Fine Structure of Vesicles Induced in Chloroplasts of Chinese Cabbage Leaves by Infection with Turnip Yellow Mosaic Virus

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SUMMARY

Examination of chloroplasts from Chinese cabbage leaves infected with turnip yellow mosaic virus (TYMV), using freeze-fracture techniques revealed, that the small peripheral vesicles induced by infection were present in clusters near the surface of the chloroplast, often being arranged in arrays that approximate to hexagonal. The small peripheral vesicles were flask shaped with their necks pointing to the chloroplast surface. The structure of the necks, which were only about 15 to 22 nm in diam., suggested but did not prove that they were open to the cytoplasmic space.

The inner membrane of the peripheral vesicles is distinguished from other chloroplast membranes in showing no particles on either the A or the B fracture faces.

In the regions where the peripheral vesicles were clustered, the number and kind of particles seen on the chloroplast membrane faces were changed compared with healthy chloroplasts or the non-vesicle bearing areas of chloroplasts from infected cells. In particular, the B face of the outer chloroplast membrane in the vesicle-bearing regions had a reduced number of particles 10 nm in diam., but in addition to these contained large numbers of particles about 5 nm in diam.

In the chloroplasts of both healthy and infected tissue we frequently observed protuberances of the stroma corresponding to the mobile phase of chloroplasts postulated from light microscopic observations.

INTRODUCTION

Infection of Chinese cabbage and other hosts by turnip yellow mosaic virus (TYMV) is followed at an early stage by the formation of numerous small vesicles, composed of two membranes, at the periphery of the chloroplast. These vesicles appear to be specific for the TYMV virus group rather than the host species. It has been suggested that they may be the site of virus RNA synthesis (Ushiyama & Matthews, 1970). Previous work on the effects of TYMV and the significance of the vesicles has been reviewed recently (Matthews, 1970, 1973).

Electron microscopic observations on thin sections of diseased chloroplasts show clearly that the outer membrane of the vesicles is continuous with the inner chloroplast membrane; and is probably formed from it by invaginations (Laflèche & Bové, 1969; Ushiyama & Matthews, 1970). Laflèche & Bové (1969) suggested that the inner membrane of the vesicles is formed by invagination from the outer chloroplast membrane. While occasional vesicles seen in thin section give the appearance of opening into the cytoplasmic space, the exact
relationship between the bounding chloroplast membranes and the vesicle membrane has not been resolved by the examination of thin sections.

In the work described here we have examined the fine structure of chloroplasts from tissue infected with TYMV using freeze-fracture methods. Freeze-fracturing is particularly useful because it reveals large areas of interior faces of membranes, and gives some indication of the disposition of particulate proteins within the lipid bilayer (Branton, 1971). It is probable that most vesicles do open to the cytoplasmic space, but it has not been possible to prove this even by the examination of complementary replicas. The main feature of the vesicles revealed by freeze-fracturing is that the inner membrane differs structurally from the other chloroplast membranes, possessing none of the particles normally seen within membranes. The vesicles are clustered over the surface of the chloroplast. In the region of these clusters the outer chloroplast membrane has an increased number of particles, indicating an increased metabolic activity.

METHODS

**Virus and plants.** TYMV was cultivated in chinese cabbage plants (*Brassica pekinensis*, Rupr., var Wong Bok) in pots in the glass house. Strains of virus were isolated from the stock culture in chinese cabbage as described by Chalcroft & Matthews (1967). For most experiments a severe ‘white’ isolate was used.

**Isolation of chloroplasts.** Systemically infected and healthy chinese cabbage plants were kept in a dark room for about 24 h to reduce the content of starch grains before isolation of the chloroplasts. Leaf tissue (1.5 g without midribs from young leaves about 10 cm long) was put in 30 ml of cold modified Millonig phosphate buffer (Millonig, 1961) (0.16 M-sodium dihydrogen phosphate, 0.08 M-glucose, 0.005 M-MgCl₂, brought to pH 7.4 with 0.12 N-NaOH). The tissue was chopped with an electrically powered razor blade at 0°C until the tissue was cut into very small fragments (4 to 5 min cutting). The suspension was filtered through two layers of Miracloth. The filtered solution was centrifuged at 1000 rev/min for 10 min in an International refrigerated centrifuge. The green pellets so obtained were examined in the electron microscope using both the freeze-fracturing and thin-sectioning procedures outlined below.

**Thin sections for electron microscopy.** Leaf tissue was fixed with 6% glutaraldehyde in Millonig buffer for 60 min at room temperature. For isolated chloroplasts this buffer was modified as noted under the isolation procedure. After washing overnight the samples were postfixed with 1% osmic acid in buffer for 2 or 3 h at room temperature. Dehydration, embedding, staining and sectioning procedures have been described previously (Ushiyama & Matthews, 1970). Thin sections were collected on unfilmed 400-mesh grids or formvar-coated Veco large hole grids. Sections were examined in a Philips EM 200 electron microscope.

**Freeze fracturing.** Before freeze-fracturing, the following pretreatments were usually carried out. Isolated chloroplasts or pieces of leaf tissue were fixed in 6% glutaraldehyde in the isolation medium or Millonig’s buffer for about 2 h at room temperature. After washing with the medium or buffer, they were soaked in 25% glycerol in the appropriate buffer for 30 min or more in a refrigerator.

The freeze-fracture method used was as described by Bullvant & Ames (1966) and Bullvant (1969). Specimens were fractured under liquid nitrogen at −196°C. Shadowing and backing were done through tunnels in a cold block, thus protecting the cold specimen surface against contamination. Since replication was carried out with the specimen temperature at about −140°C, there was no etching.
**Chloroplast vesicles induced by TYMV**

![Diagram of membrane orientations]

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**RESULTS**

*Freeze-fracture of chloroplast membranes*

Before describing the freeze-fracture appearance of the chloroplast limiting membranes and the virus-induced vesicles, it is necessary to describe the manner in which membranes freeze-fracture and the terminology to be used to denote the fracture faces produced. Membranes generally freeze-fracture along a unique hydrophobically bonded interior plane (Branton, 1966, 1971) and this splitting thus exposes two faces. For the plasma membrane of a cell, the face of that membrane portion left frozen to the cytoplasm is referred to as an A face; and the face of that portion left frozen to the extracellular space is referred to as the B face (McNutt & Weinstein, 1970, Fig. 1). Freeze-fracturing shows particles within the membrane. In general, more remain adhering to the A face than to the B face. If one proceeds inwards from the plasma membrane, alternate membranes show reversal of the A and B face direction (Branton, 1969). The plasma membrane fractures so that the A face faces out; the outer membrane of the chloroplast so the B face faces out; and the inner membrane of the chloroplast so that the A face faces out (Fig. 1). It has been pointed out that this would be the case if each successive membrane system was formed by invagination of the previous one (Bullivant, 1969). On the assumption that the vesicles are formed by invagination, then the vesicle inner membrane A face will face out, and the vesicle outer membrane B will face out (Fig. 1).
The structure and distribution of the necks of the peripheral vesicles

The illustrations of the membranes of chloroplasts with peripheral vesicles in Figs. 3 and 4 will be most readily understood if we consider first a model based on the examination of many replicas. In Fig. 2 we have drawn a peripheral vesicle assuming that the outer vesicle membrane is derived from the inner chloroplast membrane by invagination, that the inner vesicle membrane is similarly derived from the outer chloroplast membrane, and that the interior of the vesicle is connected to the extra-chloroplast space by a narrow lumen in the neck.

The legend to Fig. 2 illustrates the four different aspects the neck will present, depending upon which chloroplast membrane is fractured, and which face is viewed.

Figs. 3 and 4 illustrate the four aspects of the membranes shown diagrammatically in Fig. 2. In particular, the complementary faces of inner chloroplast membrane (Fig. 4a, b)
Fig. 3. Aspects of the chloroplast membranes: TYMV infected leaf tissue was pretreated and then freeze-fractured as described under Methods. AO, A face of outer membrane; BO, B face of outer membrane; AI, A face of inner membrane; BI, B face of inner membrane; M, "mobile phase". Arrow indicates position of an atypical outer A face vesicle neck (see text). On all micrographs a circled arrow indicates the shadowing direction. Bar markers = 1 μm.
confirm the model for this membrane. Similar complementary faces not illustrated confirm the model for the outer chloroplast membrane.

The chloroplasts shown in Fig. 3 illustrate the fact that in the majority of chloroplasts examined the vesicles were not randomly distributed but were arrayed in clusters on the surface, frequently in an hexagonal array. This clustering was seen both in isolated chloroplasts and in freeze-fractured intact leaf tissue.
Chloroplast vesicles induced by TYMV

Fig. 5. Smooth areas on B face of outer membrane. Isolated chloroplasts from TYMV infected leaf, pretreated and freeze-fractured as described under Methods. Arrows indicate relatively smooth areas above larger vesicles which have no necks. Typical arrays of depressions for B face outer membrane necks of small vesicles can also be seen. Bar marker = 500 nm.

In freeze-fracture replicas of chloroplasts we have observed protuberances of the stroma (corresponding to the ‘mobile phase’ illustrated by light microscopy and discussed by Wildman, 1967). There was no obvious difference in the nature or frequency of these protuberances between chloroplasts in healthy or TYMV-infected leaf.

Other types of vesicle

Occasionally when viewing the outer membrane B face, we have observed raised rounded areas of the chloroplast surface which are free or almost free of particles. These areas appear to lie over vesicles some or all of which do not have necks (Fig. 5).

Over these raised areas the fracture plane frequently shifts to the membrane below. This is revealed as having no particles, confirming that it is a vesicle inner membrane A face rather than a chloroplast inner membrane A face (see section below).

In thin sections vesicles are occasionally seen in which the outer membrane is clearly open to the cytoplasm and in which the vesicle formed by the inner membrane protrudes into the cytoplasm. Fig. 6 indicates sites where the shadowed replica has been lost during processing to remove the biological specimen. The most probable interpretation of these holes is that small vesicles were protruding from the chloroplast surface at these positions. Occasionally, in the A face of the outer chloroplast membrane, we have seen raised annuli which should not be present on the model presented in Fig. 2 (e.g. arrowed position in Fig. 3a). They may represent the aperture in the outer membrane through which such vesicles escape.

Vesicle structure revealed in fractures through the chloroplast

In the chloroplast illustrated in Fig. 7, the fracture plane has passed through the body of the chloroplast. For some of the vesicles the fracture has passed through the inner vesicle membrane, leaving the inner part of the vesicle itself adhering to the chloroplast. The flask-like shape of the vesicles is clearly revealed.

For other vesicles, the vesicle itself has been removed revealing the B face of the inner vesicle membrane or the A face of the outer membrane. The diam. of the necks seen in the main part of Fig. 7, measured to include two half inner membranes, is approximately
Fig. 6. Freeze-fracture of part of a cluster of chloroplasts in a cell infected with TYMV. Leaf tissue prefixed and freeze-fractured as described under Methods. Arrows indicate sites at which the shadowed replica has been lost, indicating the previous position of a vesicle protruding into the cytoplasm. The aspect seen over most of the chloroplast is outer membrane B face. Bar marker = 1 μm.

20 nm. Some of the vesicle necks seen in Fig. 7 (and in other similar fractures) appear to extend to the chloroplast surface. However, this cannot be taken as proof of patency, since we do not know whether the fracture line representing the chloroplast surface in the replicas has passed through the inner or the outer chloroplast membrane.

The membrane faces seen in Fig. 7 suggest that the A face of the inner vesicle membrane does not possess particles, contrary to what would be expected if it was an invagination of unaltered outer chloroplast membrane. This feature of the vesicles was proved by the examination of complementary replicas as is illustrated in Fig. 8.

As expected the A face of the outer vesicle membrane has many particles. Both the A and B faces of the inner vesicle membranes are entirely smooth.
Chloroplast vesicles induced by TYMV

Fig. 7. Aspects of vesicles seen when the fracture plane passes through the chloroplast. Isolated chloroplasts from TYMV-infected leaf pretreated and freeze-fractured as described under Methods. AI, A face of inner vesicle membrane; BI, B face of inner vesicle membrane; AO, A face of outer vesicle membrane. Inset: vesicle showing neck extending towards chloroplast surface. Bar markers = 100 nm.

Frequency of particles in chloroplast membrane faces

Inspection of many micrographs indicated that besides causing the development of the smooth inner vesicle membrane, TYMV brought about other changes in the distribution and nature of the particles seen in membrane fracture faces. Particle counts per unit area of chloroplast fracture face were made, care being taken to select areas that were close to horizontal in the original specimen. Results are summarized in Table 1.

As would be expected from work on other biological membranes, the B faces of almost all the membranes had significantly fewer 10 nm particles than the corresponding A faces. The only exception was the outer membrane of infected chloroplasts in non-vesicle areas. Here the number of particles on the B face was increased to about the same number as on the A face.

There were no significant differences in numbers of particles between faces of membranes of normal chloroplasts, and corresponding faces of membranes of infected chloroplasts in non-vesicle bearing areas, except that the B face of the outer membrane of diseased chloroplasts had a significantly increased number of particles (\( P \leq 0.01 \)).

The vesicle-bearing and non-vesicle-bearing areas of infected chloroplasts differed in several respects with respect to their membrane particles. (i) Vesicle areas had about half as many particles as non-vesicle areas on the A face of the inner membrane (\( P \leq 0.01 \)). (ii) They also had about 66% as many particles as non-vesicle areas on the inner membrane.
Fig. 8. Complementary replicas demonstrating the altered character of the vesicle inner membrane. Chloroplasts isolated from TYMV infected leaf and pretreated and freeze-fractured as described under Methods. The complementary replicas were photographed as described under Fig. 4. Thus to gain the correct conformational impression, (a) should be viewed with the page inverted. AO, A face of outer vesicle membrane; BO, B face of outer vesicle membrane; AI, A face of inner vesicle membrane; BI, B face of inner vesicle membrane. Bar markers = 500 nm.

B face and the outer membrane A face \( (P = < 0.05) \). (iii) The numbers given in the table for the outer membrane B face indicated that vesicle-bearing areas here also have about as many 10 nm particles \( (P = < 0.01) \). However, in addition to the reduced numbers of membrane particles of standard size, these areas bore very numerous smaller particles not seen in any other chloroplast membranes examined (Fig. 9). Occasionally (as seen in Fig. 6) the area bearing numerous smaller particles extended beyond the immediate vicinity of the
Table 1. Frequency of particles (approximately 10 nm in diam.) seen on various membrane fracture faces of healthy and TYMV-infected Chinese cabbage chloroplasts

<table>
<thead>
<tr>
<th>Membrane and face</th>
<th>Healthy (non-vesicle areas)</th>
<th>Infected (vesicle areas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner A</td>
<td>1718 (342)</td>
<td>2030 (646)</td>
</tr>
<tr>
<td>Inner B</td>
<td>516 (152)</td>
<td>554 (96)</td>
</tr>
<tr>
<td>Outer A</td>
<td>933 (223)</td>
<td>978 (477)</td>
</tr>
<tr>
<td>Outer B</td>
<td>615 (145)</td>
<td>915 (168)</td>
</tr>
</tbody>
</table>

* Each number is the mean of 6 to 8 estimates each from a different micrograph. Numbers in parentheses = standard deviation of the mean.

† In these areas large numbers of particles with diameters ≈ 5 nm were seen in addition to the 10 nm particles.

Fig. 9. Distribution of 10 nm particles and smaller particles on the B face of the outer membrane of a chloroplast isolated from TYMV-infected tissue. Chloroplast preparation prefixed and freeze-fractured as described under Methods. An area with an array of small vesicles is in centre of photograph. The A face of the inner membrane bearing many 10 nm particles can be seen at the top of the photograph. Bar marker = 500 nm.

vesicle clusters, and appeared to correspond to a flattened area of chloroplast surface that had been adjacent to another chloroplast in a clump.

DISCUSSION

As seen in thin sections of chloroplasts the small peripheral vesicles are particularly abundant beneath those areas of the chloroplast’s surface that are adjacent to other chloroplasts in a clump (Laflèche & Bové, 1969; Ushiyama & Matthews, 1970). Freeze-fracture replicas clearly demonstrate the clustered distribution of the vesicles. The clusters of vesicles may usually occupy part or all of areas of contact between chloroplasts in the intact cell.
Studies of various membrane systems have led to the generalization that there is a correlation between metabolic activity and number of particles presumably containing protein, seen in a freeze-fractured membrane (Branton, 1970). Physiologically active membranes such as chloroplast lamellae have many particles, while myelin layers which function as insulators have none.

The B face of the outer chloroplast membrane in the region of the vesicle clusters is rich in particles most of which appear to be smaller in diam. than the standard 10 nm particles. This feature may reflect enhanced metabolic activity of the membrane related to virus synthesis. It may also reflect changed physical or chemical properties of the outer membrane that lead to the clumping of chloroplasts in infected cells. In thin sections the chloroplast membranes that lie close to adjacent chloroplasts in a clump can usually be seen to be stained more heavily than elsewhere. This increase in uptake of stain may involve the particles seen on the outer membrane B face.

The clumping of the diseased chloroplasts shows organelle specificity, since the mitochondria, nucleus and the cell membranes are not included. It may be that glycoproteins or glycolipids are involved in the specific inter-organelle recognition, as has been postulated for the intercellular adhesive specificity of embryonic animal cells (Roth, McGuire & Roseman, 1971).

Thin sections show occasional vesicles which are undoubtedly patent to the exterior of the chloroplast, the inner vesicle membrane being continuous with the outer chloroplast membrane (Lafèche & Bové, 1969). These may be exceptional or abnormal vesicles. Using sections 600 to 900 Å thick it is usually impossible to observe detail in vesicle necks with an external diam. of about 200 Å. We had hoped that observations on complementary replicas might demonstrate whether or not the majority of the vesicles are patent. With hindsight, it is apparent that this technique cannot decide the issue. It would make no difference to the two fracture planes shown in Fig. 2 if the small lumen we have illustrated was in fact completely closed in the region between the two possible fracture planes.

Various electron dense markers such as ferritin (Bruns & Palade, 1968) and lanthanum salts (Revel & Karnovsky, 1967) have been used to test for the patency of vesicles, capillary vessels, etc. Ferritin, with a diam. of 105 Å, may be too large to use for the chloroplast vesicles. In experiments not reported in detail here we have attempted to use lanthanum hydroxide, but because of technical difficulties we have not obtained any decisive results.

The pinocytotic vesicles of endothelial cells of continuous capillaries are undoubtedly patent (Nickel & Grieshaber, 1969). The appearance of the peripheral chloroplast vesicles and the endothelial cell vesicles seen in freeze-fracture replicas show remarkable similarities, except that the chloroplast vesicles are more complex in having two membranes rather than one. On balance it seems likely that most of the small peripheral vesicles have a very narrow channel opening to the cytoplasmic space.

Some of the vesicles beneath the smooth raised areas illustrated in Fig. 5 appear to have no opening to the cytoplasm. They bear some resemblance to the surface views of budding influenza virus particles, where the membrane above the virus is free of particles (Bächi et al. 1969) to the budding Sindbis virus (Brown, Waite & Pfefferkorn, 1972) and to the bulges seen on the nerve cell membranes overlying the neurosecretory granules in the posterior pituitary of the rat (G. P. Dempsey, S. Bullivant & W. B. Watkins, unpublished observations). In this last example the disappearance of the membrane-associated particles may be related to the later fusion and exocytosis of the neurosecretory granule.

The interpretation of the raised vesicles without necks illustrated in Fig. 5 must await further work. They probably represent another class of vesicle, perhaps those without
electron dense contents which are larger than the peripheral vesicles and which may escape to the cytoplasm (Chalcroft & Matthews, 1966).

Almost all cell membranes that have been studied by freeze-fracturing methods show an A face covered with many particles and a B face with fewer particles (Branton, 1971). The inner membrane of the chloroplast vesicles shows no particles on either its A face or its B face. Thus it is ultrastructurally distinct from the outer chloroplast membrane from which it has been considered to be derived by invagination (Laflèche & Bové, 1969). If the inner vesicle membrane is derived from the outer chloroplast membrane, then TYMV infection must cause some change in the membrane that leads to the disappearance of the large proteins that make up the particles normally seen within membranes.

Protuberances into the cytoplasm corresponding to the ‘mobile phase’ activity postulated by Wildman (1967) on the basis of light microscope observations are seen rather infrequently in thin sections. It may be that most of them disappear during the fixation and embedding procedure. Our observations on freeze-fractured chloroplasts provide independent confirmation for the reality of these protuberances.

In experiments not detailed here using light microscopic examination of living cells we have attempted to measure the vol. and surface area of healthy and diseased chloroplasts assuming the healthy chloroplast to be a cap of a sphere, and the infected chloroplast a sphere. The means of measurements on 120 healthy and 120 diseased chloroplasts were as follows: Vol. of healthy = 48 \( \mu m^3 \); vol. of virus infected = 75 \( \mu m^3 \); surface area of healthy = 88 \( \mu m^3 \) and of virus infected, 86 \( \mu m^3 \). We further calculated that the area of membrane involved in vesicle formation would be 20 to 80% that of the healthy chloroplast. Taken at face value, and assuming the membranes do not stretch, these figures suggest that the change in shape of the diseased chloroplast from saucer-shaped to approximately spherical uses all the existing membrane, and that vesicle formation must require the synthesis of additional membrane. This conclusion is not valid unless we assume that the amount of chloroplast membrane involved in protuberances of the ‘mobile phase’ into the cytoplasm is the same in healthy and infected chloroplasts. Quantitative evidence on this point would be difficult to obtain.

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