An Electron Microscopic Study of the Infection of Isolated Tomato Fruit Protoplasts by Tobacco Mosaic Virus

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SUMMARY

Electron-microscopic evidence is presented for the infection of isolated tomato-fruit locule tissue protoplasts by tobacco mosaic virus. Uptake of tobacco mosaic virus by pinocytosis into vesicles in the cytoplasm was observed. Following the disappearance of virus from pinocytic vesicles and the regeneration of these isolated protoplasts into cells the virus appeared in the cytoplasm in aggregates characteristic of tobacco mosaic virus infection in these cells. Adequate removal of ribonucleases and purification of the pectinase enzyme employed for the isolation of protoplasts was essential for the establishment of infection.

INTRODUCTION

The use of isolated tomato-fruit locule protoplasts has provided evidence for a pinocytic uptake of tobacco mosaic virus into vesicles in the cytoplasm of these protoplasts (Cocking, 1966), but the subsequent fate of these virus particles has remained in doubt. Earlier, Mundry (1965) concluded that although the mechanism of virus uptake by plant cells was still obscure it was not incompatible with the hypothesis of virus uptake into plant cells by pinocytosis. Kassanis (1967) pointed out the advantages of using such isolated protoplasts, from which the cell wall has been removed, to follow the unaided entry of virus.

Initial studies of the infection of isolated protoplasts by tobacco mosaic virus showed that conditions favouring uptake of virus might not be ideal for virus multiplication. The finding that isolated protoplasts readily regenerated a new cell wall when maintained in suitable culture media (Pojnar, Willison & Cocking, 1967) permitted the continued culture of protoplasts, following pinocytic uptake, under conditions in which further pinocytic uptake of virus does not occur. We believed the use of purified pectinase in conjunction with an improved washing of protoplasts was essential in any study of virus multiplication in these regenerating protoplasts, in view of the known deleterious effects of ribonucleases (Hamers-Casterman & Jeener, 1957) and other enzymes. Preliminary experiments indicated that tobacco mosaic virus particles became readily attached to the newly formed cell wall. This made determinations by local lesion assay of the extent of virus multiplication unreliable. We thought that, initially, the most direct evidence for virus multiplication could be obtained from electron-microscopic observations on these regenerating protoplasts, particularly since it was known that tobacco mosaic virus particles could be identified readily in the...
cytoplasm in thin sections of protoplasts isolated from tomato-fruit locule tissue cells infected with tobacco mosaic virus (Cocking & Pojnar, 1968a). Moreover, in these observations, any attachment of virus particles to cell walls would not be a complicating factor in deciding if infection had taken place.

The present paper presents electron-microscopic evidence for pinocytic uptake of tobacco mosaic virus by isolated protoplasts, and following the formation of a new cell wall around these protoplasts for the cessation of virus uptake and for the disappearance of tobacco mosaic virus from the pinocytic vesicles. Subsequent multiplication of virus results in the formation of characteristic aggregates of tobacco mosaic virus in the cytoplasm of these cells.

METHODS

Use of tomato fruit free from tobacco mosaic virus. The tomato seed used was specially selected, virus-free seed (Dr Bewley’s ‘Gold-Label’ Hassocks Ltd, Surrey) and plants were grown with stringent precautions against virus infection. The general conditions for growth were as previously described (Cocking & Pojnar, 1968a). Before it was used for the preparation of isolated protoplasts the hormone-set fruit was checked by local lesion assay on Nicotiana tabacum (var. Xanthi) to ensure that it was free from tobacco mosaic virus infection. The leaves of the plant were also checked. Two weeks later a sample of the detached fruit, the leaves, and the fruit remaining attached on the same truss were checked again to ensure that no infection by tobacco mosaic virus had occurred.

Purification of pectinase using Sephadex G 100. The highly sensitive method of Matthaei et al. (1967) was employed to detect any ribonuclease activity in commercial pectinase preparations. Polyuridylic acid labelled with 14C (kindly donated by Dr J.H. Matthaei) was employed as the substrate. The extent of degradation of the labelled polyuridylic acid by ribonucleases was determined by liquid scintillation counting of degradation products chromatographed on filter-paper strips using the general procedure of Davies & Cocking (1966). Only a small concentration of ribonuclease was detected in the pectinase of Sigma Chemical Co. Ltd; a greater amount was present in Pectinol R. 10 (Rohm & Haas Co. Ltd). Sigma pectinase was purified using Sephadex G 100 (Pharmacia, Uppsala, Sweden). Chromatography on Sephadex G 100 not only ensured the removal of ribonucleases but also facilitated the removal of other contaminating enzymes and virus-inhibiting materials. The procedure adopted was similar to that described by Bagi & Farkas (1967). The pectinase (0.5 g.) was dissolved in 10 ml. distilled water, centrifuged and layered on the column (2.9 x 16 cm.). The Sephadex beads were equilibrated with distilled water and proteins eluted in 2 ml. fractions using distilled water. Distilled water instead of dilute buffer was used for elution to facilitate the removal of ribonucleases by adsorption on the Sephadex itself (Glazer & Wellner, 1964) as well as by the usual exclusion effects. The elution pattern is shown in Fig. 1. Most of the pectinase activity, as determined by ability to release protoplasts from tomato locule tissue (see later) was present in peak C. Twenty ml. was employed for the routine isolation of protoplasts.

Isolation and washing of protoplasts. Using the assay for ribonucleases previously described, much ribonuclease was observed in the juice from pieces of dissected locule tissue, and to avoid major contamination with these ribonucleases the tissue pieces were washed with three 100 ml. lots of modified White’s medium (Lamport,
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Fig. 1. Chromatography of Sigma pectinase (0.5 g.) on Sephadex G 100; eluted with distilled water.

1964), without 2,4-dichlorophenoxyacetic acid or coconut milk, but containing 20% sucrose. Following these washings the locule tissue pieces from three 6-week-old tomato fruits free from tobacco mosaic virus were incubated with 20 ml. of purified pectinase + 20% sucrose filtered through a 'Millipore' membrane (peak C, Fig. 1). Protoplasts were isolated using the general aseptic procedure of Pojnar et al. (1967). Isolated protoplasts were washed first in 20% sucrose containing 0.2% RNA (RNS, 15332, C. F. Boehringer & Soehne, GmbH, Mannheim) to inhibit the activity of any ribonuclease taken up into protoplasts (Brachet, 1955) and then in 20% sucrose alone.

Culture of isolated protoplasts. Washed isolated protoplasts in 20% sucrose were cultured in Conway units (No. 1, A. Gallenkamp and Co. Ltd, Widnes, Lancs). The cell-wall regeneration medium contained 9.0 g. sucrose, 2.5 g. Ficoll (Pharmacia, Uppsala, Sweden) and 5.0 g. mannitol in 100 ml. of the modified White's medium (Lamport, 1964) but without 2,4-dichlorophenoxyacetic acid or coconut milk. Three hr after the addition of tobacco mosaic virus to these protoplasts the suspending medium was routinely tested for ribonuclease activity present in the incubation mixtures at this stage. There was usually less than 30% breakdown of the [14C]polyuridylic acid in 1 hr.

Electron microscopy. Protoplasts were fixed in 6% glutaraldehyde in 0.025M-sodium phosphate buffer, pH 7.0, containing 10% (w/v) sucrose for approximately 12 hr at 4°, washed well with 0.1M-sodium phosphate buffer pH 7.0 and post-fixed in 2% osmium
tetroxide (0.1 M-sodium phosphate buffer pH 7.0 containing 10 % sucrose) for 3 hr. Protoplasts were stained during dehydration for 15 hr in 1 % uranyl acetate in 70 % ethanol at 25° and subsequently embedded in the butylmethacrylate-estrene embedding medium of Mohr & Cocking (1968). Gold sections were cut using glass knives on a Porter-Blum MT 2 ultramicrotome and collected on carbon-coated Athene type new 200 grids. They were post-stained for 3 hr with lead citrate (Reynolds, 1963) and examined in an A.E.I. EM 6B electron microscope at 60 kv using a 50 μm. objective aperture.

Quantitative sectioning methods. The number of particles of tobacco mosaic virus per isolated cell or protoplast was estimated from thin sections of cells following the method of Séchaud, Ryter & Kellenberger (1959). Serial sections showing gold interference colours (c. 1000Å thick) were mounted on carbon-coated Athene-type hexagonal grids. Nine to twelve serial sections of 10 to 12 cells were photographed for each stage of the growth cycle:

\[
\text{No. of particles/cell or protoplast} = (F/m) \times \text{av. no. of particles/section of cell or protoplast},
\]

where

\[
F = \frac{\text{av. vol. of cell or protoplast}}{\text{av. vol. of section of the cell or protoplast}}
\]

and \( m \) = average number of times the same particle of the virus appears in successive sections.

The average volume of whole cells or protoplasts was calculated from optical measurements of the diameter of the spherical cells or protoplasts floating on the surface of the culture medium, and the average volume of the sections was calculated from measurements of the average diameter of cells or protoplasts in 2 μm. sections examined by light microscopy. The average value of \( F \) was calculated to be 735. It was calculated that \( m = 3 \) for c. 1000Å sections of tobacco mosaic virus particles

RESULTS

Samples of suspensions of the well-washed, specially prepared, isolated protoplasts in 20 % sucrose were placed in the centre well of Conway units and a concentrated suspension of tobacco mosaic virus in 20 % sucrose (the VULGARE strain kindly donated by Dr J. H. Matthaei) was added to give in each Conway unit a final virus concentration of 0.1 % with isolated protoplasts. Protoplasts incubated under these conditions for 6 hr with the virus were fixed and embedded for electron-microscopic observations. After 6 hr incubation virus was observed in vesicles in the cytoplasm of these protoplasts. About 30 % of the sections of protoplasts showed some virus in vesicles but the detailed appearance of the particles differed in certain of the vesicles. About half of the pinocytic vesicles (Pl. 1 a) contained virus of about the same breadth as previously reported for tobacco mosaic virus stained by this prolonged soaking in uranyl acetate; while in others the virus was thinner in longitudinal section (Pl. 1 b) with a diameter similar to that observed with short periods of staining with uranyl acetate, in which mainly the RNA core of the virus is detectable (see Cocking & Pojnjar, 1968 b). Sometimes vesicles contained loosely coiled fibrillar material. it seemed that tobacco mosaic virus particles were being rapidly uncoated within these pinocytic vesicles (compare the appearance of virus in Pl. 1 b, c).
(a) Pinocytic vesicle containing numerous particles of tobacco mosaic virus after 6 hr incubation of protoplasts with the virus.

(b) Another pinocytic vesicle containing particles of tobacco mosaic virus after 6 hr incubation of protoplasts with the virus. The particles appear thinner and are possibly being uncoated.

(c) Tobacco mosaic virus particles attached to the newly regenerated cell wall formed around a protoplast after 54 hr in culture.

(d) Region of the cytoplasm of another regenerated protoplast after 54 hr in culture. Tobacco mosaic virus particles remain attached even to broken pieces of the cell wall (arrow). Note absence of TMV in cytoplasm. Tonoplast (T).

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(Facing p. 308)
(a) Cytoplasm of regenerated protoplast 90 hr after addition of tobacco mosaic virus. Note small aggregates of tobacco mosaic virus in the cytoplasm.

(b) Tobacco mosaic virus particles in cytoplasm of another regenerated protoplast 90 hr after addition of the virus. An aggregate of the virus is clearly visible and also single particles of tobacco mosaic virus (arrows) in the cytoplasm.

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(a) Low magnification of a region of an uninfected regenerated protoplast after 120 hr culture. The cell is plasmolysed and the cell wall (CW) is clearly visible.

(b) Low magnification of a region of the cytoplasm of an infected regenerated protoplast after 120 hr culture. Tobacco mosaic virus is clearly visible (V) and the cytoplasm shows signs of degeneration.

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(a) Higher magnification of another region of the cytoplasm of an infected regenerated protoplast after 120 hr culture. 'Thin' particles of tobacco mosaic virus are clearly visible ($V$) as well as 'thicker' particles (arrow). The cytoplasm shows signs of extensive degradation. Cell wall ($CW$).

(b) Region of the cytoplasm of an infected regenerated protoplast after 120 hr culture. Note the alignment of the virus particles ($V$) in virus aggregate. Extensive degradation of the cytoplasm, associated with the cytocidal effect of the virus, is evident.

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For more prolonged incubation protoplasts were diluted after 6 hr with four times their volume of cell-wall regeneration medium (see Methods). Protoplasts incubated for a further 48 hr under these conditions were fixed, stained and embedded as previously described. When examined in thin section the newly formed cell wall was clearly visible surrounding all nucleated protoplasts and particles of the virus were seen attached to this wall but did not penetrate it (Pl. I c). Virus remained attached even to broken pieces of the wall (Pl. I d). Although these regenerating protoplasts were incubated initially with tobacco mosaic virus for the same time and under similar conditions to the 6 hr sample in which pinocytic uptake of the virus had been observed, no virus was detected in any vesicles following this longer incubation. This would be expected if the protein coat of the virus particles was being removed within the pinocytic vesicle permitting the release and uncoiling of the RNA core. No virus particles were observed in the cytoplasm. However, when protoplasts were incubated for a further 84 hr under these conditions of cell-wall regeneration and then similarly fixed, stained, embedded and examined in thin section, aggregates of particles were readily discernible in the cytoplasm. The nature of these aggregates and the characteristic appearance and dimensions of individual particles were the same as those of tobacco mosaic virus in isolated protoplasts from infected fruit (Cocking & Pojnar, 1968 b). A general view of a region of the cytoplasm of an infected cell is shown in Pl. 2 a and a higher magnification of a small aggregate of tobacco mosaic virus in the cytoplasm of another infected cell is shown in Pl. 2 b. About 20 % of the sections of these cells showed similar aggregates of virus in the cytoplasm. No virus was present in vesicles.

In other experiments protoplasts isolated under similar conditions were incubated either in 20 % sucrose or in the cell-wall regeneration medium for 48 hr before the addition of the same concentration of the virus. Following incubation for a further 48 hr the samples were fixed, stained, embedded and sectioned as before. Virus particles were seen attached to the newly formed cell walls to about the same extent as previously. The newly formed wall around protoplasts in sucrose was, however, much thinner than that around protoplasts cultured in the cell-wall regenerating medium. No virus particles were present either in vesicles or in the cytoplasm, and, even when present at high concentrations at the cell wall, no particles were detected between the cell wall and the plasmalemma even when plasmolysis had occurred and even when the wall was thinner than usual.

In order to quantify the uptake of virus and the extent of its multiplication in these cells, serial sectioning of cells was employed. Isolated protoplasts were incubated with virus under conditions similar to those previously described except that after 6 hr incubation protoplasts were washed four times with cell-wall regeneration medium to remove as fully as possible any residual virus before culturing them under aseptic conditions in the cell-wall regenerating medium. Samples were taken at zero time (no virus added) 6 hr, 30 hr, 54 hr, 90 hr and 120 hr. A control culture was set up in which isolated protoplasts to which no virus had been added were similarly incubated for 120 hr. The average number of particles of tobacco mosaic virus per cell in pinocytic vesicles or in the cytoplasm was estimated at various times during the growth cycle (Table I). After 120 hr extensive multiplication of the virus had taken place; on average, 40 % of the cells had become infected. Uninfected cells appeared healthy but often showed marked plasmolysis (Pl. 3 a). Plasmolysis was much less evident in
infected cells. The cytoplasm of these infected cells after 120 hr showed signs of extensive degradation and a general cytocidal effect of the virus was clearly evident (Pl. 3b, 4a, b). Moreover, in these infected cells the virus appeared considerably thinner than at 90 hr (compare Pl. 2 with Pl. 4). This suggests that associated with cell degeneration there was a progressive thinning of the particles of tobacco mosaic virus, probably as a result of uncoating of the virus particles, so that often it was mainly the densely stained RNA core of the virus which could be seen.

Table 1. Average numbers of particles of tobacco mosaic virus in isolated protoplasts or cells during the growth cycle

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Average number of virus particles in vesicles per protoplast or cell</th>
<th>Average number of virus particles in the cytoplasm per protoplast or cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>239</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
<td>1,730</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>262,000</td>
</tr>
<tr>
<td>120 (no virus added)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Although pinocytic uptake of virus could be consistently shown without control of ribonucleases or purification of pectinase (Cocking, 1966) there was no evidence either for a disappearance of virus from pinocytic vesicles or for any formation of virus aggregates in the cytoplasm. Adequate removal of ribonucleases and purification of the pectinase used to isolate the protoplasts is clearly an essential factor in enabling infection to become established.

DISCUSSION

Several investigations have indicated that release of virus RNA from the virus protein is one of the first processes occurring in the cell after entry of the plant virus (Mundry, 1963), and it seems likely from the present electron-microscopic observations that following uptake of tobacco mosaic virus into pinocytic vesicles the protein coat is removed from this virus. It is unlikely that the resultant change in the appearance of the virus can be attributed to sectioning or staining artifacts. However, definite conclusions regarding removal of the protein coat from virus particles within these pinocytic vesicles will only be possible if ribonuclease digestion was shown to remove 'thin' but not 'thick' particles and ferritin tagged antibody was shown to attach only to 'thick' and not to 'thin' particles. The phosphotungstic acid staining procedure of Mayo & Cocking (1968) in which the edge of the protein coat of tobacco mosaic virus is more clearly defined than when using uranyl acetate (Cocking & Pojnar, 1968b) could also be usefully employed to detect early stages in any removal of the protein coat of the virus. It is particularly interesting that degradation of virus in the cytoplasm of degenerating infected cells, in which the virus is having a cytocidal effect, also appears to involve a removal of the protein coat of the virus particle. Disappearance of the virus from these vesicles would apparently occur when the RNA becomes uncoiled since the staining procedure employed would not readily detect uncoiled
RNA in these vesicles in thin section. The pinocytic vesicles in which removal of the protein capsid of the virus is probably occurring are likely to be cellular infective centres, but the possibility cannot be excluded of undetected infecting particle(s) entering isolated protoplasts by a route other than the pinocytic vesicle. More information about the fate of these vesicles could be obtained by marking them with colloidal thorium dioxide (Holmes & Watson, 1963).

The rate of infection of these regenerated protoplasts by tobacco mosaic virus is rather slow (Table 1) and it is possible that the plasmolyzing conditions are somewhat inhibitory to the multiplication of the virus in this system. It is also likely that the culture medium employed is not the best for virus multiplication in this single cell system (Kassanis, 1967).

There is perhaps a parallel between pinocytic uptake of virus, cell-wall regeneration and the initiation of virus infection in these isolated protoplasts and the processes involved in the mechanical inoculation of leaves with plant viruses. Mundry (1965) concluded from indirect evidence that virus uptake by the protoplasm of leaf epidermal cells resembles pinocytosis. Probably in leaves breakage of regions of the cell wall exposes the plasmalemma to virus, allowing pinocytic uptake to occur, but to a more limited extent than when the cell wall has been completely removed as in isolated protoplasts. Rapid cell-wall regeneration at these points of breakage is known to occur and here again there is a parallel with cell-wall regeneration by isolated protoplasts following uptake of virus. Pinocytic uptake of virus ceases with the onset of cell-wall regeneration and the cell wall acts as a very effective barrier to the entry of tobacco mosaic virus into the cell. The attachment of virus particles to this newly formed cell wall (Pl. 1, c, d) may, however, be a special characteristic of these newly formed cell walls and not characteristic of most other plant cell walls.

As pointed out by Siegel (1966), one of the principal difficulties in the study of plant virus infection has been the lack of a model system in which cells under study can be simultaneously exposed to virus particles and become infected. This difficulty has now been overcome by initially exposing isolated fruit protoplasts to tobacco mosaic virus under conditions in which pinocytic uptake of the virus occurs. Isolated cells containing pinocytosed virus are then formed as a result of the regeneration of these protoplasts and virus infection becomes established. It is clearly now possible to begin to utilize this isolated protoplast system, and also the isolated protoplast system from tobacco leaves recently described by Takebe, Otsuki & Aoki (1968), for a comprehensive study of the infection of plant cells by viruses and to obtain a fuller understanding of the pathways involved. The cytocidal effect of the virus in these infected regenerated protoplasts could perhaps form a basis for the development of an assay for plant virus infectivity comparable to the plaque assay for animal viruses.

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