Freeze-etching Observations on Tobacco Leaves Infected with Tobacco Mosaic Virus

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SUMMARY

Tobacco leaf mesophyll cells infected with tobacco mosaic virus were freeze-etched. Virus particles frequently fractured during the freeze-etching process and the implications of this observation are discussed. Freeze-etching is useful in investigations of the fine structure of virus-infected leaf cells and the general application of freeze-etching as an ancillary preparative technique to that of thin sectioning for the study of virus-infected cells is outlined.

INTRODUCTION

Since the initial freeze-etching studies by Steere (1957) the application of freeze-etching methods in virology has been largely neglected even though the introduction of a commercial freeze-etching instrument (Moor et al. 1961) has enabled such studies to be made more easily. Freeze-etching has developed into a preparative method for the electron microscopic study of biological material of comparable importance to thin sectioning (Moor, 1966). To enable careful comparisons to be made between the results obtained using these two different, yet complementary, preparative procedures tobacco leaf material infected with tobacco mosaic virus was chosen for freeze-etching studies since the disposition and detailed appearance of the virus in these leaf cells in thin section has already been described (Kolehmainen, Zech & von Wettstein, 1965; Milne, 1966). These thin section observations have been recently further amplified by Esau & Cronshaw (1967) who observed virus particles in the nuclei and the chloroplasts of parenchyma cells.

METHODS

Freeze-etching procedures. A Balzers unit (Balzers High Vacuum Ltd, Northbridge Rd, Berkhamsted, Hertfordshire) BA 360 M was used for freeze-etching. The general procedure for the preparation of replicas was similar to that described by Moor & Mühlethaler (1963) with slight modifications. Specimens for freezing in liquid Freon 22 were mounted in 3 mm. collared gold specimen holders (Balzers 11-3654 Pt) and a routine etching time of 75 sec. was employed. Particular attention was given to the adequate cleaning of replicas (Moor, 1967). The following procedure was found to be suitable for the cleaning of replicas of freeze-etched leaf tissue pieces. After freeze-etching the tissue with attached replica was removed from the collared gold specimen holder by floating it off on the medium in which the tissue was originally frozen (see later), and then transferring the replica and associated tissue using a platinum loop to the surface of a commercial cellulase mixture (‘suc digestif d’Helix
pomatia' from L'Industrie Biologique Française, 35 à 49 Quai du Moulin de Cage, Gennevilliers, France) for several days at room temperature. Subsequently replicas were transferred and floated on 20%, 40%, 60%, and 80% sulphuric acid, each for 10 min., and then treated with a few drops of a potassium permanganate + potassium dichromate mixture (0.075 g. KMnO₄ + 0.075 g. K₂Cr₂O₇ in 4.5 ml. H₂SO₄) in a small crucible. Removal of the partially degraded tissue from the replica was completed in this digestion mixture. Most of the tissue was removed from the replica in about 10 min. and removal was complete after a further 10 min. During this process the replica sank and good illumination was essential in order to recover it. The replica was then re-floated in 80% sulphuric acid and transferred through graded sulphuric acid solutions to distilled water, taken up on Formvar-covered Athene type 200 copper grids and examined in an AEI EM6B electron microscope using a 25 μm. objective aperture. All the photographs were processed so as to show shadows in white.

Leaf material for glutaraldehyde fixation and glycerol treatment before freeze-etching. Leaf material was selected from a tobacco plant (Nicotiana tabacum L. var. Havana) systemically infected with the VULGARE strain of tobacco mosaic virus. Young leaves showing typical mosaic symptoms were cut into strips approximately 1 mm. wide and fixed in 2% glutaraldehyde in 0.02 M-sodium phosphate buffer pH 7.0 for 3 hr and then transferred to 20% glycerol in 0.2 M-sodium phosphate buffer pH 7.0 for at least 2 days. One mm. cubes of selected areas were used for freeze-etching.

Leaf material for glycerol treatment before freeze-etching. A small shoot approximately 80 mm. in length was detached from the infected tobacco plant and kept supported in sand for 3 days with added 10% glycerol. After 3 days a young leaf, comparable in size and mosaic appearance with that used for glutaraldehyde fixation, was removed from the shoot system, quickly cut up into approximately 1 mm. cubes in 10% glycerol, and immediately frozen before freeze-etching.

Direct freeze-etching of leaf material. A young leaf of comparable size to those previously employed and showing typical mosaic symptoms was cut into approximately 1 mm. cubes in water, immediately frozen and then examined by freeze-etching.

RESULTS

Freeze-etching of glutaraldehyde fixed, glycerol treated, tobacco mosaic virus infected leaf tissue

Examination of replicas of this freeze-etched leaf tissue showed clearly the presence in the cytoplasm of numerous small semi-crystalline aggregates of tobacco mosaic virus (Pl. 1a). Not infrequently similar small aggregates of the virus were visible in the chloroplasts and were lacking a bounding membrane (Pl. 1b). There was displacement of chloroplast grana near the aggregates (cf. Esau & Cronshaw, 1967). Sometimes aggregates of virus were present in protrusions from the cytoplasm into the vacuole of the leaf cells (Pl. 2a). Single particles of virus could be readily identified when in the cytoplasm and frequently free virus particles were also present in the vacuole (cf. Cocking & Pojnar, 1968); and while it was often difficult to detect any break in the tonoplast which would allow this release of virus from the cytoplasm into the vacuole, occasionally definite breaks in the tonoplast were detected (Pl. 2b). A freeze-etched particle of isolated, purified tobacco mosaic virus showing the typical fracturing of virus particles in longitudinal fracture which occurs during freeze-etching is shown in
(c) Freeze-etched, glutaraldehyde-fixed, virus-infected tobacco leaf mesophyll cell impregnated with 20% glycerol showing an aggregate of tobacco mosaic virus (arrow) in the cytoplasm near a plastid (P). Cell Wall (CW), tonoplast (T) and vacuole (V).

(b) Freeze-etched, glutaraldehyde-fixed, virus-infected tobacco leaf mesophyll cell impregnated with 20% glycerol showing a small aggregate of tobacco mosaic virus in a chloroplast (arrow). Cell wall (CW), tonoplast (T), vacuole (V) and plastid envelope (PE).

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(a) Freeze-etched, glutaraldehyde-fixed, virus-infected tobacco leaf mesophyll cells impregnated with 20% glycerol showing aggregates of tobacco mosaic virus forming ingrowths (I) into the central vacuole of one of the cells. In the other cell, virus particles are visible in the central vacuole and a break in the tonoplast can be seen (arrows). Tonoplast (T) and cell wall (CW).

(b) Freeze-etched, glutaraldehyde-fixed, virus-infected tobacco leaf mesophyll cell impregnated with 20% glycerol. A distinct break in the tonoplast is visible and tobacco mosaic virus particles are present in the vacuole. A possible pinocytotic vesicle is being formed (PV). Plasmalemma (PL). Virus particles showing surface and internal fractures are arrowed.

Inset. Enlargement of one of these virus particles showing both surface and internal fracture.

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Freeze-etched, glutaraldehyde-fixed, virus-infected tobacco leaf mesophyll cell impregnated with 20% glycerol showing large aggregate of tobacco mosaic virus in the cytoplasm. The fractured virus is variously orientated. In longitudinal fracture the virus has fractured in a similar manner to that shown in Pl. 2b. Transverse fracture (TF), longitudinal fracture (LF) and oblique fracture (OF).

Inset. Freeze-etched particle of tobacco mosaic virus from purified suspension of the virus in 20% glycerol. Fracture along the length of the particle is visible and some suggestion of the internal structure is evident.

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(a) Ice crystal formation within the vacuole of a freeze-etched leaf cell in the absence of added glycerol.
(b) Region of freeze-etched plasmolyzed cell from leaf of infected shoot system grown in 10% glycerol for 3 days. Ice crystal formation is considerably reduced. Ice crystal formation between plasmalemma and cell wall (IC\textsubscript{1}) and within the cytoplasm (IC\textsubscript{2}). Cell wall (CW) and plasmalemma (PL).
(c) Another cell from freeze-etched leaf replica as in Pl. 4b. Good preservation of fine structure is evident and there is no ice crystal formation in the cytoplasm. Ice crystals in intercellular space (IC), cell wall (CW), plasmalemma (PL), mitochondria (M), surface view of nuclear envelope (N) nuclear pores (NP).
(d) Region of freeze-etched cell from leaf of shoot system grown in 10% glycerol for 3 days. A large crystalline aggregate of tobacco mosaic virus is visible in the cytoplasm. Surface view of nuclear envelope (N). Transversely fractured virus (TF), region of cut rather than fractured cytoplasm with virus (C).

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(a) Higher magnification of a region of transversely fractured virus from Pl. 4d.

(b) Tobacco mosaic virus particles lying on a membrane surface. The leaf tissue was unfixed and no glycerol was added before freeze-etching.

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(a) Crystal of tobacco mosaic virus in leaf cell showing characteristic 'herring-bone' appearance. The leaf tissue was unfixed and no glycerol was added before freeze-etching.

(b) Higher magnification of region of Pl. 6a. Showing orientation of virus particles.

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(a) Higher magnification of a region of Pl. 6 (b) A. Surface fractures of the virus particles are visible (SF arrows).

(b) Higher magnification of a region of Pl. 6(b) B. Longitudinal internal fractures are visible (LF arrows).

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Freeze-etching of tobacco mosaic virus

Pl. 3. Aggregates of virus in infected leaf cells were made up of particles showing similar fracture appearances (Pl. 3), and in this particular aggregate transverse fractures of the virus are also visible.

Freeze-etching of glycerol-treated leaf tissue infected with tobacco mosaic virus

When glutaraldehyde-fixed material was incubated in 20% glycerol for several days and then freeze-etched ice crystal formation was negligible (Pl. 1-3). When, however, glycerol was absent considerable ice-crystal growth during freezing was evident (Pl. 4 c). Growth of the infected shoot system in glycerol for several days before the detachment of leaves for freeze-etching studies considerably reduced the extent of ice-crystal growth during freezing (Pl. 4 b). Marked ice-crystal damage to the cytoplasm was, nevertheless, evident in most cells observed, but occasionally a few cells were detectable in which ice-crystal growth in the cytoplasm was negligible with consequent good preservation of cell structure (Pl. 4 c). A large crystalline aggregate of tobacco mosaic virus in one of these cells is shown in Pl. 4 d. The appearance of the virus fractured transversely is clearly seen in Pl. 5 a.

Freeze-etching of leaf tissue infected with tobacco mosaic virus (without the addition of glycerol and without fixation)

When fresh, unfixed leaf tissue was freeze-etched without the addition of glycerol as an antifreeze agent considerable ice-crystal damage occurred. This resulted in the general disruption of cytoplasmic material and sometimes free virus particles were found lying on membrane surfaces (Pl. 5 b).

Frequently, however, the crystalline aggregates of tobacco mosaic virus remained intact and were readily detected when the fracture plane resulted in the appearance of the characteristic ‘herring-bone’ structure in the virus crystal (Pl. 6 a). The detailed appearance and orientation of virus particles within these fractured crystals is illustrated in Pl. 6 and in Pl. 7.

DISCUSSION

The appearance of virus-infected cells following freeze-etching although generally similar to that obtained by thin sectioning is, nevertheless, different in certain important respects. In freeze-etched material not only is one observing a shadowed, replicated surface, but the surface observed is a fractured, not a cut, surface. It is likely that when biological specimens are broken in freeze-etching they fracture along the path of least resistance. In the case of virus particles this is probably in between the protein subunits leaving the subunits themselves entire. When particles of tobacco mosaic virus lie with their long axis parallel to the plane of specimen fracture they appear to fracture over the surface or along an approximately median plane leaving the hollow centre exposed. Sometimes fracture is both over the surface and within the particle (Pl. 2 b). It is clear from Plate 3 that evaporated material has accumulated on exposed surfaces of the protein subunits; however, it is not possible to determine the precise nature of the fracture since the resolution of the platinum-carbon replica is close to the size of the protein subunits of the virus.

The large crystals of virus observed in cells which had not been fixed in glutaraldehyde resemble the ‘herring-bone’ appearance of freeze-etched tobacco mosaic virus crystals observed by Steere (1957). Internally fractured particles within these crystals
(Pl. 7 b) were not apparent in Steere's replicas (cf. Steere 1957, Plate 12, fig. 5). In agreement with the earlier results of Steere (1957) the ends of the virus particles are not separated by any appreciable gap and their length is close to 3000 Å. This suggests that dehydration of tobacco mosaic virus during embedding of material for thin sectioning or for negative staining causes little change in the length of the particles (Markham et al. 1964).

The detection of breaks in the tonoplast and the general absence of large crystalline aggregates of tobacco mosaic virus in leaf cells fixed in glutaraldehyde before freeze-etching serve to substantiate the thin section observations of Warmke & Edwardson (1966) that glutaraldehyde fixation can cause virus crystal breakdown. While it should be noted that Matile & Moor (1968) detected no significant effect of glutaraldehyde on the ultrastructural characteristics of freeze-etched corn roots and that Branton & Park (1967) came to similar conclusions in their studies of chloroplast structure, freeze-etching of virus-infected cells without prior fixation may be particularly instructive in studies of virus disposition in cells. Freeze-etching of leaf cells at early stages of infection, without prior fixation, might detect any special arrangement of virus particles which is significantly altered by the usual fixation methods (Matsui & Yamaguchi, 1966) and freeze-etching of isolated protoplasts taking up virus by pinocytosis (Cocking 1966) would be particularly instructive. The difficulty of readily infiltrating glycerol into leaf cells might possibly be overcome by isolating cells from leaves using pectinase (Takebe et al. 1968) and incubating these isolated leaf cells in glycerol before freeze-etching. Animal materials appear to take up an antifreeze substance very much more rapidly (Haggis, 1961); a solution of 20% glycerol in a buffered isotonic solution of sodium chloride can serve as a standard (Moor, 1966), and, as a result, glycerol treated, unfixed, virus infected animal cells can be readily freeze-etched.

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REFERENCES

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