ULTRASTRUCTURAL FEATURES OF *BETA* LEAVES INFECTED WITH BEET YELLOWS VIRUS

J. CRONSHAW, L. HOEFERT, and K. ESAU

From the Department of Biological Sciences, University of California, Santa Barbara

ABSTRACT

A cytochemical and electron microscope study has been made of leaves of sugar beet infected with beet yellows virus. Inclusions of particles, which agree in size with beet yellows virus particles isolated by other investigators, have been localized in the ground cytoplasm, in the chloroplasts, and in the nuclei. These particles are circa 100 A in diameter and have an electron-transparent core of 30 to 40 A. Use of acridine orange, azure B, and pyronine Y has revealed that the cytoplasmic inclusion bodies, which consist wholly of the elongate particles, have a strong RNA reaction removable by RNase pretreatment. Particles observed in the chloroplasts may or may not be associated with lipid spheres. If they are, the particles are confined to the periphery of the spheres. In this position the particles are arranged tangentially and are further arranged parallel into groups which lie at various angles to one another. Within the groups the particles are regularly spaced in a three dimensional lattice. Particles located free in the stromal regions are often arranged regularly in curved rows which lie parallel to one another so that a three dimensional lattice is formed. The dispersed and compact forms of virus inclusions are described and related to the condition of the associated cytoplasm. The ground cytoplasm of cells associated with the sieve elements contains numerous ribosomes. A decrease in the number of ribosomes is concomitant with the increase in size of virus aggregations in a cell. Vesiculation of some component of the cytoplasm occurs during the period of virus replication. The vesicles are approximately 100 mμ in diameter and could be derived from the dictyosomes. At later stages of infection these vesicles collapse and convoluted membranous material appears.

INTRODUCTION

The beet yellows virus is usually transmitted by an aphid vector and is transported within the plant in the phloem. The infection does not remain confined to the phloem cells but appears also in the mesophyll where it gives rise to localized pin point clear spots, irregular chlorotic spots (flecking), and eventually to necrotic lesions. Specific intracellular inclusions, visible in the light microscope, develop in many of the infected cells. These inclusions (8) have been shown to consist of filamentous particles that agree in size and structure with particles identified by several authors as the virus particles (2, 3, 4, 12, 18, 23). The organization of the particles within the inclusion bodies varies in degree from random to apparently crystalline.

The present paper gives the results of a cytochemical study of the inclusion bodies and of an electron microscope investigation of the organization of the cytoplasm and the virus in cells at various stages of infection. Normal *Beta* leaf cells were previously described and illustrated (7).

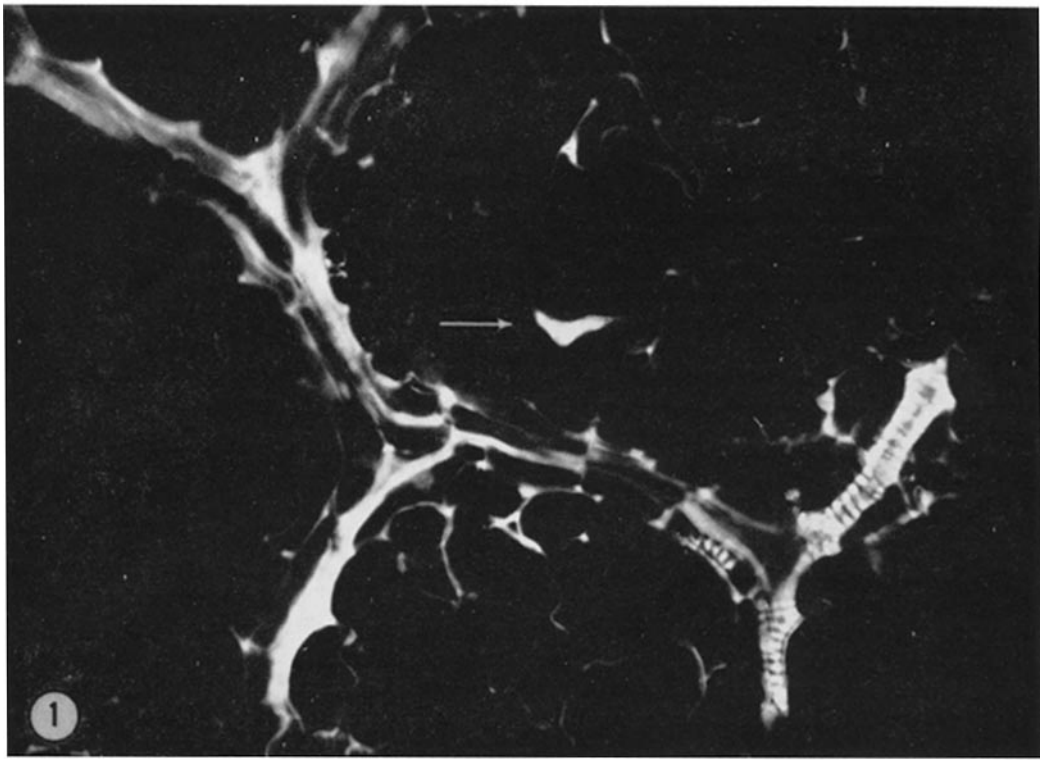


FIGURE 1 *Beta vulgaris.* Fluorescence microscope picture of a paradermal section of a leaf infected with beet yellows virus. The section was stained in acridine orange to localize the nucleic acids. The vascular bundle is highly fluorescent. The fluorescence was yellow from the lignin (right), and orange-red from the RNA (left). The RNA in the phloem cells pertains to ribosomal nucleic acid component of the cytoplasm itself as well as the virus. Large virus inclusion bodies could be identified by their characteristic forms, and all showed the orange-red fluorescence of RNA. The micrograph includes a large inclusion body somewhat removed from the vascular bundle (arrow). × 500.

MATERIALS AND METHODS

Infected and noninfected control seedlings of sugar beet, *Beta vulgaris* L., were kindly supplied by Dr. C. W. Bennett of the United States Agricultural Research Station at Salinas, California. The test plants were inoculated by the use of the aphid vector, *Myzus persicae* Sulz., with one of the virulent strains of beet yellows virus (Bennett's isolate 5, "Brawley strain").

For the cytochemical studies at the light microscope level, leaves of various ages were fixed in formalin-acetic alcohol, dehydrated in a tertiary butyl alcohol series, and embedded in paraplast. Sections were cut on a Spencer rotary microtome.

RNA Localization

AZURE B STAINING: Sections were stained with a 0.2 mg/ml azure B in 0.05 M phthalate buffer at pH 4.0 (9).

ACRIDINE ORANGE STAINING: Sections were stained with a 0.01% solution of acridine orange in 0.15 M phosphate buffer at pH 6.3. The acridine orange was excited with an osram HB0200 mercury lamp in a Zeiss fluorescence microscope (11).

PYRONIN STAINING: Sections were stained in a solution of pyronine Y and methyl green in phosphate buffer at pH 5.3 (1).

Enzymic removal of RNA was accomplished by incubating duplicate slides in a solution of 0.2 mg RNase/ml at pH 6.0 for 2 hr at 40°C.

Electron Microscopy

Specimens were cut from leaves of various ages, from infected and noninfected plants, and fixed in 3% glutaraldehyde buffered in pH 7.0 phosphate buffer. After washing for 3 hr in phosphate buffer, the specimens were post fixed in 2% phosphate-buffered osmium tetroxide. The material was dehydrated through a series of acetone solutions and embedded in Epon epoxy resin. Sections were cut with

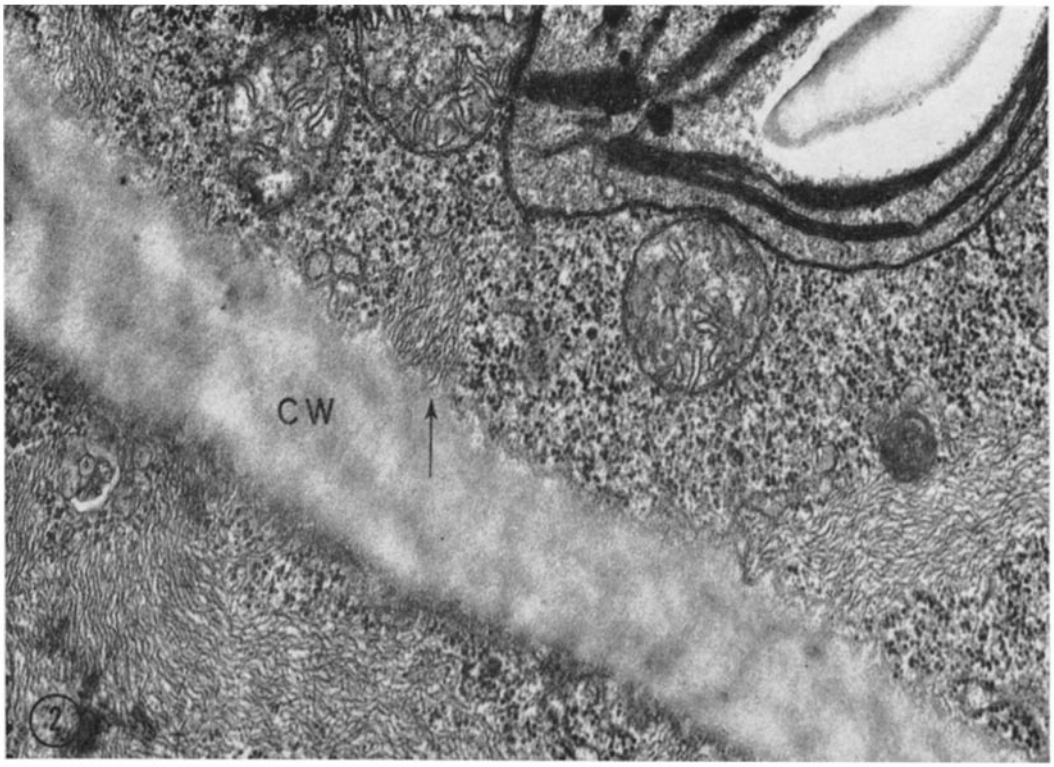


FIGURE 2 *Beta vulgaris.* Electron micrograph of a portion of two cells from the vascular bundle of a leaf infected with beet yellows virus. The cells contain ribosomes and groups of virus particles. One of the two cells also contains mitochondria and a chloroplast. Where the virus particles are near the plasma membrane, many of them are oriented perpendicular to the cell surface. In certain areas the virus particles appear to be penetrating the cell wall (arrow). *CW*, cell wall. × 38,000.

a diamond knife, stained with uranyl acetate and lead, and viewed and photographed with a Siemens Elmiskop I.

RESULTS

RNA Localization

The distribution of RNA in the infected leaves was studied by staining with azure B, acridine orange, and pyronine Y. Acridine orange, as an RNA stain, would be expected to stain normal cell components (nucleoli, ribosomes) and virus material. In the section shown in Fig. 1, orange-red fluorescence appeared in the phloem parenchyma cells of the vein (left), while yellow fluorescence, characteristic of lignin, was present in the xylem (right). Orange-red fluorescence was present also in the inclusion body seen at some distance from the vein in a mesophyll cell in Fig. 1 (arrow). The orange-red fluorescence distributed as in Fig. 1 was not present in sections treated with RNase and stained with acridine orange. Presence of RNA in normal cell components and virus inclusion material was indicated also by staining with azure B and pyronine Y. The stainable material was absent after RNase enzyme treatment.

In material stained with acridine orange, intense fluorescence appeared in necrotic areas of the leaf. The necrotic areas continued to fluoresce after RNase treatment.

Electron Microscopy

As was presented in the first paper of this series (8), the beet yellows virus located in the cytoplasm of infected cells may occur in a more or less completely dispersed state or in the form of compact inclusion bodies, some of which are so large that one body fills the cell. The present paper deals with the dispersed and the compact forms of virus inclusions and relates them to the condition of the

associated cytoplasm. Inclusions were found also in chloroplasts and nuclei. Those in the chloroplasts may or may not be virus inclusions. Some of those found in the nuclei were virus particles; others were unidentified inclusions.

CYTOPLASMIC INCLUSIONS: The majority of the cytoplasmic inclusions have been observed in nucleate cells associated with the phloem sieve elements. Typically the ground cytoplasm of these cells contains numerous ribosomes which are closely packed (Fig. 2). The inclusions consist of aggregations of elongate virus particles which may be observed in small groups or compact bodies, in some regions of which the particles are organized. In the previously described larger inclusion bodies, the particles are also arranged with differing degrees of order (8). Identifiable cytoplasmic components of cells are usually excluded from the aggregations of virus particles although small groups of ribosomes may be present (Fig. 4). The number of ribosomes decreases concomitantly with the increase in size of virus aggregations in a cell.

In some electron images the virus particles appear to be arranged in groups with the particles oriented perpendicular to the plasma membrane, as is illustrated in Fig. 2. This view shows portions of two cells in which the virus particles occur in groups scattered throughout the cytoplasm. Where the groups are near the cell surface, many of the individual particles are oriented perpendicular to the wall and some appear to be penetrating the wall. At these regions the plasma membrane is not evident. The plasma membrane is discernible as a shadow along the wall in the portion of the cell shown in Fig. 5. The virus particles forming several groups are oriented at right angles to the plasma membrane and seem to be attached to it.

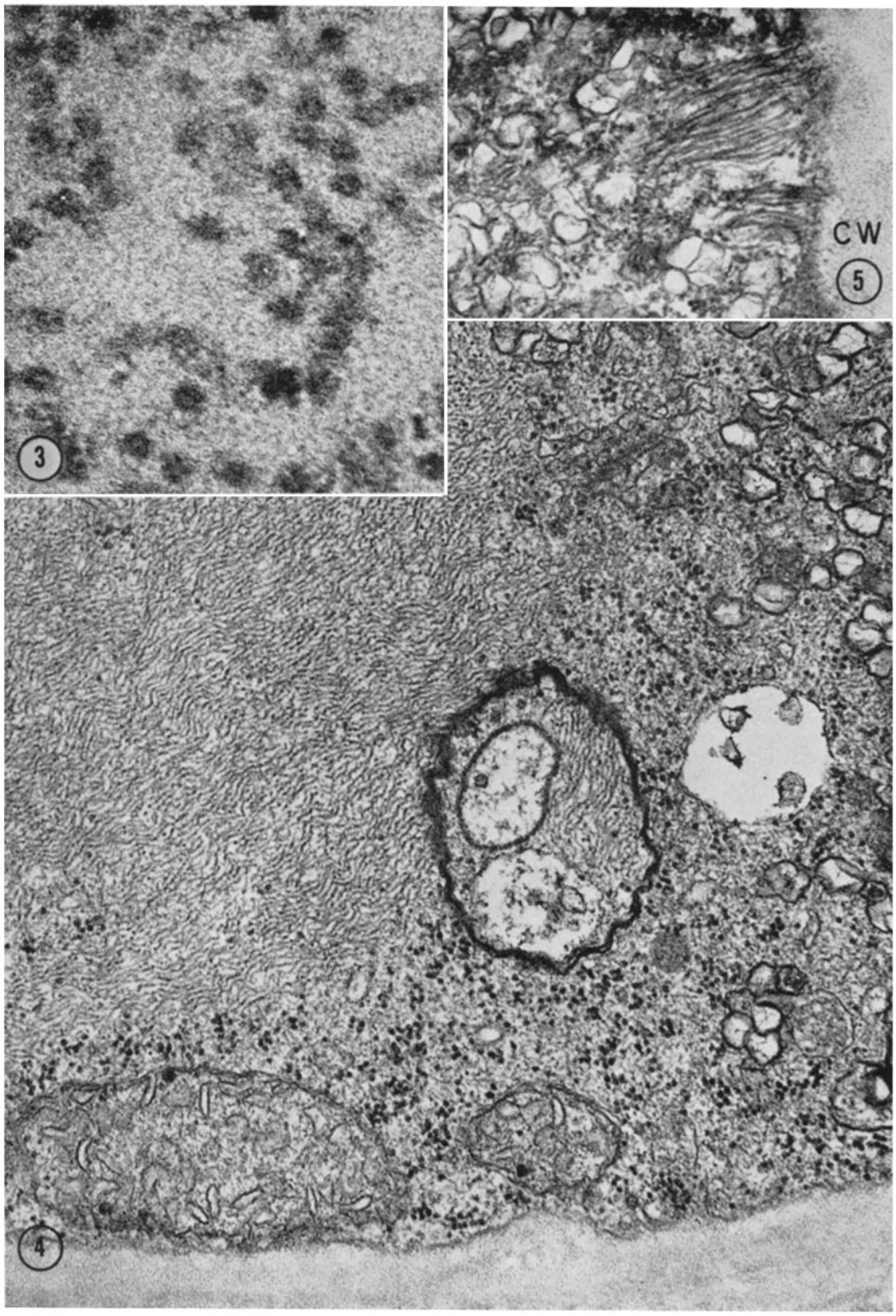
The beet yellows virus particle is known to be a flexuous rod (cf. 8). The curved and contorted form of many of the particles (Fig. 4) may be the cause for the apparent low degree of crystallinity of the aggregates. The mean diameter of the rods in the present micrographs is about 100 A. Many of the electron-opaque particles that are cut transversely appear to have an electron-transparent core of the order of 30 to 40 A (Fig. 3). These dimensions agree with those obtained by Horne, Russell, and Trim (12) and Russell and Bell (23) who measured isolated beet yellows virus particles treated according to negative staining techniques.

A characteristic feature of infected cells is a vesiculation of some component of the cytoplasm. The majority of the vesicles are circa 100 mμ in diameter and contain small fibrils which often radiate from an electron-opaque center (Figs. 4 and 6). Larger vesicles, which may contain particles identical in structure with those of the virus inclusions, are also present (Fig. 4, structure with heavily stained membrane). The 100 mμ vesicles are often arranged in characteristic groups which, in many electron images, appear to have a bounding, single-layered membrane (Fig. 6, arrows). These vesicles resemble those associated with the dictyosomes and possibly originate as such. At the time that the 100 mμ vesicles are formed, the mitochondria, plastids, dictyosomes, and nuclei are intact so that the vesicles are presumably not degeneration products of these organelles. At some later stage, however, these organelles do degenerate although, as evidenced

FIGURE 3 *Beta vulgaris.* Section of a portion of a virus inclusion. Some of the virus particles are cut transversely and can be resolved into an electron-opaque outer region and an electron-transparent core. × 400,000.

FIGURE 4 *Beta vulgaris.* Section of a portion of a cell from a leaf infected with beet yellows virus. The cell has a large aggregation of virus particles with a few ribosomes included among them. The cytoplasm contains mitochondria and ribosomes, both apparently normal, and numerous vesicles. Many of the vesicles are about 100 mμ in diameter. The majority of these vesicles contain fine fibrils. This figure also shows a large membrane-bounded vesicle containing virus particles. There is no limiting boundary between the virus inclusion and the cytoplasm. × 45,000.

FIGURE 5 *Beta vulgaris.* Section of a portion of a cell infected with beet yellows virus. Groups of virus particles appear to be attached to the plasma membrane and are oriented perpendicular to it. *CW*, cell wall. × 52,000.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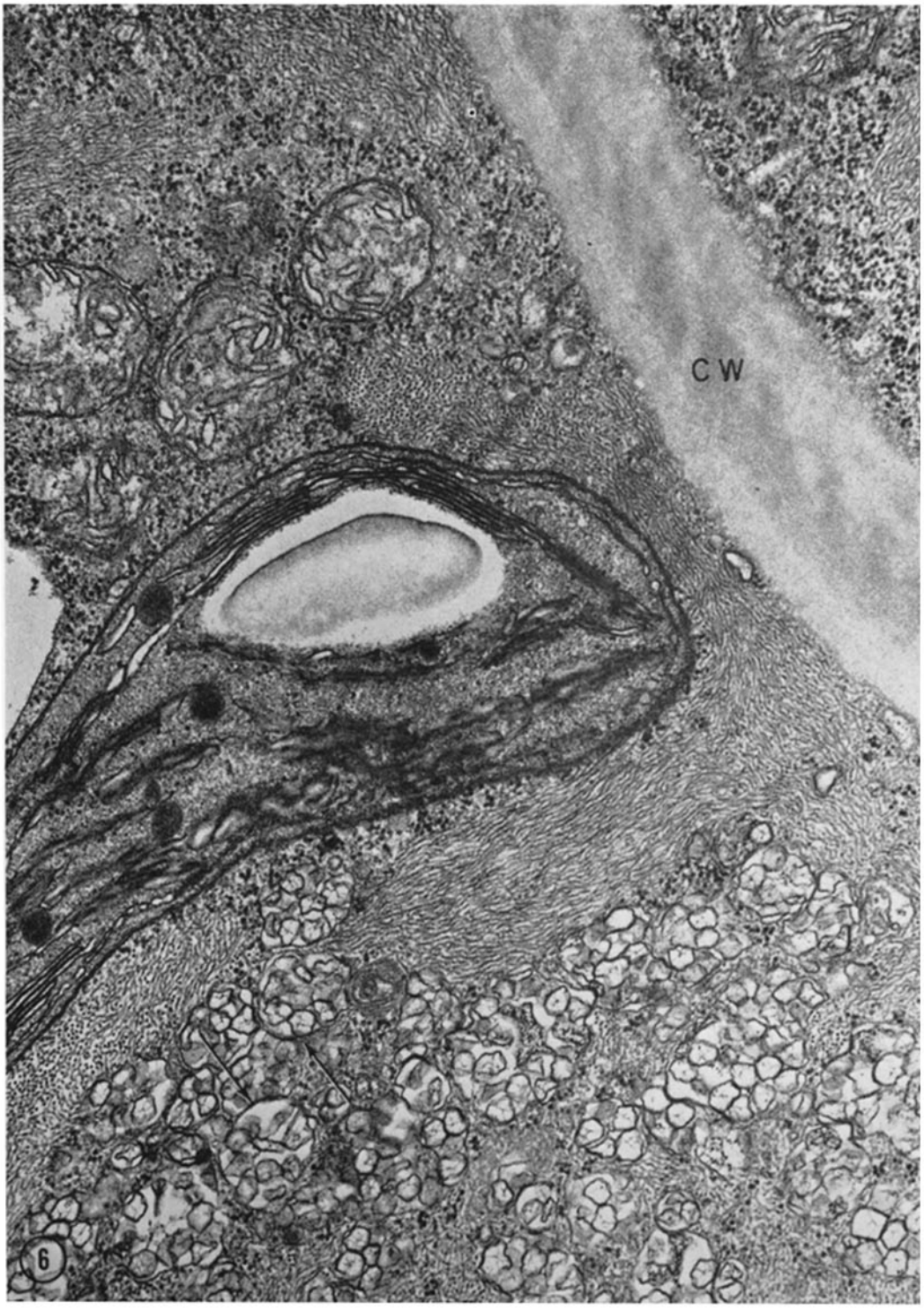

FIGURE 6 *Beta vulgaris.* Section of portions of two cells infected with beet yellows virus. Typical virus inclusions are present. The cytoplasm contains a large number of vesicles about 100 mμ in diameter. These vesicles contain fine fibrils, and many of them are arranged in groups with a membrane surrounding them (arrows). *CW*, cell wall. × 38,000.

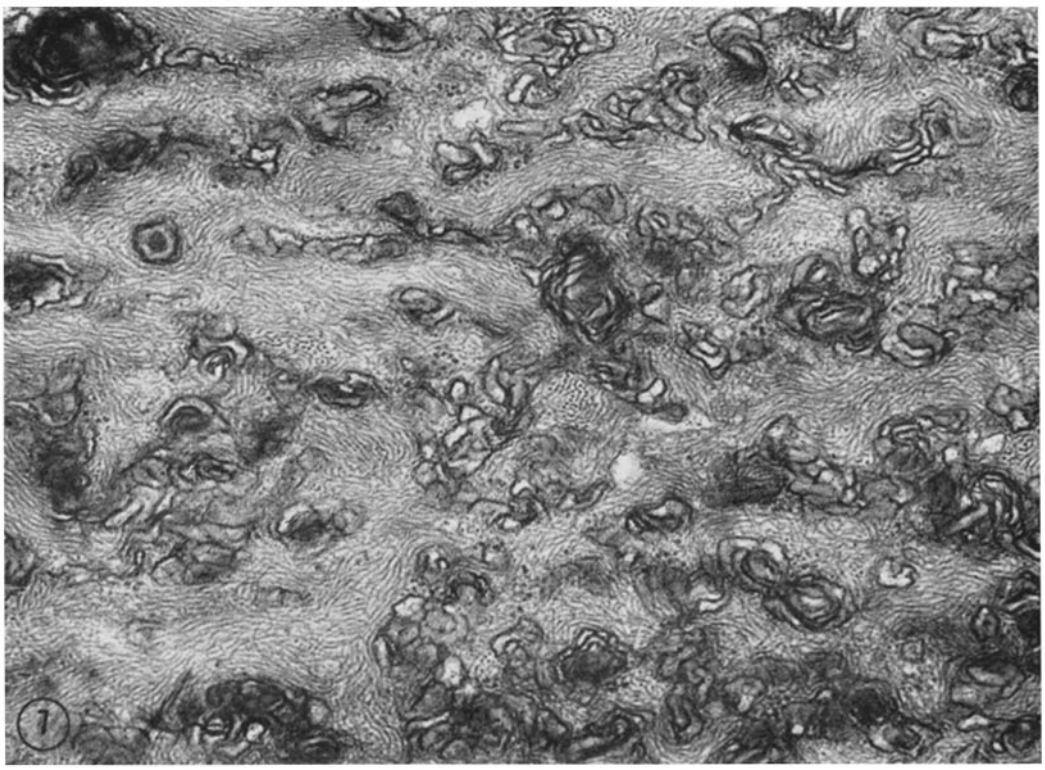

FIGURE 7 *Beta vulgaris.* Section of a portion of a leaf cell at a late stage of infection with beet yellows virus. The vesicles present in earlier stages of infection appear to have fused to give aggregations of contorted membranous material intermixed with virus particles. × 42,000.

from light microscopy, the nucleus may persist until the stage when the cell is filled with an organized inclusion. The endoplasmic reticulum is a possible source of the 100 mμ vesicles, but the cells associated with the sieve elements contain very few elements of this membrane system.

At later stages of infection, as judged by the high density of virus particles in such cells, the cytoplasmic 100 mμ vesicles collapse and convoluted membranous material appears in their stead (Fig. 7). Apparently, this membranous material is derived from the membranes of the vesicles and vesicle groups. As the density of the virus particles rises further, the vesicles disappear entirely and the amount of convoluted membranous material increases. This material was the last cytoplasmic component recognized before the cell disorganized completely and the virus particles were compacted into the gross inclusion bodies. Fig. 8 shows part of a cell with a relatively large fibrous inclusion body surrounded by amorphous material derived from the disorganized cytoplasm. Within the inclusion body the virus particles are oriented parallel to one another and show a three dimensional, ordered arrangement in certain regions.

NUCLEAR INCLUSION: As shown in Figs. 9 and 10, some inclusions have been observed in the nuclei. The cytoplasm surrounding the nucleus in Fig. 9 appeared normal with plastids, numerous mitochondria, and ribosomes. The nuclear membrane appeared intact and normal. The inclusion in Fig. 9 consists of a group of parallel fibrils which are much finer than the virus particles in the cytoplasm. These fibrils are surrounded by a region of low electron opacity, and this region, in turn, is surrounded by densely packed ribosomelike particles. Other ribosomelike particles are scattered throughout the nucleus. The inclusion in the nucleus in Fig. 10 consists of a group of particles similar to the virus particles in the cytoplasm. The particles in the nucleus may consist, therefore, of the nucleic acid core and the protein capsid. If this interpretation is correct, it implies either that the particles have migrated into the nucleus or

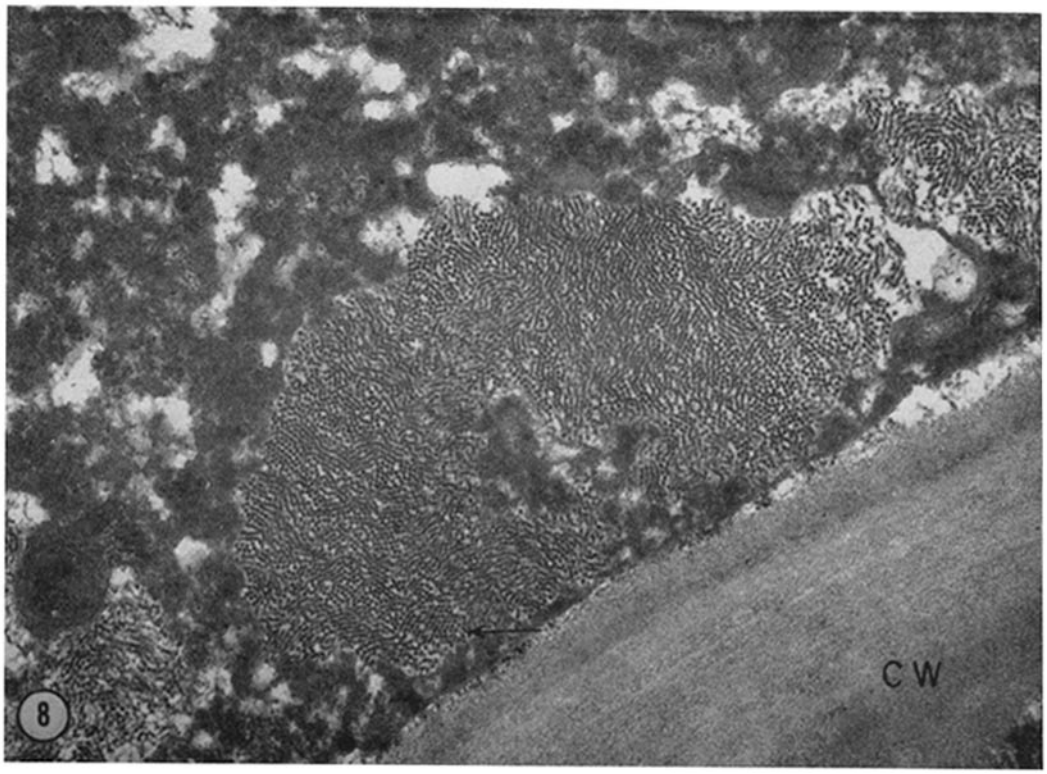

FIGURE 8 *Beta vulgaris*. Section of a leaf cell infected with beet yellows virus at a stage when recognizable cytoplasmic components are no longer evident. The virus particles form aggregations which have crystalline areas (arrows) and are surrounded by amorphous material. *CW*, cell wall. × 50,000.

that complete fabrication of the virus particles is possible within the nucleus.

These observations are of interest in view of the evidence that, in tobacco mosaic virus (TMV) infections, TMV-RNA is manufactured in the nuclei and migrates to the cytoplasm (19, 27, 28). Whether the manufacture of the protein coats takes place within the nucleus is still controversial (27, 21, 22, 10, 20, 5).

CHLOROPLAST INCLUSIONS: The chloroplasts of sugar beet have a structure typical of these organelles in higher plants, i.e. they are surrounded by a double membrane and have granal and stromal regions. The lipid inclusions are sometimes unusually large, and starch grains may or may not be present. Two types of inclusions have been observed in the chloroplasts of infected cells, both consisting of elongated particles. One type occurred free in the stromal region, and the other was associated with the lipid droplets.

Arrays of particles, often arranged in a regular manner, may be observed in the stromal regions of the chloroplasts (Figs. 11 to 13). The particles are rodlike and have an outer electron-opaque region and a central electron-transparent core (Fig. 12). The mean diameter of the particles is 100 A and of the core 30 to 40 A. The particles are arranged in curved rows, and these lie parallel to one another (Fig. 13) so that a three dimensional lattice is formed. Fig. 13 suggests that alternate rows of particles are arranged at an angle to one another. The almost solid lines, however, show the cross-sectional outlines of the component particles and indicate that this angle is small.

Particles similar to those found in the stroma are observed associated with the lipid droplets of the chloroplasts. These particles also have a mean diameter of 100 A and contain an electron-transparent core 30 to 40 A. Sections near to the center of the lipid sphere (Figs. 14, left, and 16) show that the particles are confined to a peripheral layer. Sections, which include the surface of a lipid

sphere (Figs. 14 and 15), show that most of the particles are arranged tangentially, i.e. with their long axes parallel with the surface of the droplet. The tangentially oriented particles are further arranged parallel into groups which lie at various angles to one another. Within the groups the particles are regularly spaced in a three dimensional lattice (Fig. 16, arrow).

DISCUSSION

Particles that agree in size and morphology with isolated beet yellows virus particles (2–4, 12, 18, 23) have been localized in the ground cytoplasm, in the chloroplasts, and in the nuclei of cells of sugar beet plants infected with beet yellows virus. These particles are 100 A in diameter and have an electron-transparent core of 30 to 40 A. RNA staining procedures have revealed that the cytoplasmic inclusion bodies, which consist wholly of the elongate particles, have a strong RNA reaction removable by RNase pretreatment. It seems reasonable to assume that these particles are indeed the virus. One might postulate that the particles in the chloroplasts also are viral in nature, even though we have not as yet been able to demonstrate the nucleic acid content of the chloroplast inclusions. Another point against this postulate would be that the characteristic arrangement of the particles in the chloroplasts has not been seen in the ground cytoplasm and it is possible that the

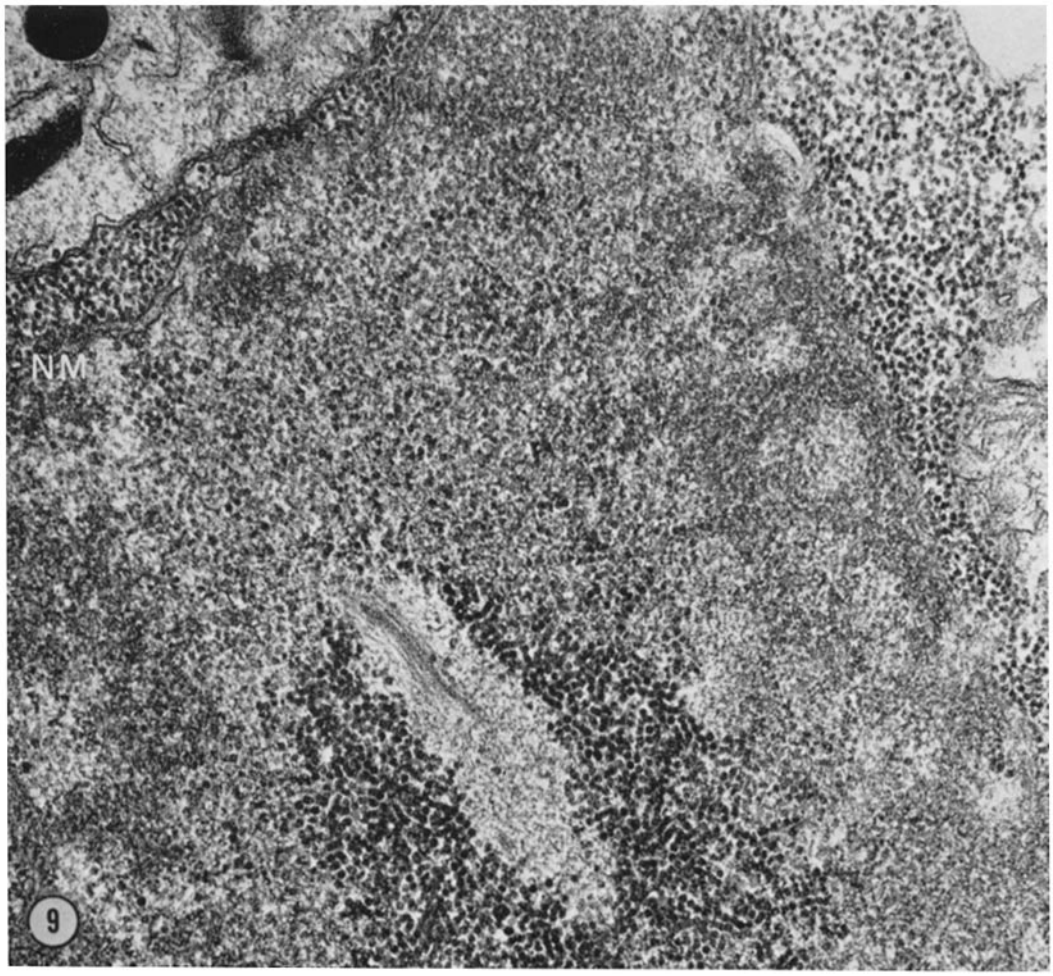

FIGURE 9 *Beta vulgaris.* Section of a nucleus from a leaf cell infected with beet yellows virus. The nucleus has an inclusion of fine fibrils which have a parallel arrangement. These are surrounded by a clear region which, in turn, is surrounded by a region containing ribosomelike particles. *NM*, nuclear membrane. × 50,000.

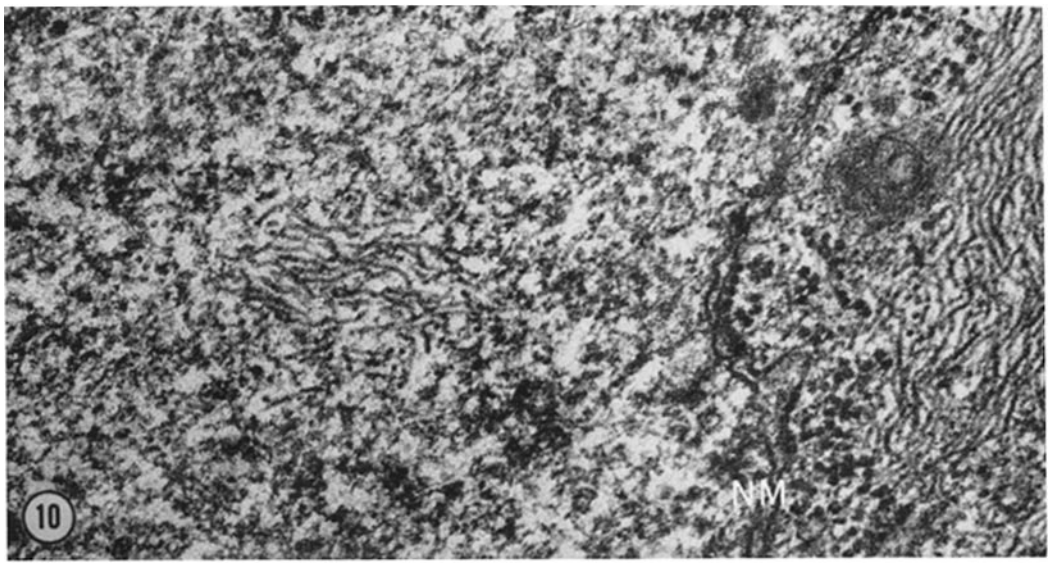

FIGURE 10 *Beta vulgaris.* Section of a portion of a nucleus (left) and some adjacent cytoplasm from a leaf infected with beet yellows virus. Complete virus particles are evident within the nucleus. *NM*, nuclear membrane. × 67,000.

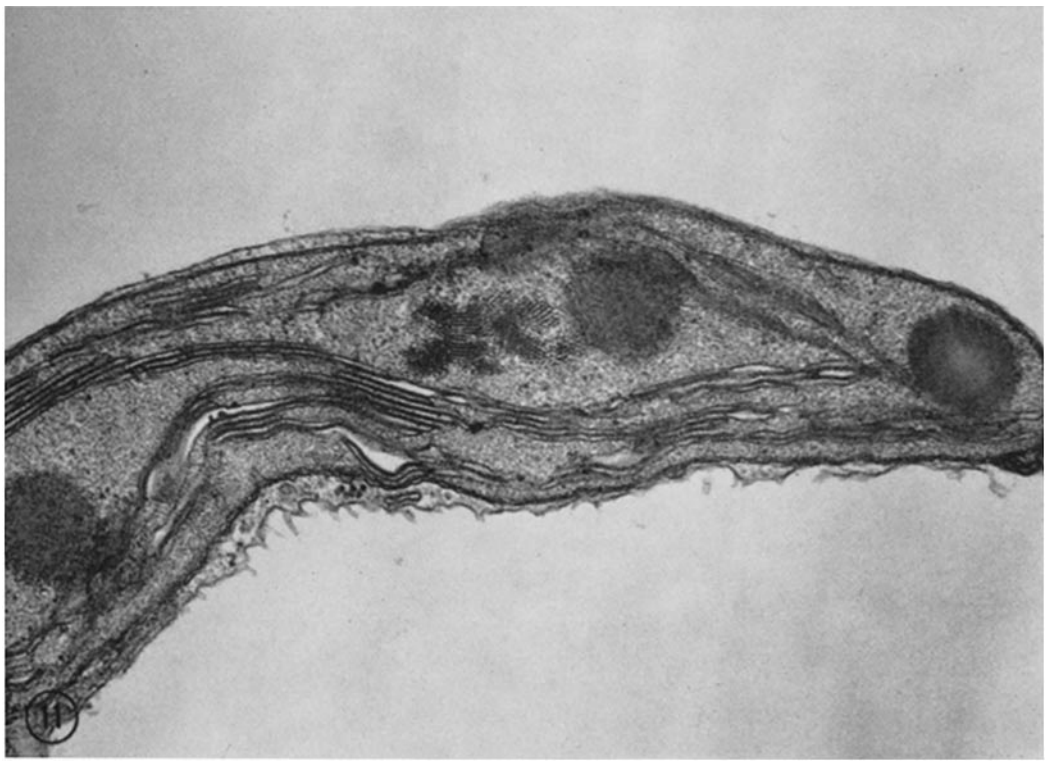

FIGURE 11 *Beta vulgaris.* Section of a chloroplast from a mesophyll cell of a leaf infected with beet yellows virus. The chloroplast contains an inclusion of viruslike particles and large lipid bodies in the stromal region. × 42,000.

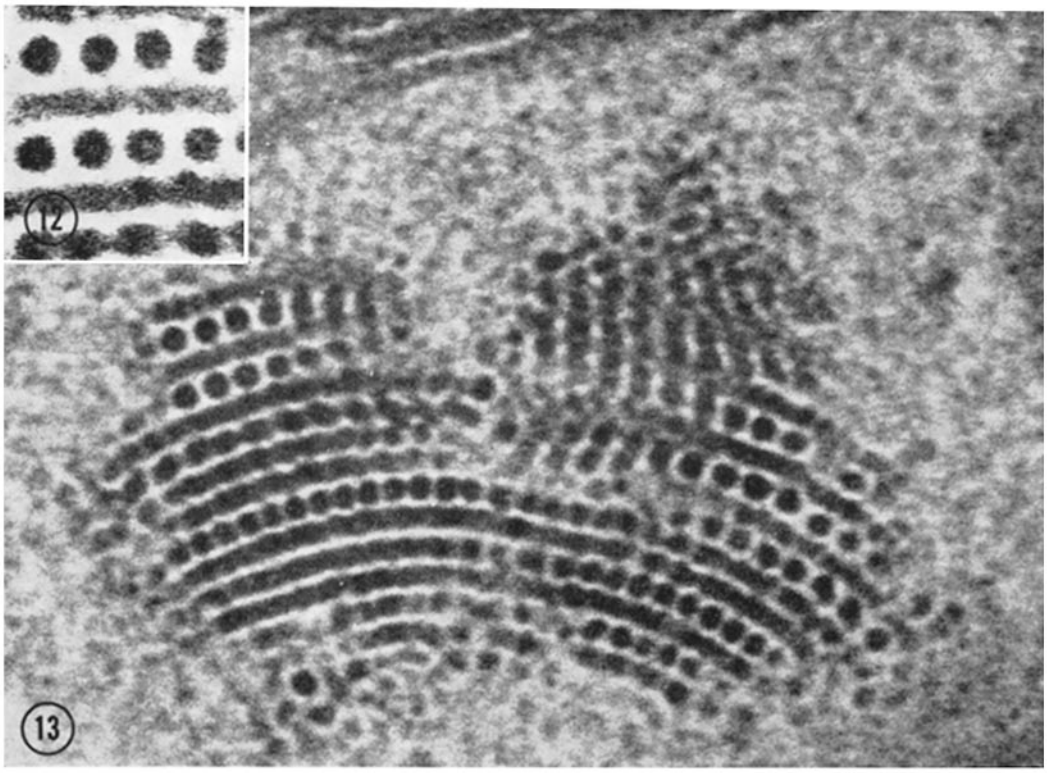

FIGURE 12 *Beta vulgaris.* High magnification micrograph of a part of the chloroplast inclusion shown in Fig. 13. × 525,000.

FIGURE 13 *Beta vulgaris.* Section of an inclusion from the stroma of a chloroplast of a mesophyll cell infected with beet yellows virus. The inclusion consists of many rod-shaped particles. The particles are arranged in curved rows oriented parallel to one another. In transection the particles are electron-opaque with an electron-transparent core. × 310,000.

particles have been formed by the chloroplasts as a result of the virus infection.

The observation of particles in the chloroplasts is of interest in view of the yellowing symptoms of the disease and of the possibility that the replicating machinery of the plastids is being used by the virus. TMV particles have been observed apparently in the chloroplasts of cells of tomato (26). However, these particles were enclosed in a vacuole and it was not ascertained whether the vacuoles were enclosed in the plastid or were cupshaped invaginations of cytoplasmic vacuoles. Milne (17) reported that similar inclusions in the chloroplasts of palisade cells in tobacco leaf were invaginations open to the cytoplasm. In TMV-infected tobacco, Kolehmainen et al. (13) could not unequivocally identify in the plastids any inclusions that resembled the presumed virus material observed in other parts of the cell.

Leyon (15, 16) observed filamentous particles associated with fragments of chloroplasts in sap from beet infected with beet yellows virus and suggested a possible relationship between these particles and the chloroplasts. Leyon (16) suggested that formation of the virus takes place in the stroma of the chloroplasts. In the present study, inclusions in the chloroplasts of infected beets have been shown to consist of particles similar in size and morphology to particles observed in the cytoplasm and to particles isolated from infected plants by other authors. Arrays of particles similar to the ones observed in the stroma of the chloroplasts of beet infected with beet yellows were observed by Schnepf (24) in *Passiflora* chloroplasts.

Later, Schnepf and Brandes (25) examined several *Passiflora* plants of different species and varieties and found that all contained presumed virus particles, but only in the cytoplasm. The authors also examined the sap from the plants under study and observed particles of the same diameter as that of the particles included in the cells. It is possible that the inclusions observed in the plastids (24) of *Passiflora* were also associated with viral infection.[1] In beet yellows-infected beet leaves, many of the particles are arranged around the lipid spheres of the chloroplasts. This association may be biological or may be explainable in physical terms. The lipid spheres would tend to "gather" the viral rods coated with lipophilic protein if these rods and spheres moved about in the chloroplasts as a result of normal chloroplast movements.

The observations of Engelbrecht and Esau (6) of inclusions with a regular crystalline pattern in the chloroplasts of sugar beet plants infected with beet yellows virus or western yellows virus could not be correlated with any structure which pertained to the virus infection. Crystalline inclusions have been observed by us in the chloroplasts of both infected and uninfected control plants.

The occurrence of virus particles oriented with respect to the cell surface is of interest in a consideration of cell to cell movement of the virus. In a study of wheat plants infected with wheat streak mosaic virus, Lee (14) observed platelike inclusions oriented approximately in a direction perpendicular to the surface of the plasma membrane in places where plasmodesmata occurred. In the published micrographs, the particles composing the plates appear to be attached to the plasma membrane. It seems likely that cell to cell movement of virus material can take place via the plasmodesmata. From the observations of beet cells infected with the beet yellows virus, one could assume also that the virus can cause a breakdown in the plasma membrane and penetrate the cell wall material, at least partially, as intact particles.

[1] *Note added in proof.* Hyde et al. (30) consider that the material illustrated by Schnepf (24) may be phytoferritin. Similar material interpreted as phytoferritin was illustrated in plastids of *Acer pseudoplatanus* by Catesson (29).

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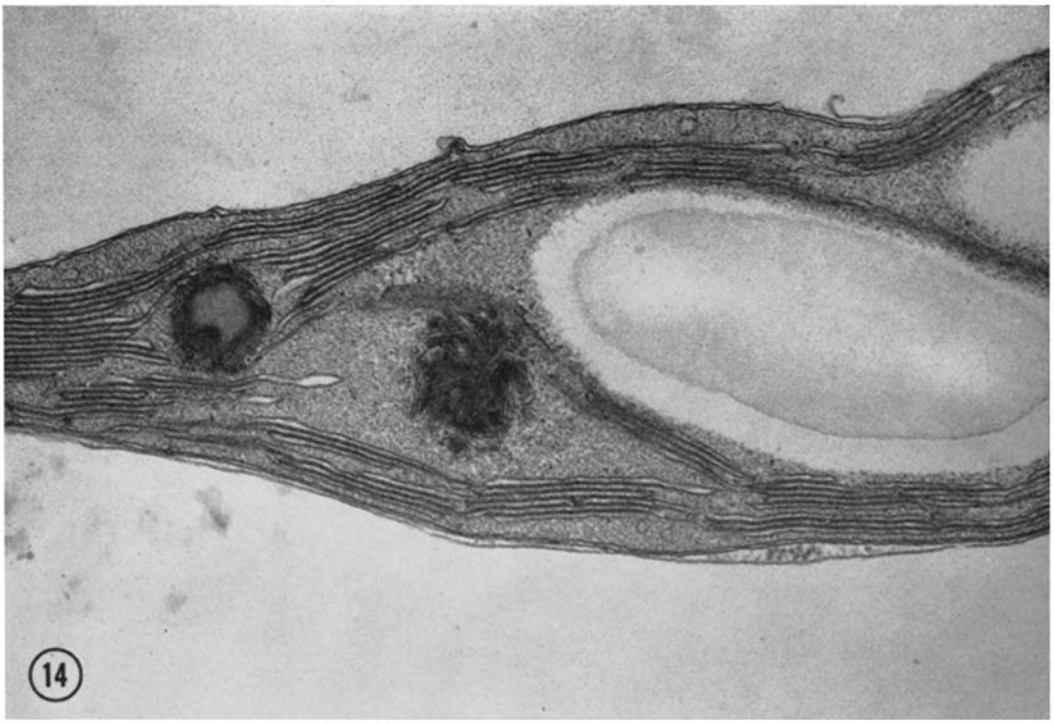
FIGURE 14 *Beta vulgaris.* Section of a portion of a chlorolpast with inclusions from a leaf infected with beet yellows virus. The inclusions consist of lipid globules covered with presumed virus particles. One inclusion is seen in transection; the other is sectioned near its surface. × 42,000.

FIGURE 15 *Beta vulgaris.* Higher magnification micrograph of the surface of a chloroplast inclusion from a leaf infected with beet yellows. The particles show a considerable degree of organization on the surface of the lipid sphere. × 176,000.



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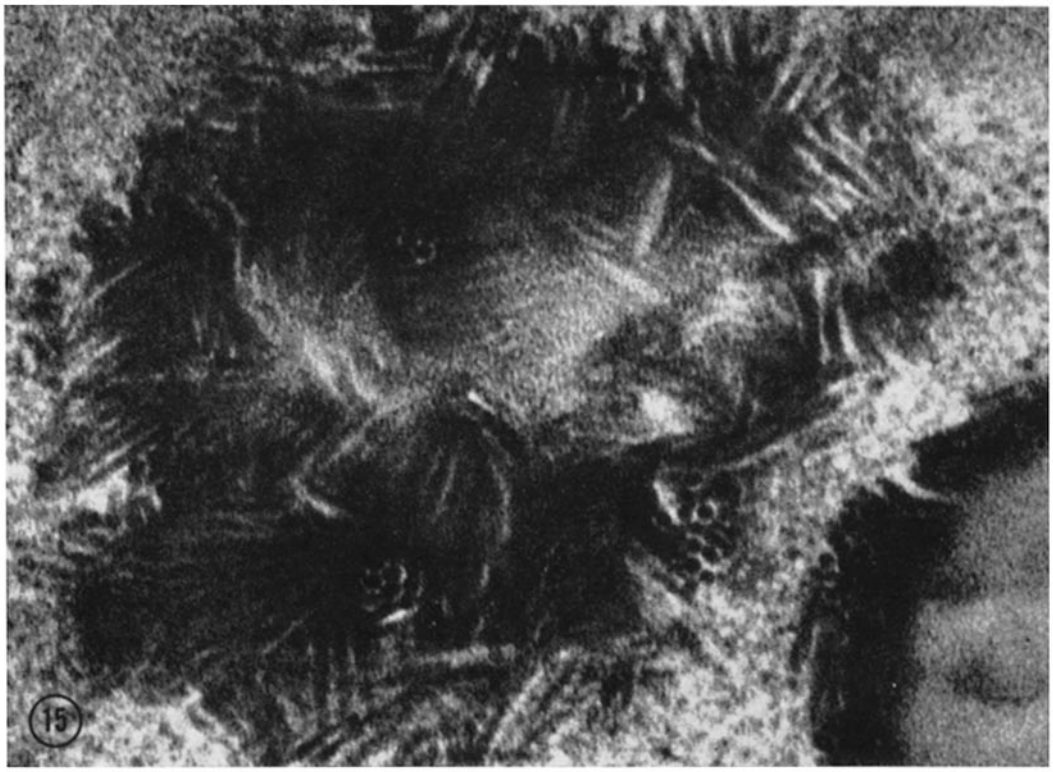

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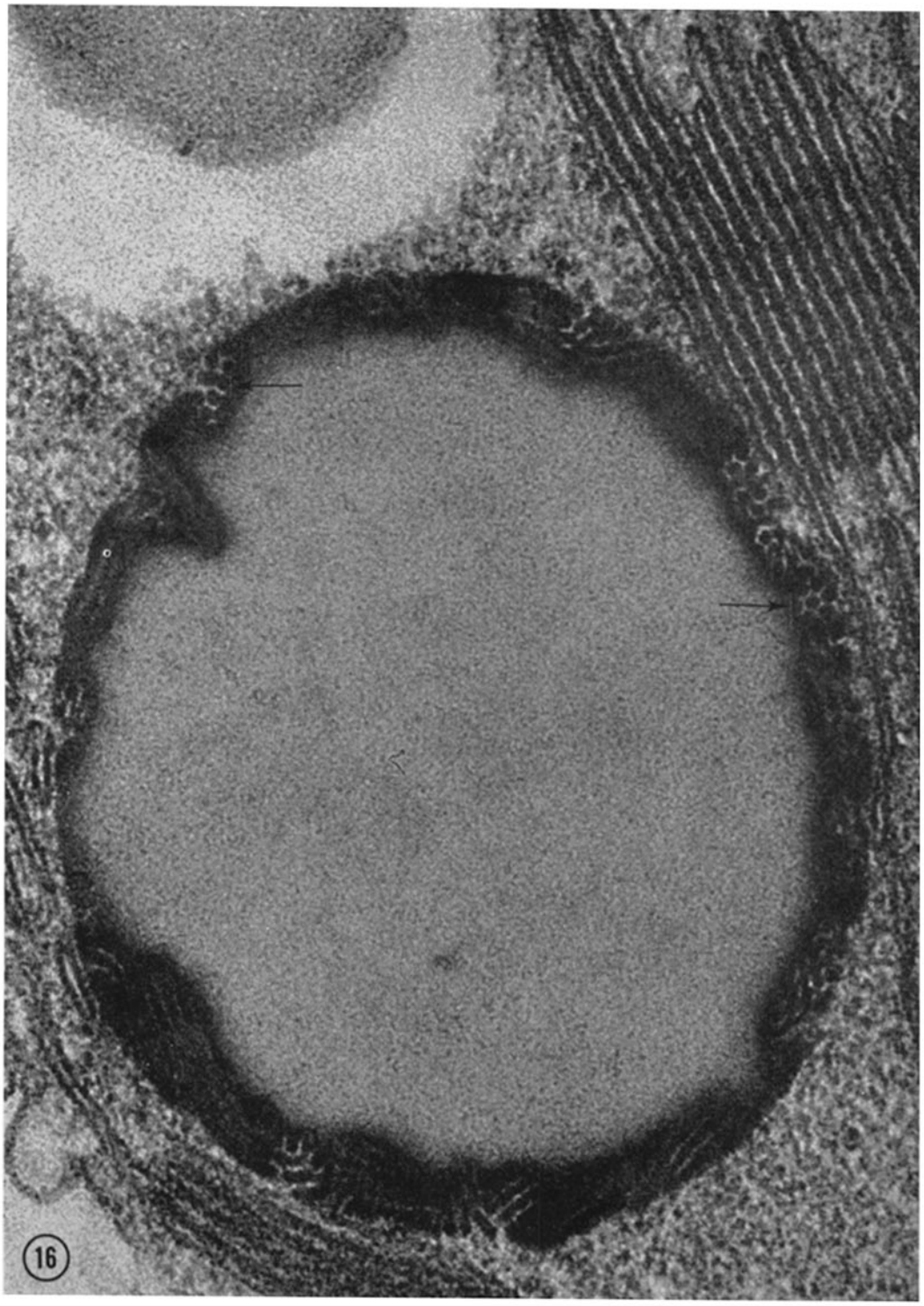

FIGURE 16 *Beta vulgaris*. Beet yellows-infected leaf. Higher magnification micrograph of a transection of an inclusion, a lipid globule, with particles attached to its surface. The core appears structureless and the presumed virus particles are confined to a narrow peripheral region. In transection, the particles appear electron-opaque with an electron-transparent core. The particles within the groups are regularly spaced in a three dimensional lattice (arrows). × 162,000.

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