a shell of dense matter surrounded by a further membrane. Both membranes appeared to have the usual triple structure, and each of the three layers was about 2·5 nm. thick. The thickness of the outer shell or envelope, from the inner membrane to the outer one, was 12·5 nm. Enveloped particles most often had dense cores and dense envelopes (Fig. 4*c*), but some had hollow cores and dense envelopes (Fig. 3 *a*), or dense cores with hollow envelopes (Fig. 4*a*, bottom).

Parallel membranes

Pairs of parallel membranes 12·5 nm. apart (Fig. 3) were often seen near viroplasm and enveloped particles. The space between the membranes was usually filled with densely staining material. The origin of the membranes is unknown but they did not seem to be derived from Golgi apparatus, endoplasmic reticulum or other usual cell membranes. Fig. 3 and 4 illustrate configurations suggesting that enveloped particles grew from parallel membranes by budding.

Sectioned local lesions

All four local-lesion hosts examined gave similar results, though *Nicotiana clevelandii* was studied most and this account refers to it. Little detail was discernible in the central necrotic part of the lesion but outside this there were many mature particles. A little further out, in cells probably still living at the time of fixation, there was a region containing mature particles, viroplasm, enveloped particles and small amounts of paired parallel membrane. Further still from the lesion centre, cells appeared normal.

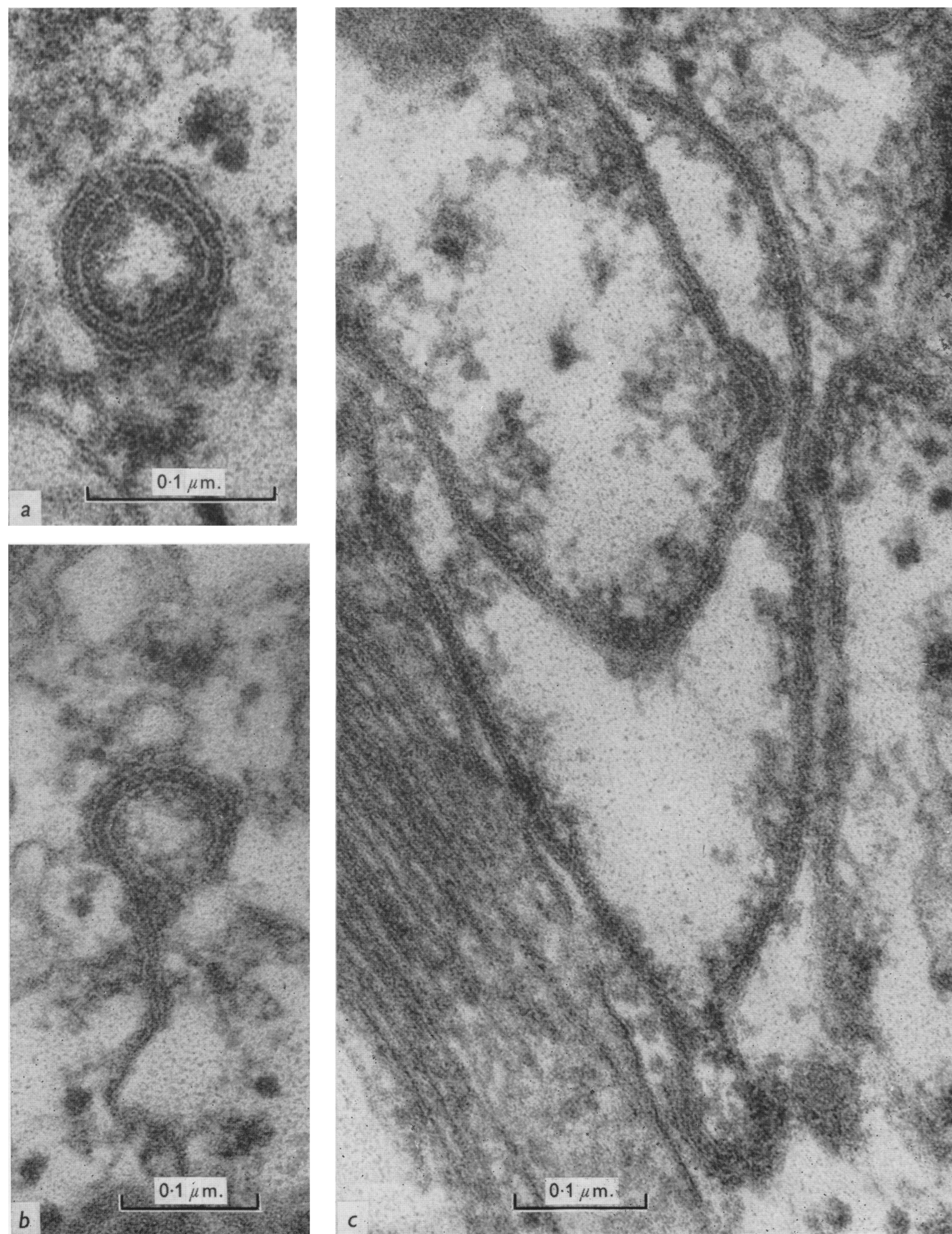

Fig. 3. (*a*) Section of an enveloped particle showing its two membranes. In this example the core is hollow. Fig. 4(*a*) and (*c*) show enveloped particles with dense cores. (*b*) and (*c*) Sections of infected cytoplasm containing paired parallel membranes from which enveloped particles seem to originate.

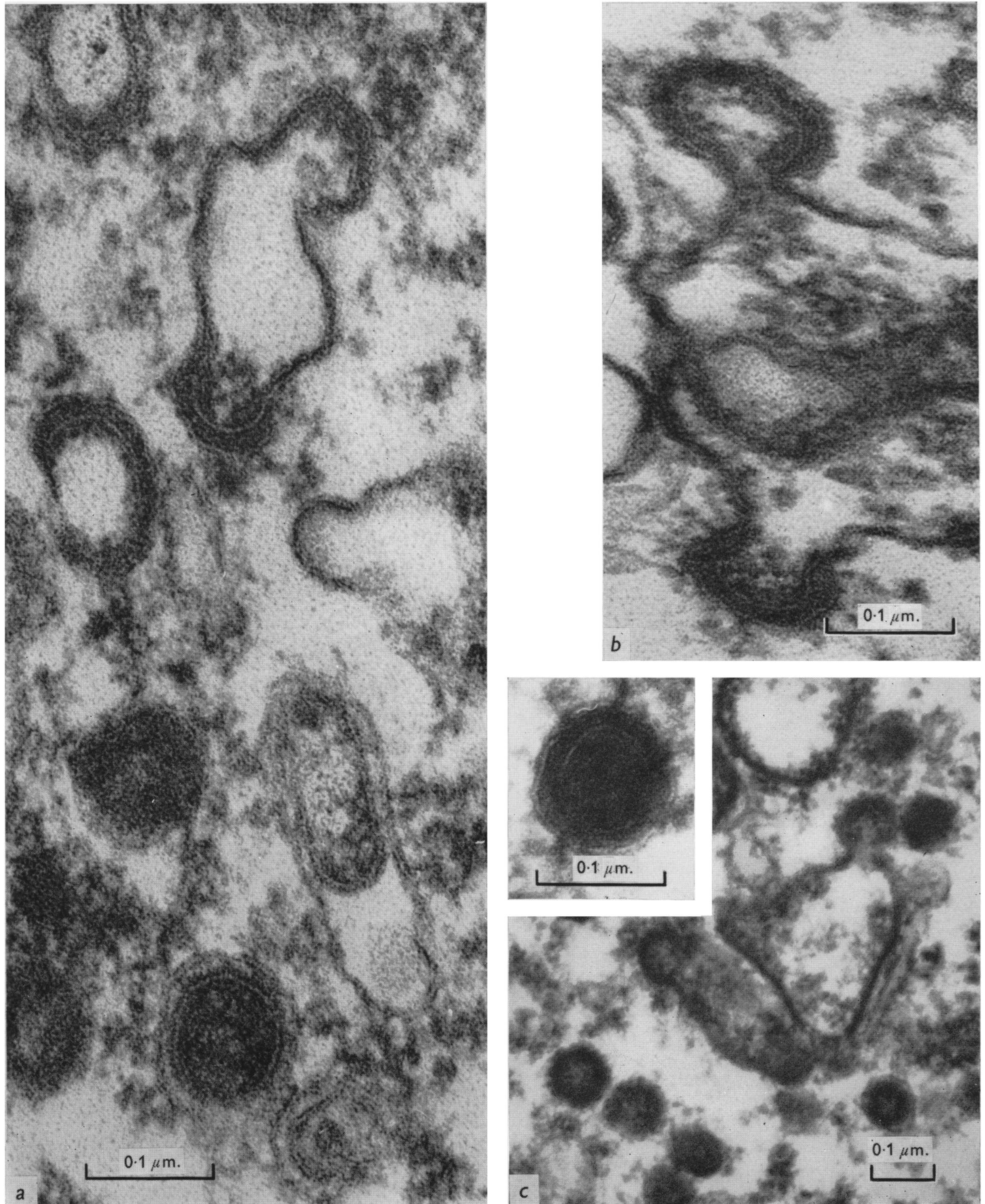

Fig. 4. Sections of infected cytoplasm suggesting that enveloped particles grow from parallel membranes by budding. In (*a*) and (*c*) enveloped particles can be seen.

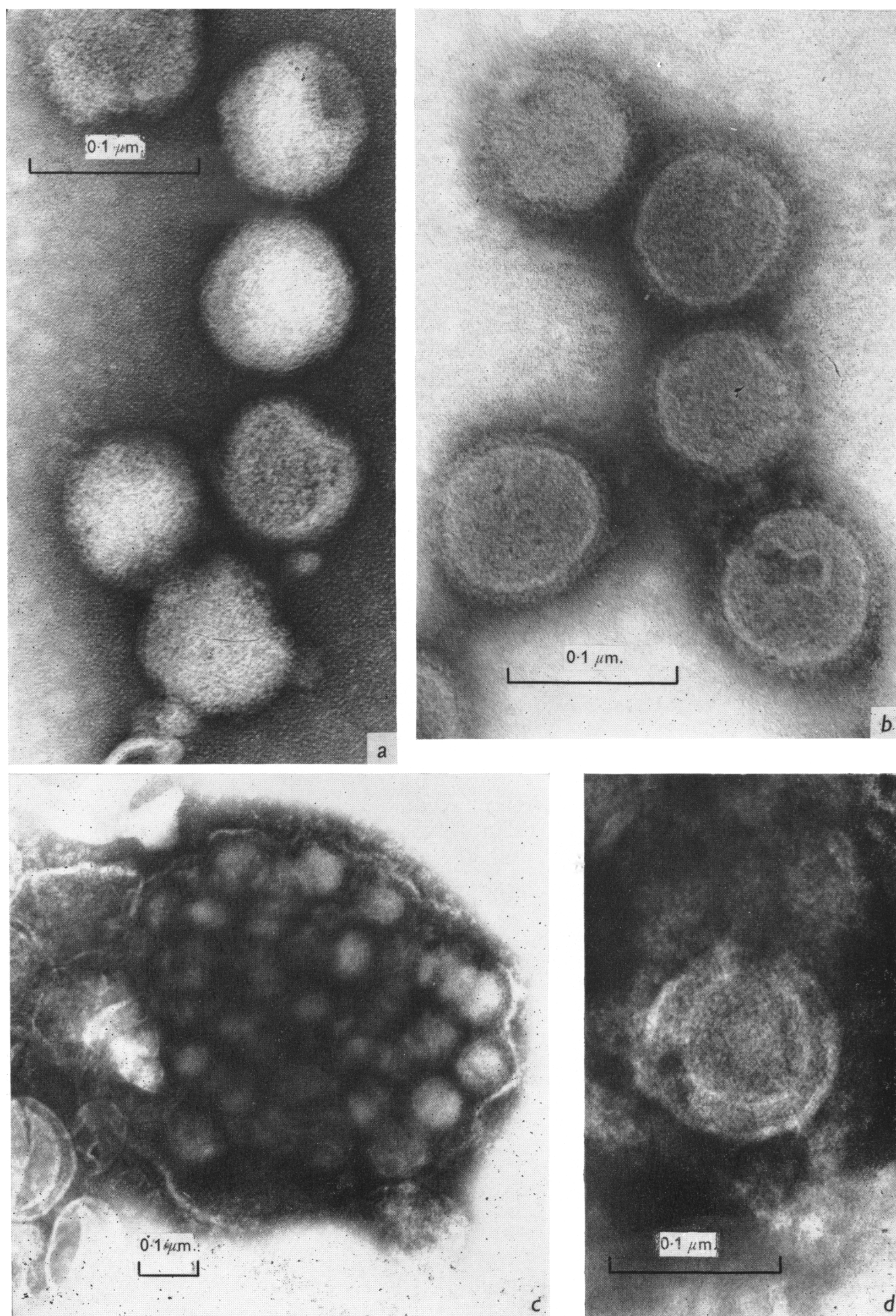

Fig. 5. (*a*) TSWV particles spread on 5% glutaraldehyde and negatively stained with uranyl acetate at pH 4·2. (*b*) A similar preparation stained with neutral sodium phosphotungstate. (*c*) A preparation similar to (*b*). Here, some 50 virus particles have remained in their membrane-bound cluster. (*d*) Part of the contents of a leaf cell 75 hr after inoculation, spread on glutaraldehyde and negatively stained with phosphotungstate. Such particles were seen in preparations from early infections but not in those from late infections or healthy tissue. Compare with Fig. 3*a*.

Negative staining

Particles thought to be those of the mature virus were seen in infected but not in healthy tomato leaf tissue crushed and spread on glutaraldehyde (Fig. 5). The diameter of the particles in negative stain was 85 to 100 nm., i.e. greater than in sections and also more variable, probably because of flattening. Tailed or dumb-bell-shaped particles were never seen; nor any particles 55 nm. in diameter as described by Best & Palk (1964).

In uranyl acetate (Fig. 5*a*) or uranyl formate, the particles either appeared almost smooth and were not penetrated by the stain, or were completely penetrated and became positively stained. In neither case was internal structure apparent. In phosphotungstate (Fig. 5*b* and *c*) or ammonium molybdate the core of the particle was penetrated but showed little structure. Near the periphery, a pale ring probably indicated the position of the membrane and, outside this, a mottled layer 6 or 7 nm. thick was probably homologous with the outermost layer seen in sectioned particles. Negative stain preparations made from tomato leaves 75 and 100 hr after inoculation revealed structures that were probably enveloped particles (Fig. 5*d*). They were not seen in long-infected or uninfected leaves.

DISCUSSION

It did not prove possible to separate the steps in virus growth, either by sequential sampling in a systemic host or by sectioning expanding lesions from the centre outwards. A virus-maturation sequence cannot therefore be presented unequivocally but the following series of events seems plausible.

(1) Viroplasm appears in the cytoplasm. Its structure, composition and function are unknown but it may consist of ribonucleoprotein and is probably involved in the early stages of forming virus particles.

(2) Within the viroplasm, paired parallel membranes appear and bud to form enveloped particles. Both the material sandwiched between the membranes and that incorporated into the enveloped particle are probably derived from the viroplasm. Enveloped particles may also be built directly from the viroplasm, the parallel membrane stage being telescoped or suppressed (see Fig. 2, arrows, where incomplete enveloped particles occur without any parallel membrane).

(3) The shell of dense material between the two membranes of the enveloped particle is absorbed by the core or condensed in a thin layer around it, i.e. particles such as those in Fig. 4*c* change to those in Fig. 4*a* (bottom).

(4) The outer membranes of a group of enveloped particles such as those in Fig. 4*a* (bottom) join up to form a cisterna and the inner membranes plus cores of these particles are released into it to form mature particles (Fig. 1). Hollow-cored enveloped particles (Fig. 3*a*) give rise to hollow-cored mature particles (Fig. 2, upper left), probably not infectious. Hollow and solid-cored particles are almost certainly not section artifacts produced from a homogeneous population, though it remains possible that the difference is produced by poor fixation.

The mature TSWV particle resembles influenza virus (a myxovirus) in thin sections (see, for instance, Apostolov & Flewett, 1969) and also contains lipid and RNA in addition to protein (Best, 1968). However, no helical nucleoprotein component (such as myxo- and paramyxoviruses have) has been seen during this work or elsewhere, in sections or negative-stain preparations; nor has budding characteristic of myxoviruses been observed at cell membranes. The budding and the enveloped particles here described more nearly

resemble what occurs in leukovirus infections (see Fenner, 1968), though probably the parallel is not close. The RNA of myxoviruses has a molecular weight of 2 to 3×10^6 and is probably in several pieces, whereas the RNA of leukoviruses is a single piece of molecular weight and is about $9{\cdot}5 \times 10^6$. To know the size and disposition of the RNA of TSWV would therefore be of great interest.

The 55 nm. particles described by Best & Palk (1964) were not seen and their status remains obscure. No tailed particles were seen and it seems certain that those described were artifacts caused by extrusion of core material. Dumb-bell-shaped particles (Best, 1968) may have been enveloped particles in process of budding, or simply a misinterpretation of two closely associated spherical particles.

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