# ELECTRON MICROSCOPY OF APHID-TRANSMISSIBLE AND NONAPHID-TRANSMISSIBLE VARIANTS OF PEA ENATION MOSAIC VIRUS IN PEA APHID, ACYRTHOSIPHON PISUM (HARRIS), AND GARDEN PEA, PISUM SATIVUM L.

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YA-CHU JUDY KAO
1974

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# ABSTRACT

## ELECTRON MICROSCOPY OF APHID-TRANSMISSIBLE AND NONAPHID-TRANSMISSIBLE VARIANTS OF PEA ENATION MOSAIC VIRUS IN PEA APHID, *ACYRTHOSIPHON PISUM* (HARRIS), AND GARDEN PEA, *PISUM SATIVUM* L.

By

Ya-Chu Judy Kao

Comparative studies were made between aphid transmissible (T) and nonaphid transmissible (NT) pea enation mosaic virus (PEMV) variants in their plant host, *Pisum sativum* L., and in the pea aphid, *Acyrthosiphon pisum* (Harris), by using ultrathin sectioning, negative staining and particle-counting techniques of transmission electron microscopy.

High concentrations of virions (ca. 24-28 nm) were found in infected pea tissue collected 10 days after mechanical inoculation either scattered in the nuclei, vacuoles and cytoplasm or crystalized in the cytoplasm. Virion aggregates were found in the nuclei, central vacuoles and cytoplasm of NT-PEMV infected leaves but not the T-PEMV infected tissues. Vacuolelike membranous structures, ruptured chloroplasts and nuclear vesiculations in association with virus infection were found in the infected cell cytoplasm. X-tubules were observed in the cytoplasm of NT-PEMV but not in T-PEMV infected leaves either scattered individually or formed into aggregates. These phenomena did not appear in healthy pea tissue.

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Two types of particles were observed in highly purified preparations from infected plant tissue. One type had a distinct hexagonal outline while the other was relatively electron opaque and spherical in shape. The size measurements of each particle type in each virus preparation gave diameters of 24.4 ± 1.6 nm and 29.8 ± 2.1 nm in T-PEMV preparation and 27.4 ± 1.7 nm and 28.8 ± 1.6 nm in NT-PEMV preparation for hexagonal and spherical particles, respectively. The ratio of these two types of particles varied in these two purified preparations.

Particles of PEMV were commonly found in the midgut of the pea aphids. However, virions could not be located with certainty in other tissues or organs. In a few cases, viruslike particles were found in hemocytes and degenerated fat body cells of T-PEMV infected aphids but did not occur in NT-PEMV infected cells. In the midgut, virions mostly accumulated around the margin or in the central region of the food material in gut lumen. Virions also were found in the microvillous border and appeared more frequently in T-PEMV than NT-PEMV infected adults. Viroplasmlike and membrane bound structures were the detectable structures associated with viral infection in the midgut epithelial cells. They were found more frequently in T- than in NT-PEMV infected tissues and more in adults than in nymphs.

PEMV viral densities in the viruliferous aphid midgut lumen were determined in specimen samples obtained from various experiments, i.e., the treatment combinations of acquisition access periods (AAP's, 1 vs 5 days), holding periods (0 vs 3 days) and vector life stages (adult vs

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By

Ya-Chu Judy Kao

A THESIS

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# TABLE OF CONTENTS

Table

# LIST OF TABLES

Figure

1. A midgut
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# LIST OF FIGURES

Figure | Page

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# INTRODUCTION

Pea enation mosaic virus (PEMV) is one of the persistent, circulative aphid-borne viruses. Extensive studies have been conducted on PEMV not only because it is economically important in California, Wisconsin, New York and some of the European countries but also a suitable tool for studying the vector-virus relationship of persistent aphid-borne viruses since many of its transmission characteristics have been defined. In addition, vector-virus specificity of PEMV has been demonstrated between the virus and vector species and also between virus isolates or variants and vector biotypes. An understanding of the mechanism of vector-virus specificity should enhance the development of efficient control methods.

It has been reported that the propagative potato yellow dwarf and wound tumor viruses lose their ability to be transmitted by their leafhopper vectors to various degrees after repeated replication in plant hosts without passage through their vectors (Black, 1953; Wolcryz and Black, 1957; Black _et_ _al_., 1958; Whitcomb and Black, 1969; and Liu _et_ _al_., 1973). By the infectivity assay technique on vector cell monolayers, Liu _et_ _al_. (1973) suggested that loss of infectivity is due to accumulated mutations in the plant host, or to mutation followed by selection in the plant when no counterselection occurs by passage through the vector. Pea enation mosaic virus (PEMV) probably

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is the first aphid-borne persistent virus which lost its aphid transmissibility after long duration of mechanical inoculation (Tsai and Bath, unpublished). However, the mechanism for loss of transmissibility is still unknown. So far, only preliminary characterization studies of nonaphid transmissible PEMV strains has been reported (French, 1973). It is hoped that further information of vector-virus specificity will be achieved through studies of the fate of aphid-transmissible (T) and nonaphid-transmissible (NT) PEMV variants in infected pea plants and viruliferous pea aphids.

It was the objective of this study to: (1) determine the fate of T- and NT-PEMV variants and compare the viral density in midgut lumen of both nympth and adult aphids which were given various acquisition access periods on virus source plants and holding periods on noninfected broad beans; (2) localize T- and NT-PEMV variants in infected pea leaf tissues and compare the size and shape of virions in the highly purified preparations.

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# REVIEW OF THE LITERATURE

Mechanism of plant virus transmission by insects. Several orders of insects are involved in the spread and development of plant virus diseases. Among them the Homoptera is the most important, particularly the leafhoppers (Cicadellidae) and the aphids (Aphididae). The mechanism of virus transmission by these insects has been reviewed by several researchers (Bawden, 1943; Sylvester, 1962, 1969b; Black, 1962; Maramorosch, 1963; Bath, 1964; and Harris, 1971).

Watson and Roberts (1939) first classified vector-virus relationships into two categories, the nonpersistent which were usually lost by the vector after a short period of time (mins-hrs), and the persistent which were retained for longer periods (days-weeks). However, there was no established dividing line between these two categories. Sylvester (1956) added another category, the semipersistent, to include those which had relatively brief period of virus retention but was considerably longer than that of nonpersistent viruses. Kennedy *et al*. (1962) proposed a new classification system based on the fate of virus within the insect vector. In this system vector-virus relationships were termed as stylet-borne, circulative, or circulative-propagative. The stylet-borne viruses were of nonpersistent type and transmitted exclusively by aphids with the exception of rice tungro virus which is transmitted in a nonpersistent manner by its leafhopper vector, *Nephotettix impicticeps*

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(Ling, 1966); whereas the circulative or circulative-propagative viruses were the persistent types and transmitted either by aphids or leafhoppers. It was not determined whether the semipersistent viruses were stylet-borne or circulative, or both.

Nonpersistent viruses are generally flexuous and rod shaped. They produce mosaic type diseases and can be transmitted by mechanical inoculations of crude sap to the epidermis of the host plants and by aphid vectors. These viruses are able to be acquired and inoculated within a few seconds (Sylvester, 1949). A short preliminary fasting period was reported to increase the probability of acquisition (Watson, 1938) while a prolonged acquisition probe decreased the transmission efficiency. The latter phenomenon was thought to be caused by less accessible virus being present in the sub-epidermal tissues (Bawden *et al*., 1954), or by the continuous flushing and scouring activities occurring during the formation of the stylet sheath (Bradley, 1956). The nonpersistent viruses have a short virus retention period and exhibit a low level of vector specificity. Several hypotheses have been proposed to explain vector specificity (see review by Sylvester, 1962 and 1969b).

Persistent viruses are carried in the circulatory system of the vectors before being transmitted to plants via saliva. In addition to being circulative, some viruses propagate within their vector. One of the characteristics of a persistent virus is that its inoculativity can be retained transstadially. It takes a relatively long time to acquire virus from source plant (ca. 1-2 hr). The concentration of virus acquired and the inoculativity of the vector are directly proportional to the length of acquisition access period on the virus source plant. This may be due to low virus titers in the plant (Simons, 1954). Inoculation of plants is accomplished by vectors in a relatively short feeding time (ca. 30 min). Pea enation mosaic virus has been reported inoculated to pea seedlings after a 1-min inoculation feeding (Bath, 1964). Transmission of persistent viruses is not affected by either pre- or post-acquisition starvation (Sylvester, 1950; Kassanis, 1952; and Simons, 1954). A definite latent or incubation period (ca. days to weeks) occurs for persistent viruses and represents the time required to move through the vector's body to salivary gland and/or to propagate in the vector. Persistent viruses have long retention periods, i.e., for days, weeks or even for the remainder of vector's life. They also exhibit a high level of vector specificity. The degree of specificity varies in different viruses (see review by Bath, 1964).

Most leafhopper-borne viruses have been shown to be propagative whereas most of aphid-borne viruses are nonpersistent, stylet-borne viruses; some are persistent, circulative but only 3 are propagative. Transovarial transmission, serial passage or heat treatment techniques have been used to demonstrate propagative nature of wound tumor, rice dwarf and potato yellow dwarf viruses in leafhopper vectors and potato leafroll, sowthistle yellow vein and lettuce necrosis yellow viruses in aphid vectors (Fukushi, 1935, 1939; Black and Brakke, 1952; Maramorasch, 1952; Black, 1953a; Stegwee and Ponsen, 1958; Fukushi and Shikata, 1963; Nasu, 1965, 1969; Sylvester, 1969a; and Sylvester and Richardson, 1969).

In addition to being acquired directly from the diseased plants by insect vectors, the persistent, circulative or circulative-propagative

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viruses have been artificially introduced into insect vectors by injection and membrane-feeding techniques. Injection of infectious plant extracts, honeydew, hemolymph or extracts from viruliferous vectors has been reported in maize streak (Storey, 1933, 1939), barley yellow dwarf (Mueller and Rochow, 1961), potato leafroll (Day, 1955; Heinze, 1955a; Harrison, 1958; and Stegwee and Ponsen, 1958) and pea enation mosaic (Schmidt, 1959; Nault _et_ _al_., 1964; Richardson and Sylvester, 1965; Schmutterer, 1969a; Sylvester, 1969b; and Clarke and Bath, 1973) viruses. Membrane-feeding, which is thought to be more closely approximate conventional transmission than does injection, has been used successfully as the infectivity assay for curly top (Carter, 1927), barley yellow dwarf (Rochow, 1960; and Rochow and Brakke, 1964), beet western yellow (Duffus and Gold, 1965, 1967, 1969; and Duffus, 1969), potato leafroll (Peters and Van Loon, 1968; and Duffus and Gold, 1969) and pea enation mosaic (Thottappilly _et_ _al_., 1972) viruses.

_Properties of pea enation mosaic virus_. The physical, chemical and biological properties of pea enation mosaic virus (PEMV) have been extensively studied. The symptomatology, host range and the aphid vector species have been reviewed (Harris, 1971) and the vector-virus relationships have been demonstrated (Heinze, 1959a, b; Hinz, 1964, 1966, 1969; Ehrhardt and Schmutterer, 1964, 1965; Nault _et_ _al_., 1964; Schmutterer and Ehrhardt, 1964; Bath and Chapman, 1966, 1968; Kvicala, 1966; Tsai, 1967; Chapman and Bath, 1968; Bath and Tsai, 1969; Kyriakopoulou and Sylvester, 1969; and Schmutterer, 1969a, b, c). PEMV has also been characterized by physical, serological and electron

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Shepherd et al. (1968) found that PEMV virion was composed of single-stranded RNA and protein, 28% and 72% of virus particle by weight, respectively. The protein coat contained about 199 amino acid residues, and had a molecular weight of 21,800.

Many researchers reported that purified PEMV preparations contained two distinct nucleoprotein components. However, Mahmood and Peters (1973) reported that the bottom (heavier) component was not always entirely free from top (lighter) component material. Discrepancies existed in the calculated sedimentation coefficients of these two components. By using a comparative method in density gradient columns, Izadpanah and Shepherd (1966) reported the values of 106 S and 122 S for the lighter and the heavier component of their isolate, respectively. Shepherd et al. (1968) obtained the values after analytical centrifugation and were of 90 S and 107 S, respectively. In other isolates, the respective values reported for these two components were 94 S and 113 S (Bozarth and Chow, 1966; Musil et al., 1970), 95 S and 115 S (Gibbs et al., 1966; Gonsalves and Shepherd, 1972), 99 S and 112 S (Hull and Lane, 1973), or 91 S and 107 S (Mahmood and Peters, 1973).

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The infectivity of the two nucleoprotein components is still uncertain. Izadpanah and Shepherd (1966), Gonsalves and Shepherd (1972) and Mahmood and Peters (1973) suggested that both PEMV nucleoprotein components were infectious and the top (lighter) component had a higher infectivity than the bottom (heavier) one. However, Bozarth and Chow (1966), Sehgal et al. (1970) and Hull and Lane (1973) reported only the bottom (heavier) component to be infectious.

The nucleoprotein components were found enclose three species of nucleic acid (RNA). The molecular weights of these nucleic acids were reported to be $1.74 \times 10^6$, $1.44 \times 10^6$ and $0.28 \times 10^6$ daltons, respectively, by Gonsalves and Shepherd (1972) while were $1.42 \times 10^6$, $1.15 \times 10^6$ and $0.20 \times 10^6$ daltons, respectively, by Hull and Lane (1973). Gonsalves and Shepherd (1972) suggested that only RNA species 2 was infectious and the function of species 1 and 3 was not known. They concluded that both nucleoprotein components contained RNA species 2 whereas the bottom (heavier) had two particle types, one containing RNA species 1 alone and the other containing RNA species 2 and 3. However, Hull and Lane (1973) found that the bottom (heavier) component of their PEMV isolate did not contain RNA species 2.

Hull and Lane (1973) assayed the infectivities of individual and mixed species of nucleic acids and nucleoprotein components by means of gel electrophoresis. They found that mixed RNA species 1 and 2 or nucleoprotein components were more infectious than they were individually, and that addition of species 3 actually reduced the infectivity. However, Gonsalves and Shepherd (1972) and Mahmood

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and Peters (1973) found no enhancement of the infectivity in mixtures of both nucleoprotein components so that they suggested that the complete genome of PEMV seemed to reside in a single virion particle.

Like potato leafroll virus (Peters, 1967) and barley yellow dwarf virus (Rochow and Brakke, 1964), PEMV has been purified from viruliferous aphid vectors (French, 1973; French _et_ _al_., 1973). The UV scanning pattern of this preparation was similar to those obtained from plant sources. The UV absorbance peak consisted of a low intensity of top (lighter) nucleoprotein component and a high intensity of bottom (heavier) component. French (1973) reported that after this aphid-source purified virus was established in host plants, it was transmitted by pea aphids with remarkably high efficiency.

Infectivity of this virus was reported to be influenced by the pH of buffer used in extraction of PEMV. According to Izadpanah and Shepherd (1966), it was generally inactivated by buffers of pH 5.0 or below and decreased considerably at pH 7.0 or above. They suggested that 0.1-0.3 _M_ acetate buffer at pH 6.0 was suitable for extraction and maintaining the infectivity. Musil _et_ _al_. (1970) obtained the highest infectivity in the extract prepared with phosphate buffer at pH 6.5.

Multiplication of PEMV in the infected peas, _Pisum_ _sativum_ L., was shown by infectivity tests (Shepherd _et_ _al_., 1968). The titer of PEMV in infected pea tissue was relatively low at the early stage of symptom development and then reached a maximum at about 10-12 days after mechanical inoculation (Shepherd _et_ _al_., 1968; and French, 1973). This phenomenon was confirmed by serological tests which showed high dilution end point and stron

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According to Izadpanah and Shepherd (1966), Shepherd (1970) and Mahmood and Peters (1973), highly and partially purified PEMV and the infected pea crude saps reacted well with absorbed antiserum in agar-gel diffusion tests and might form two specific precipitation bands. The quantity of antigen did not affect the pattern of the bands (Mahmood and Peters, 1973). Mahmood and Peters (1973) reported that both components were antigenically identical when testing by gel double diffusion, but different in their electrophoretic mobilities when testing by immunoelectrophoresis. Izadpanah and Shepherd (1966) and Mahmood and Peters (1973) also reported the occurrence of soluble antigen with a low molecular weight in sap from infected plants, which was antigenically related to, but not identical with the virus. It was found that ring precipitin tests gave a microprecipitin titer of 1:512 (Izadpanah and Shepherd, 1966).

Both nucleoprotein components in purified virus preparation from infected pea tissues were found by electron microscopy to be composed of small polyhedral particles. It was reported that the PEMV particles were hexagonal or pentagonal in profile (Mahmood and Peters, 1973). However, Musil _et al_. (1970) suggested that the structural units of the PEMV virions were not arranged into hexamers and pentamers, but seemed to form trimers on the triangular facets. Izadpanah and Shephard (1966) reported that no detectable difference in size and shape of the particles composing the top (lighter) and bottom (heavier) components.

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However, Gibbs et al. (1966) found that purified virus preparations appeared to contain two types of particles. Though both types were isometric and about 30 nm in diameter, one type appeared irregular and distorted unless previously treated with 1% formaldehyde while the other exhibited a more regular appearance. Mahmood and Peters (1973) also reported that pentagonal particles were found more frequently in the top zone fractions than in the bottom. The sizes of virus particles obtained by different researchers varied from 20 to 36 nm depending upon the technique used to prepare the specimens (Bustrillos, 1964; Shikata and Maramorosch, 1965, 1966; Shikata et al., 1965, 1966; Bozarth and Chow, 1966; Gibbs et al., 1966; Izadpanah and Shepherd, 1966; Musil et al., 1970; French, 1973; French et al., 1973; Hull and Lane, 1973; and Mahmood and Peters, 1973).

The question as to whether PEMV propagates in its aphid vector is still unanswered, although it has been reviewed extensively (Sylvester, 1969b). Transovarial and serial passage techniques are generally used by many researchers to demonstrate the propagative nature of plant viruses in their insect vectors. Experimental evidence showed the transovarial transmissible nature of rice dwarf virus (Fukushi, 1969; and Nasu, 1965, 1969), European wheat striate mosaic virus (Watson and Sinha, 1959), wound tumor virus (Sinha and Shelley, 1965) and sowthistle yellow vein virus (Sylvester, 1969a) and the serial passability of potato leafroll virus (Stegwee and Ponsen, 1958), wound tumor virus (Black and Brakke, 1952) and sowthistle yellow vein virus (Sylvester and Richardson, 1969). However, PEMV has not been shown to transovarially pa

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be transovarially passed. Attempts to successively pass PEMV from aphid to aphid by injection of hemolymph has also failed (Heinze, 1955a; Schmidt, 1959; Nault _et al_., 1964; Richardson and Sylvester, 1965; Schmutterer, 1969a; and Clarke and Bath, 1973). Since it was found by electron microscopy (Harris and Bath, 1972) that pea aphid hemolymph was a poor virus carrier, failures in serial passage does not necessarily preclude multiplication. Clarke and Bath (1973) suggested that PEMV may propagate in pea aphid but its titer in the hemolymph may not be sufficiently high for successful serial passage.

By electron microscopic investigation of the fate of PEMV in the pea aphid vector, Harris and Bath (1972) suggested that PEMV multiplication may occur in the vector. When reviewing previous researches, Matthews (1970) concluded that PEMV is in fact a propagative virus in _Acyrthosiphon pisum_ (Harris). However, most of the evidence available to date may indicate that only limited multiplication does occur in the aphid vector (Sylvester, 1969b).

NT-PEMV variant (nonaphid transmissible) used in this study was only recently derived from a T-PEMV (aphid transmissible) California isolate (Tsai and Bath, unpublished) in our laboratory and consequently only limited physical characterizations of these two variants have been completed. French (1973) reported that the NT-PEMV variant had a consistently higher nucleoprotein yield than that obtained from T-PEMV infected tissue. Like other isolates noted earlier, two nucleoprotein components appeared in either highly purified variant preparations. T-PEMV contained a much higher proportion of bottom (heavier) component the top (lighte ighter) component r sucrose-containin at the presence of mponent ratios. I eavier) component esulted in an UV-sc ith a higher propor ighter). However, y the presence or a

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By using el 966) and Shikata e irions in the cyto nations. Similar iseased plant sap than the top (lighter) whereas NT-PEMV contained a higher ratio of top (lighter) component than did T-PEMV. When resuspending virus pellets in sucrose-containing potassium phosphate buffer, French (1973) found that the presence of sucrose increased the nucleoprotein yields and the component ratios. It was observed in NT-PEMV that the level of bottom (heavier) component increased sharply but not the top (lighter) and resulted in an UV-scanning profile pattern similar to that of T-PEMV with a higher proportion of bottom (heavier) component than the top (lighter). However, the pattern of T-PEMV was not appreciably changed by the presence or absence of sucrose in the resuspending buffer.

Electron microscopic studies of PEMV in its host plant and its aphid vector. Host Plants. Pea enation mosaic virus (PEMV) is the first circulative aphid-borne virus localized by electron microscopy in plants and aphids *in situ* (Shikata and Maramorosch, 1965, 1966; Shikata *et al*., 1965, 1966; Harris, 1971; Harris and Bath, 1972). Prior to application of electron microscopy, Tsao (1962) and Tsao and Hagedorn (1962) studied the anatomical and histopathological changes in the stem and leaf of infected pea plant, *Pisum sativum* L., by light microscopic technique. McWhorter (1949, 1950, 1965) also showed nuclear changes in the infected cells.

By using electron microscopy, Shikata and Maramorosch (1965, 1966) and Shikata *et al*. (1965, 1966) were the first to find PEMV virions in the cytoplasm and the central vacuoles of pea leaf and pod enations. Similar particles were observed in dip preparations of diseased plant sap. They also found large accumulations of virions in the nuclei of bo

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Membranous structures were observed in both leaf and pod enations (Shikata et al., 1966) with PEMV virions being aligned along these structures in rows. Similar virion alignment also reported in the leaf enations caused by radish mosaic virus on Chinese white winter radish (Hooper et al., 1972).

Cytoplasmic inclusion bodies such as microcrystals (24-27 nm) were seldom found in the infected pea tissue (Shikata et al., 1966). These microcrystal formations were thought to be the result of high viral concentrations. They occurred only in the cytoplasm of PEMV infected pea leaf tissue. However, they also were reported in the nuclei of plants infected with pelargonium leaf curl virus, tomato bushy stunt virus and beet western yellow virus and in the vacuoles of artichoke mottled crinkle virus infected plant leaves (Russo et al., 1968; Esau and Hoefert, 1972; Martelli and Russo, 1972; and Russo and Martelli, 1972).

Amorphous inclusion bodies associated with virus infections have been observed in several plant-plant virus associations including papaya mosaic and ringspot viruses (Zettler et al., 1968), tobacco mosaic virus (Essau, 1968), maize dwarf mosaic virus (Langenberg and Schroeder, 1973), but not in the PEMV infected tissues.

Shikata and Maramorosch (1966) investigated the distribution of PEMV during the sequential invasion of the host cells. At an early stage of infection (chlorotic spots) by New York PEMV isolate, pea tissues showed degradation of chromatin material in the nuclei. Later, the virions almost completely occupied the nucleoli (Shikata and Maramorosch, 1965, 1966). The nuclear membrane sometimes ruptured and virions escaped into the cytoplasm. Virions in nuclei were slightly smaller than those in the cytoplasm and the vacuoles.

Due to the high density of PEMV virions located within the nuclei during the early stages of infection, Shikata and Maramorosch (1966) suggested that PEMV may multiply in the nuclei of the host cells and then dispersed into the cytoplasm.

Some cytological changes associated with PEMV infection have been observed in diseased plants. De Zoeten _et al_. (1972) found vesicles, which contained DNase-digestible fibrillar material, in the perinuclear space in the cytoplasm of parenchymatic cells and phloem elements of PEMV infected _Pisum sativum_ L. This vesiculation was thought to originate from the outer membrane of the nuclei.

_Aphid vectors_. Infection of aphid vector by PEMV has been shown directly be electron microscopy (Shikata _et al_., 1966; Harris, 1971; Harris and Bath, 1972; Seryczynska and Wegorek, 1972). Large numbers of virus particles were usually found in the lumen of mid- and hind-guts but not in the fore-gut. The virions tended to aggregate along the peripheral margins of food material. Harris and Bath (1972) found that the highest concentration of virus appeared in midgut where they presumed the first contact between the ingested virus and the absorptive tissue of the vector occurred. Virions also were found within the midgut microvillous borders and epithelial cells. Virions in the

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epithelium appeared in viroplasmlike areas or some defined structures in the cytoplasm. In very rare cases, PEMV virions were detected in nuclei of midgut cells. Virus particles also were observed in fat body and blood cells but never in epidermis, mycetomes, ovaries, tracheal system, skeletal musculature, eye tissues, nervous system and salivary glands.

Infection of salivary glands has been reported in vectors of wound tumor (Maramorosch et al., 1969), sowthistle yellow vein (Sylvester and Richardson, 1970), maize mosaic (Herold and Munz, 1965), rice dwarf (Shikata and Maramorosch, 1969), and also St. Louis encephalitis (Whitfield et al., 1973) viruses. However, PEMV has not been seen in the salivary glands of aphid vector. Harris and Bath (1972) suggested that low virus titer in the blood may give a proper explanation for the failure of finding PEMV virions in salivary glands.

Preliminary studies on ultrastructural changes associated with PEMV infection in aphid midgut were reported by Seryczynska and Wegorek (1972). They found that enlargement occurred in the canals of the vacuolar system in the cytoplasm of midgut cells and suggested that it was a pathogenic effect to the aphid vector. No effect of viral infection on the ultrastructure of the microvillous border was observed.

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# MATERIALS AND METHODS

Maintenance of virus culture and aphid colony. Two variants of a California isolate (Bath and Chapman, 1967) of pea enation mosaic virus (PEMV) were used in this study. An aphid transmissible variant (T-PEMV) was derived from the California isolate through biological transmission by pea aphids, while a non-transmissible variant (NT-PEMV) was selected through continuous sap inoculations from infected to healthy pea seedlings (Tsai and Bath, unpublished). Both variants are mechanically transmissible and the symptomatology in plants is identical.

The pea aphid, *Acyrthosiphon pisum* (Harris), used in this study was collected in East Lansing, Michigan in 1964, identified and reared on broad beans, *Vicia faba* L. in a controlled-environment chamber under conditions described by Tsai (1967).

The garden pea, *Pisum sativum* L., variety Midfreezer served as source plant for virus acquisition and as test plant for transmission trials. Source plants were chosen 10 days after virus inoculation and consisted of those which showed the most intense symptoms; the virus yield in infected peas has been shown to be at maximum titer at this stage (French, 1973). Only the apical portion of an infected plant was used as the PEMV source. The test seedlings were grown in the manner described by Tsai (1967), and pre-leaf stage plants were used for T-PEMV aphid inoculation and for NT-PEMV mechanical inoculation.

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The T-PEMV variant was maintained in pea plants by means of viruliferous insects. Pea aphid nymphs (1st instars), which are the most efficient transmitters of PEMV (Bath and Chapman, 1968) were fed on source plants for 24 to 48 hr. These aphids were then transferred to healthy pre-leaf-stage test plants. Aphids were allowed to feed on the test plants for 2 days in a controlled-environment chamber at 22°C, RH 60-70%, and 14 hr photophase. Plants were then fumigated with insecticide and moved to a greenhouse for incubation.

The NT-PEMV variant was maintained by rubbing infective crude sap onto pea seedlings. The inoculum was prepared by grinding infected pea leaf tissue in water with a sterile mortar and pestle, and then rubbed onto the carborundum-dusted young seedlings with a finger. The inoculated pea plants were then moved to a greenhouse for symptom development.

Tissue preparation for electron microscopy. At the start of each experiment, T- and NT-PEMV were concomitantly mechanically inoculated onto pre-leaf-stage pea seedlings. These inoculated seedlings were then grown under greenhouse conditions. In this way, uniform aged source plants were produced. To insure that the nymph and adult test aphids were also of uniform age, a number of mature apterous adults were collected from the aphid rearing colony and placed on healthy broad beans. The newly deposited 1st instars were then collected from these plants and reared to the designated nymphal stages which were necessary for a particular experiment. All the aphids were reared in controlled-environment chamber, 22°C, 14 hr photophase and RH 60-70%.

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To obtain viruliferous aphids for electron microscopic studies, aphids acquired T- and NT-PEMV variants as described earlier (Tsai, 1967; and Harris, 1971). Throughout the acquisition access periods (AAP), source plants were kept in the aphid rearing controlled-environment chamber. Test aphids were allowed to have AAP's from 1 to 5 days. After acquiring virus, the test aphids were removed from the source plants with a brush and either transferred to healthy broad beans for different holding periods, or processed immediately for electron microscopy.

Both whole aphids and excised organs were processed in the same manner as described by Harris (1971). Specimens were dissected in cold fixative under a dissecting stereoscope and placed into vials containing cold, fresh 6% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, or potassium dichromate-acrolein mixture (2%:3%, pH 7.35) for 3 to 4 hr. After this primary fixation, specimens were rinsed twice for 30 min with 0.1 M Sorensen's phosphate buffer at pH 7.2. Specimens were then postfixed in 1% 0.1 M phosphate buffered osmic acid for 3 or 4 hr at room temperature. After rinsing with 2 changes of buffer, specimens were dehydrated in a graded series of ethanol (25, 50, 70, 95, and 100%), 30 min in each, followed by several changes in 100% ethanol for at least 24 hr.

Specimens were infiltrated with Spurr's (1969) low viscosity standard embedding medium. The infiltration process was accomplished by gradually changing 100% ethanol dehydrating agent into 100% embedding medium, i.e., after dehydration the specimens were replaced to a 1:1

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mixture of embedding material and 100% ethanol for 4 hr, and then transferred to 75% embedding medium in absolute ethanol for 4 hr and to 95% medium for another 4 hr. Finally, the specimens were held in 100% embedding medium overnight. Throughout the dehydration and resin infiltration procedures, the specimens were kept in tightly sealed vials and subjected to continuous agitation.

The specimens were placed in fresh 100% embedding medium the following morning. To insure complete resin infiltration, the specimens were held under vacuum for about 30 to 60 min, then placed in a desiccator for 4 hr before being transferred to flat embedding molds. Penciled, oven-dried paper labels were placed into the molds and specimens in the molds were returned to the desiccator for an hour. Specimens were oriented to their proper positions and cured at 70°C for 2 days.

Throughout the experiments, the embedding media were prepared one day prior to conducting resin infiltration and embedding. Samples were taken from these media and polymerization was completed in an oven overnight. The hardness of the sample was checked the following morning. If the sample blocks were too brittle or too soft, the prepared media were discarded. Otherwise, they were used in that day's infiltration and embedding experiments.

Ultrathin sections of whole aphids or excised organs were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome and placed on formvar or collodion coated grids. These sections were stained for 2 hr in freshly prepared, filtered and saturated uranyl acetate in a 1:1

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solution of 25% methanol and 70% ethanol, followed by rinsings with 1:1 absolute methanol-ethanol mixture and then distilled water. Next, the sections were stained for 10 min in 0.4% lead citrate prepared in boiled, double distilled water. The pH of the stain was adjusted to be less than 12 by gradual addition of 10 N NaOH. After staining, sections were rinsed with 0.02 N NaOH and then distilled water. The grids were placed in a petri dish and dried overnight.

The double stained specimen sections were examined with a Philips EM-300 transmission electron microscope (TEM). Kodak electron microscope films (3 1/4 x 4 in) with Estar thick base were used and developed with Arkay EM 410-4 Processor (Arkay Corporation, Milwaukee, Wisconsin). Contact prints were made on Ilford photographic paper and processed with an Ilfoprint Processor 951 (Photo Reproduction Materials, Inc., England).

Negative staining. For examining highly purified virus preparations and extracts of excised organs with TEM, a negative staining technique was used. Phosphotungstic acid (PTA, 2%, pH 7.0) was used as the stain. Soluble starch (0.1%) was also added to help the stain spread evenly on the film supported grids. Grids were coated with 0.25% formvar or collodion solution and after drying coated with ca. 50-100 Å of carbon using a Ladd vacuum evaporator (Ladd Research Industries).

The preparation of highly purified virus and extracts of excised organs will be described later. A small droplet of a preparation was placed on top of a carbon coated grid which was held by a pair of forceps. After about 2 min, the preparation was drained off from the edge of the grid wit

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edge of the grid with a small piece of filter paper. Only a thin layer of the preparation was allowed to remain on the grid. The grid was allowed to air dry. Next, a small droplet of PTA was added on top of the preparation layer for 30 to 40 sec and then drained off, and the grids were allowed to dry overnight before TEM investigation. Throughout the staining procedure, the grids were covered with a petri dish lid to prevent dust from settling on them.

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# EXPERIMENTS AND RESULTS

## A. Evaluation of Several Specimen Preparation Techniques

Pressure treatment. In the course of this study, it was found that resin infiltration was difficult to accomplish in whole aphid specimens. Even though appendages were removed and wounds were made intentionally in each specimen, adequate infiltration was still not achieved. The impermeability of the aphid cuticle to the resin was thought to be a possible explanation. A pressure treatment technique (Hooper and Bath, 1973) was used in this experiment in an attempt to resolve this problem.

Pea aphid, Acyrthosiphon pisum (Harris), were given an acquisition access period (AAP) on PEMV-infected pea plants. Specimens were fixed, dehydrated and infiltrated with a series of Spurr's low viscosity embedding media. Pressure was then applied with $CO_2$ gas in a Bomar SPC-900 critical point apparatus. Specimens in 100% embedding material were set at 800 to 1,200 psi overnight. The following morning, pressure was released very slowly (ca. 2 hr) to avoid resin bubbling and destruction of the specimens. Specimens were then transferred to fresh embedding medium and cured in an oven.

The device used in this experiment was not designed specifically for such a specialized procedure. Due to bubbling during the exhaust phase many specimens were destroyed. Specimens which were not damaged

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during decompression showed that resin infiltration was satisfactory in head and thoracic regions, where openings were made, but was still incomplete in the large abdomen. Sections of well-infiltrated aphid tissues showed that the cytological background was so darkly stained and unclear that recognition of organelles became difficult at higher magnificantions (Fig. 1). Air bubbles were found in the tissue (Fig. 2). In most of the specimens, the gut lumen of test aphids was closed and the microvilli border clustered (Fig. 3). This made recognition of PEMV particles in epithelial cells and gut lumen almost impossible. In a few cases, virus particles were found oriented along the microvilli.

Critical point drying. In most of the adult specimens, resin infiltration in the abdomen was incomplete even after pressure treatment. A soft, uncured body cavity was usually found while trimming the specimen block. This was thought to be caused by incomplete replacement of ethanol dehydrating agent with resin in the abdominal cavity. Critical point drying, which allows specimens to be brought to a dry state without a disruptive effect on surface tension, was tried to enhance complete dehydration of tissue preparation.

Nymph and adult pea aphids were given a 1-day AAP on T-PEMV source plants and then fixed and dehydrated. Specimens were then rinsed in a series of graded iso-amyl acetate (10 through 100%), 2 min in each, with several additional rinses in 100%. Next, specimens were placed in a Bomar SPC-900 critical point apparatus. Liquid $CO_2$ was heated to a critical point at which all the liquid in the specimens became gas and was bled off. The dried specimens were treated with pressure

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pressure in 100% Spurr's embedding medium overnight, then polymerized, sectioned, stained and examined under TEM.

Following this treatment, a completely dried, hollow abdominal cavity was found in the specimen block. This indicated that the dehydration by critical point drying was complete in the abdomen while resin infiltration was still insufficient. The aphid tissues sectioned from the thoracic area showed good contrast when viewed under TEM. Mitochondria were stained well yet ribosomes became clumped. This was thought to be caused by the critical point drying treatment. Artifacts of assorted types appeared in the cells.

Negative staining of PEMV in extracts of excised organs. Because difficulties arose during the resin infiltration step of the ultrathin sectioning technique, attempts were made to locate PEMV in excised tissues of aphid vector by negative staining technique.

Alimentary tracts and salivary glands were dissected from viruliferous aphids which had been given a 2-day AAP on T-PEMV infected peas. About 65 to 85 pea aphids were used in each trial. Extracts of the excised organs were obtained by using a modified version of the procedure developed by French (1973) for purifying PEMV from aphid vectors. Excised organs were fixed for 1 to 2 hr in cold 3% glutaraldehyde in 0.1 M potassium phosphate buffer and then homogenized with a glass tissue homogenizer in 0.2 ml of 0.1 M potassium phosphate buffer (pH 6.0) and 0.2 ml chloroform-butanol (1:1) mixture. The homogenate was held over ice for 60 to 90 min and then centrifuged at 9,000 rpm for 15 min on a Sorvall SS-1 centrifuge. The upper aqueous phas

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aqueous phase was collected and negatively stained with 2% PTA (pH 7.0) and then examined by the TEM.

Several trials using the above procedure to locate PEMV in the crude extracts by negative staining met with limited success, particularly in the salivary gland extracts. Due to the small volume of aqueous supernatant obtained after low speed centrifugation, it was easy to mix it with the interface of insect debris while collecting for negative staining. Also, because of the small volume, additional cleanup procedures such as filtration could not be used to eliminate this problem. Consequently, much debris on the grids interfered with visualizing virus particles under TEM. Virus particles, approximately 22-24 nm in size, were found in gut extracts, while only a few viruslike particles were found in salivary gland extracts.

## B. Evaluation of Methods to Distinguish PEMV Particles *In Situ*

*Using colloidal iron hydroxide*. By electron microscopic examination, neuraminic acid which exists in microorganisms and animal cells was found capable of binding with colloidal iron hydroxide (CIH) (Gottschalk, 1960; and Klenk *et al*., 1970). This technique has been used to determine the presence or absence of neuraminic acid in several enveloped animal viruses (Klenk *et al*., 1970). Lee *et al*. (1972) found that the aphid-borne sowthistle yellow vein virus (SYVV) was stained heavily by iron granules after treatment with CIH. Since it is difficult to distinguish PEMV particles from ribosomes *in situ*, I tested the possibility of binding CIH to the surface of virus particles and thereby enhancing the identity of the virus *in situ*.

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EL biotype pea aphids were allowed a 2-day AAP on T-PEMV source plants. The excised alimentary tracts were fixed for 2 to 3 hr in 4% cold glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2) and then washed for 5 min in 5% acetic acid. Next, the fragmented guts were treated with CIH (12 ml $FeCl_3$ in 750 ml boiling distilled water, pH less than 2) for 1 to 2 hr, and then rinsed with 5% acetic acid for 10 min. Postfixation, dehydration and embedding of the specimens were carried out as described earlier. A control group was prepared in the same way except the treatments with CIH and acetic acid were omitted.

After CIH treatment, the tissue stained darkly. Iron granules were found attached to the surface of the basement membrane of the guts (Fig. 4), but were not in the nontreated ones (Fig. 5). Accumulation of iron particles also was occasionally observed in a few organelles in epithelial cells of CIH-treated specimens.

Virus particles observed in the gut lumen were free of iron granules. Contamination of iron to virus particles at the periphery of food material were observed in a few cases. It was assumed to be nonspecific to virus particles. This experiment indicated that neuraminic acid does not exist in the PEMV viral protein coat, and, consequently, the attempt to label PEMV with CIH was unsuccessful.

Treatment with ethylene dinitrilo tetraacetic acid (EDTA)--destroying ribosomes in vivo? Both PEMV (27 nm, French, 1973) and ribosomes (25 nm x 15 nm, De Robertis *et al.*, 1970; and 15-25 nm, Esau and Hoefert, 1972a) are roughly round in shape when viewed under TEM. This causes some difficulty for the researcher attempting to distinguish

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PEMV particles from ribosomes in aphid tissue. It is known that the structural cohesion of ribosomes depends on the concentration of magnesium. At low concentrations of magnesium, ribosomes are dissociated into subunits (De Robertis _et al_., 1970). Ethylene dinitrilo tetraacetic acid (EDTA) which chelated free divalent cations _in vitro_ was used in this experiment to see if ribosomal dissociation would occur in vector tissue and thus allow PEMV particles, if scattered in tissue cells, to be easily detected with TEM.

Alimentary tracts from viruliferous aphids which had a 24-hr AAP on T-PEMV infected peas were dissected in 0.1 _M_ Sorensen's phosphate buffer and cut into small pieces. These gut fragments were then treated with 0.01 _M_ EDTA for 2 hr either on ice or at room temperature. Specimens were fixed in 6% glutaraldehyde in 0.1 _M_ phosphate buffer and postfixed in 2% buffered osmic acid for electron microscopy. Control gut sections were processed in the same manner only without the EDTA treatment.

Fewer ribosomes were observed in EDTA-treated gut tissue, in both the ice and room temperature treatments (Fig. 6) than in control group (Fig. 7). In the EDTA-treated tissue, ribosomes were dissociated and resulted in a relatively clear cytoplasmic background for mitochondria and endoplasmic reticulum. Some single ribosomes were found attached to the membrane of the endoplasmic reticulum (Fig. 8). Chromatin material in nuclei was less dense in the EDTA-treated epithelial cell (Fig. 9) than in the nontreated one (Fig. 7).

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Neither PEMV virus particles nor viroplasms were found in the epithelial cells of guts in either treated or nontreated specimens. However, large numbers of virus particles were found in the gut lumen of the control and treated aphids (Figs. 10 and 11). These virus particles were darkly stained and appeared to be well preserved.

## C. Morphology of PEMV Variants

Particle structure in infected pea tissue. A comparison of the T- and NT-PEMV variants in infected pea tissue by electron microscopy has not been done. It was the objective of this experiment to determine the morphology and localization of PEMV in the plant tissue infected by these two variants.

Both T- and NT-PEMV source pea plants were very intense chlorotic symptoms were selected 10 days after mechanical inoculation. Infected pea leaves were cut into small pieces (ca. 0.1 cm x 0.3 cm) and prepared for electron microscopy. Glutaraldehyde (4-5%) in 0.1 M Sorensen's phosphate buffer or potassium dichromate-acrolein mixture (2%:3%, pH 7.35) were used as primary fixatives. Health pea leaves were processed in the same manner.

Localization and morphology of PEMV were found quite similar in both T- and NT-PEMV infected pea tissues. PEMV virions were observed in the mesophyll cells, vascular bundle sheath cells and sieve tubes. Large numbers of virions of both variants accumulated in nuclei where multiplication was assumed by Shikata and Maramorosah (1966) to occur. However, NT-PEMV seemed to be more densely packed (Fig. 12) than T-PEMV (Fig. 13). Figure 12 shows an almost completely destroyed nucleolus.

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Chromatin material was found at the periphery of the nucleus and part of the nuclear envelop was disintegrated. A special type of virion aggregation (Fig. 14) which was similar to the aggregation bodies in alfalfa mosaic virus (AMV) infection (Hull et al., 1970), was observed occasionally in the nucleus of NT-PEMV infected pea leaves. These virus particles were packed side by side to form bands which were arranged in a subparallel manner through the nucleoplasm.

Virions were commonly scattered in the vacular sap (Fig. 15) of the tissues infected by both PEMV variants. However, virion aggregates also were found in the vacuoles of NT-PEMV infected tissues (Fig. 16) yet never in the T-PEMV infected cells. Virus particles were often located in the cytoplasm of the infected peas and tended to clump along the cell walls. Figure 17 shows a large membrane-bound virion aggregate in the cytoplasm of NT-PEMV infected leaf. Along with some virus associated structures, the virion aggregates which were observed in the NT-PEMV infected nucleus also were seen in the cytoplasm of the NT-PEMV infected tissue (Fig. 18).

In some instances, vacuolelike membranous structures (Shikata et al., 1966; and Hooper et al., 1972) of varying sizes and shapes appeared in the infected pea leaves. Virus particles aligned along the membrane forming single, double, or even triple rows, as shown in Figures 19 and 20. PEMV particles also were found either scattered or aggregated inside the vacuoles. The amount of virus in the vacuoles varied; some vacuoles contained relatively few virus particles while others were filled with virions (Fig. 20). Comparatively speaking, such vacuoles in NT-PEMV infected tissues seemed to be at an earlier stage of structure formation than those in T-PEMV infected peas (Figs. 19 and 20).

Some microtubulelike structures were found in the vacuoles and the cytoplasm of NT-PEMV infected tissues (Figs. 18, 21 and 22). Similar structures also were observed in tobacco mosaic virus (TMV) infected plants (Esau, 1968) and were referred to as X-tubules. Aggregates of X-tubules associated with PEMV infection appeared in the cytoplasm. They were morphologically variable. A very different and unusual arrangement of X-tubules which occurred in close association with one another in groups is shown in Figures 18 and 23. Some transverse sections of the tubules were found both situated in the center of the aggregates and scattered in the cytoplasm nearby (Figs. 18 and 23). The diameter of these X-tubules was approximately 19-22 nm. Other aggregates showed that X-tubules were flexuously, irregularly scattered in the cytoplasm (Fig. 24). So far, none of the X-tubules was observed in T-PEMV infected pea leaves.

Unlike tobacco mosaic virus (Shalla, 1968; and Otsuki *et al*., 1972) and pelargonium leaf curl virus (Martelli and Russo, 1972), PEMV virions have not been reported in intact chloroplasts of infected tissues. However, ruptured chloroplasts resulting from virus infection were often observed in NT-PEMV infected mesophyll cells (Figs. 24, 25 and 26) and occasionally in T-PEMV infected peas. Figure 26 shows malformed chloroplasts with virions scattered inside and with large virus aggregates in the cytoplasm.

Crystalline material, a characteristic inclusion associated with PEMV infection found by Shikata et al. (1966), was consistently observed in the cytoplasm of both T- and NT-PEMV infected vascular bundle sheath cells and sieve tubes. It was not likely to occur in the vacuoles as reported in artichoke mottled crinkle virus infection (Russo et al., 1968) and in the nuclei as found in pelargonium leaf curl virus (Martelli and Russo, 1972), tomato bushy stunt virus (Russo and Martelli, 1972) and beet western yellow virus (Esau and Hoefert, 1972) infections. Aggregation of these virus crystals varied in size and appearance. Most of them were arranged in hexagonal pattern (Fig. 27). However, a few of them seemed to be arranged more or less in a square pattern (Fig. 28). In any case, the single particles constituting the crystal lattice were always well defined and easily recognizable. Each individual particle was about 24-28 nm in diameter. Structures associated with PEMV infection were not observed in the healthy pea leaves.

Nuclear vesiculation which was thought to be a cytological effect of PEMV infection in plants (De Zoeten et al., 1972) was visualized frequently in both T- and NT-PEMV infected pea leaves (Fig. 29). Vesicles were found in the perinuclear spaces of nuclei, and it was assumed to have originated from the nuclear envelope. Virions could be seen scattered in the nucleus and among the vesicles (Fig. 29).

Comparison of particle shape and size in highly purified virus preparations. The characteristics of purified T- and NT-PEMV from infected pea tissues were studied by French (1973) and differences in nucleoprotein yield and component ratios were detected. Attempts were made in this experiment to examine the morphological differences in the viral protein capsid in highly purified and negatively stained preparations of these two variants.

Ten days after mechanical inoculation of pea seedlings, T- and NT-PEMV were purified by the procedure described by French (1973). After rate-zonal density gradient centrifugation, the virus zones were collected and negatively stained with 2% PTA (pH 7.0). The sizes of virus particles were measured on the negatives which were randomly recorded from TEM at the magnification of 40,000 X and the ratios of virus particle types also were calculated.

Two types of virus particles were observed in the highly purified T- and NT-PEMV preparations. One type had distinct outlines and was hexagonal and the other was relatively electron opaque and spherical in shape. However, the ratio of these two types of particles varied in these two preparations. The T-PEMV preparation contained more hexagonal particles than spherical ones (Fig. 30) and in a ratio of 1.6:1 whereas the NT-PEMV preparation contained about 28 times as many spherical particles as hexagonal ones (Fig. 31).

Measurements of 96 particles which were randomly sampled from each particle type in each virus preparation gave diameters of 24.4 nm and 29.8 nm in T-PEMV preparation and 27.4 nm and 28.8 nm in NT-PEMV preparation for hexagonal and spherical particles, respectively (Table 1). Difference in the sizes seemed to be small in spheroids, but was significant in hexagonal particles in both preparations. The differences in the particle type ratio and the sizes between T- and

Table 1. Measurements of particle size in T- and NT-PEMV preparations

| | Hexagonal particles | Spherical particles |
|---|---|---|
| T-PEMV | 24.4 ± 1.6 nm | 29.8 ± 2.1 nm |
| NT-PEMV | 27.4 ± 1.7 nm | 28.8 ± 1.6 nm |

NT-PEMV may give some explanation for the different aphid transmission characteristics.

According to the data obtained from this study, one would presume that hexagonal particles (24.4 nm) are the original intact virions, the spherical particles are the disrupted ones, and the other hexagonal particles (27.4 nm) are the intermediate forms. Perhaps, the composition and/or the structure of the protein capsid of these 27.4 nm particles is different from that of the intact virions. Since the size of hexagonal particles (27.4 nm) is quite close to that of spherical ones, it is possible that the spheroids are derived from hexagonal particles. Presumably, these three various sized particles (24.4, 27.4 and 28.8 or 29.8 nm) may exist with different ratios in both T- and NT-PEMV preparations.

It is possible that the aphid transmissibility characteristic of T-PEMV is afforded by the abundance of intact hexagonal particles and that the scarcity of those particles in NT-PEMV accounts for nonaphid transmissibility. This possibility warrants considerable additional study.

## D. Localization of PEMV Variants in the Pea Aphid

The localization of PEMV virions in the aphid vector, *Acyrthosiphon pisum* (Harris), has been studied by means of electron microscopy (Shikata *et al*., 1966; Harris, 1971; and Harris and Bath, 1972). Virions of PEMV were detected mainly in the midgut lumen and also in the epithelial cells, fat body and hemocytes of viruliferous aphids. Since T- and NT-PEMV exhibit totally different aphid transmissibility characteristics, attempts were made to determine the fate of these two variants in both nymph and adult aphid vectors. The effects of various acquisition access periods and holding periods after AAP's also were studied.

Uniform aged T- and NT-PEMV source plants, as well as first instar nymph and young adult test aphids were obtained as described earlier. Acquisition access periods were completed in a controlled-environment chamber (Tsai, 1967). Test aphids were allowed to have a 1-day or 5-day acquisition access period (AAP) and were either processed immediately for electron microscopy or after a 3-day holding period on noninfected broad beans. Treatments were designated as 1 + 0, 1 + 3, 5 + 0 and 5 + 3 for AAP and holding period, respectively. In addition to these two factors, virus variants (T- and NT-PEMV) and vector life-stages (adults and 1st instar nymphs) also were tested in each AAP and holding period combination and resulted in 16 treatment combinations. Approximately 20 aphid specimens were collected from each treatment and prepared for electron microscopy. Control aphids were processed in the same manner.

Virus localization. Midgut lumen. PEMV virus particles of both variants were commonly found in the lumen of the test aphids in close association with plant materials ingested from the PEMV source plants. Virions were either aggregated along the margin or scattered within the ingested food materials (Figs. 32 and 33). Those which were observed at the periphery of the food material were of a distinct viral shape and scattered individually (Fig. 32) or they formed small aggregates (ca. 3-10 virions/clump) with deformed morphology. Occasionally, virions in the lumen were found aligned in rows and bound with a membrane. The titer of virion in the lumen differed in the various treatments and individual specimens within a treatment.

I found that aphids which were processed after the 1-day AAP's (1 + 0 and 1 + 3 treatments) tended to contain numerous small masses of food materials in the lumen (Fig. 32). However, those in the 5 + 0 and 5 + 3 treatments tended to contain larger masses of food material (Fig. 33). It was reported that the ingested materials might be concentrated and gradually accumulated (Moericke and Mittler, 1965) in the stomach by the removal of water (Treherne, 1967). This may provide an explanation for the formation of large accumulated food material after the longer AAP's. In some instances, these large food balls were surrounded by a membranous layer. At times portions of the membrane were ripped and virions appeared to be relased from the food material into gut lumen. PEMV virions appearing within the large masses of food materials were generally well preserved.

High titers of both T- and NT-PEMV virions were found in adults after the 1 + 0 treatments. Most of the virus particles were in aggregate forms and scattered around small masses of food material. Some of them seemed to be free in the lumen. T-PEMV virions also were found accumulated at the periphery or in the center region of large food masses. However, it was obvious that the virus titer in gut lumen was reduced in the 1 + 3 treatments. Ingestion of healthy broad bean sap during the 3-day holding period may result in washing out or diluting the virions in the midgut lumen. I found in the 1 + 3 treatments that NT-PEMV infested aphids contained more small masses of food materials than did the T-PEMV infested insects and these small food mateials were either free of virions or with few virus aggregates scattered around. In nymphs, high concentrations of well preserved T-PEMV virions were occasionally located in the center or at the peripheral area of large food materials in the lumen.

T-PEMV infested nymphs and adults in 5 + 0 and 5 + 3 treatments contained mostly large food masses with virions which were well preserved. Very few small food materials also were observed and they were generally free of virions. In the 5 + 3 T-PEMV treatments, the viral concentration inside the large masses of food material was higher in the viruliferous adults, and was apparently much higher in the nymphs than in the 5 + 0 treatments.

On the contrary, nymphs and adults in 5 + 0 and 5 + 3 NT-PEMV treatments showed only a few distorted virions in the large food materials. However, large numbers of small virion aggregates appeared again along the margin of food materials or sometimes free in the lumen of aphids in 5 + 3 treatments. This happened especially in the virulferous nymphs. In a few cases, these small viral aggregates also appeared inside the large food masses.

Midgut microvilli. Both T- and NT-PEMV virions were found in the microvillous borders of the aphid stomach. Virions were generally scattered in the striated areas or formed small aggregates in the vicinity (Fig. 34). However, viral aggregates occasionally were found at the base of the microvilli next to the epithelial cells. I also noticed that PEMV virions were found more frequently in T-PEMV infected adults than in NT-PEMV infected ones and also in 1 + 0 more than in other treatments.

Midgut epithelial cells. As mentioned earlier, due to similarities in size and shape to ribosomes, PEMV virions were difficult to recognize in the midgut epithelial cells unless they were located in viroplasmlike or distinctly defined structures in the cytoplasm. In the viroplasmlike structures, virions were generally found at the periphery of electron-dense areas and some appeared in the central regions (Fig. 35). Mostly, these viroplasms occurred in the apical part of the epithelial cells, just next to the striated border and with some found throughout the cell cytoplasm. No viral infections were detected in the nuclei. Those which were not located in viroplasmlike, electron-dense areas were generally bounded by a membranous layer and either formed aggregates or were aligned in rows (Figs. 36, 37, 38 and 39). These defined structures were scattered randomly in the cytoplasm of stomach cells. In some instances, virions were found located in some unidentified structures (Fig. 40) in the cytoplasm. All of these three types of structures associated with viral infections were observed in the T-PEMV infected aphids. However, only the viroplasmlike structures were observed in the NT-PEMV infection. These viroplasms seemed to be at a very early stage of formation and consequently contained only a few virions (Fig. 41). Due to the small number of detectable virions in the viroplasm, it was difficult to determine viral infection in most NT-PEMV infected tissues.

Results of this study indicated that the infections of California PEMV isolate were less common in midgut tissues than in New York isolate which was studied by Harris (1971). As shown in Table 2, viral infection in the midgut epithelial cells seemed to occur about twice as frequently in T-PEMV than in NT-PEMV infected aphids. Out of 50 examined T-PEMV infected aphids, viroplasmlike and other associated structures were found in 10 of the viruliferous aphids; whereas only 4 out of 40 showed viral infection of NT-PEMV.

Among the examined T- and NT-PEMV infected aphids, viroplasmlike structures were found in 1 out of 8 T-PEMV infected adults, while none in the 1st instar viruliferous nymphs in 1-day AAP (1 + 0) treatments. However, they were detected in 5 out of 9 and 3 out of 5 T- and NT-PEMV infected adults, respectively, and also 1 out of 6 T-PEMV infected nymphs in 5 + 3 treatments. The appearance of T- and NT-PEMV associated viroplasmlike structures in the examined nymphs was rarely found and only one case in the 5 + 3 and 5 + 0 treatments, respectively.

Table 2. Numbers of aphids showing viral infections in the midgut epithelial cells in various treatments[a]

| | | Treatments | | | | | |
|---|---|---|---|---|---|---|---|
| Variants | Aphid stage | 1+0 | 1+3 | 5+0 | 5+3 | Sub-total | Total |
| T-PEMV | Adult | 1/8 | 2/8 | 1/6 | 5/9 | 9/31 | 10/50 |
| | 1st instar nymph | 0/5 | 0/5 | 0/3 | 1/6 | 1/19 | |
| NT-PEMV | Adult | 0/9 | 0/5 | 0/7 | 3/5 | 3/26 | 4/40 |
| | 1st instar nymph | 0/3 | 0/4 | 1/3 | 0/4 | 1/14 | |

[a] Numerator = number of aphids showed viral infection in midgut tissues; denominator = number of aphid specimens examined.

<u>Other organs and tissues</u>. Attempts also were made to locate PEMV virions in other aphid tissues including muscle fibers, hemocytes, fat body, mycetomes and salivary glands. In rare instances of T-PEMV infection, viruslike particles were found aggregated or scattered individually in the muscle fiber cells surrounding the gut tissue. Figure 42 shows those viruslike particles scattered in the vacuole of a ruptured muscle fiber cell. Similar particles also were observed in a hemocyte which was adjacent to the gut muscle fibers (Fig. 43). High concentration of viruslike particles occupied the whole nucleus. An aggregate was formed in the nucleus. These kind of particles also were found located in the pinocytic vesicles (Fig. 43) which scattered

through the cytoplasm of the hemocyte. Degenerated fat body cells appeared in the hemocoel. Viruslike particles aggregated in the vacuoles or in the cytoplasm of these cells (Fig. 44). Some of the particle-containing vacuoles seemed to be released from fat body cells (Fig. 45). Due to low frequency of occurrence in the examined specimens, no conclusion could be drawn with certainty that these particles represented virions of PEMV. No detectable virus particles were seen in the mycetomes and salivary glands of viruliferous aphids.

## E. Quantification of T- and NT-PEMV in the Midgut Lumen

Pea enation mosaic virus (PEMV) has been the subject of intensive studies for several years. However, quantitative studies of this virus in its pea aphid vector have not been reported. It was the objective of this study to compare T- and NT-PEMV after various treatments by means of particle-counting. Electron microscopic particle-counting technique has been applied by several researchers to study purified plant viruses, e.g., wound tumor virus (Sharp, 1965; Strohmaier, 1967; Gamez and Black, 1967, 1968; and Streissle _et al_., 1968). This technique has not been successfully applied to plant viruses _in vivo_. In the present experiment, some of Weibel's (1969) stereological principles for morphometry in electron microscopic cytology were applied and a method for viral density determination in the midgut lumen of the pea aphid vector was established.

Gut tissues of T- or NT-PEMV infected aphid adults and nymphs were obtained from the 1 + 0, 1 + 3, 5 + 0 and 5 + 3 treatments described in the section of localization of PEMV variants in the pea aphid vector. From a pool of these gut tissues, a random sample of 3 specimen blocks was collected from each treatment and ultrathin sections were uniformly obtained from similar region of the stomach. While scanning under TEM, five electron micrographs in which fields were sampled systematically by a modified method described by Weibel et al. (1966) were recorded at 16,000 X magnification for each tissue sample. Virus particle counting was conducted and the volume of food material was estimated directly from the negatives by using a 18 x 23 unit (0.16 $cm^2$/unit) test screen. The viral density (particles/unit of food material) was then calculated and analyzed by statistical methods.

The mean viral density obtained from 15 observations for each treatment combination is listed in Table 3. The results were analyzed by using a mixed model nested analysis of variance technique. Due to poor resin infiltration, no individuals were available from the 1 + 0 nymphal treatments and thus a completely randomized analysis of variance also was applied. The statistical results of these experiments are presented in Table 4. Acquisition access period (AAP) effects on the adults appeared to be significant at the 5% level in both T- and NT-PEMV experiments. The holding period effect on the adults in 1-day AAP treatments as well as the vector stage effect on 1 + 3 treatment showed at 1% level in T-PEMV experiments. In addition to these treatment effects, sampling effects within each treatment also were analyzed. Significant differences (1%) were detected among samples within treatment in 5-day AAP treatments in both T- and NT-PEMV experiments.

Table 3. Mean density of PEMV virions in midgut lumen of viruliferous aphids

| Virus variants | Treatments[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1+0 Adult | 1+0 Nymph | 1+3 Adult | 1+3 Nymph | 5+0 Adult | 5+0 Nymph | 5+3 Adult | 5+3 Nymph |
| T-PEMV | 1.666 ± 0.5871 | --- | 0.642 ± 0.1933 | 1.660 ± 0.9732 | 0.715 ± 0.5019 | 1.103 ± 0.7668 | 0.901 ± 0.4256 | 2.337 ± 1.7433 |
| NT-PEMV | 2.685 ± 1.3591 | --- | 1.387 ± 0.4318 | 1.957 ± 1.1677 | 0.520 ± 0.5597 | 1.134 ± 0.5126 | 1.388 ± 1.1814 | 2.734 ± 0.2570 |

[a] Treatments were designated as 1+0, 1+3, 5+0 and 5+3 for acquisition access period (AAP) on virus source plants and holding period on noninfected broad beans, respectively, i.e., the test aphids were allowed to have a 1-day or 5-day AAP and were with or without 3-day holding periods.

Table 4. Significance of treatment effects in T- and NT-PEMV infections[a]

| Treatments | Virus variants | |
|---|---|---|
| | T-PEMV | NT-PEMV |
| AAP's[b] | | |
| Adults | +[c] | +[c] |
| Nymphs | - | - |
| Holding periods | | |
| Adults | | |
| 1-day AAP[b] | +[d] | - |
| 5-day AAP[b] | - | - |
| Nymphs | | |
| 5-day AAP[b] | - | - |
| Vector stages | | |
| 1+3 | +[d] | - |
| 5+0 | - | - |
| 5+3 | - | - |

[a] - = nonsignificant; + = significant.

[b] AAP = acquisition access period.

[c] Significant at 5% level.

[d] Significant at 1% level.

Sampling effects also appeared on adults in 1 + 3 T-PEMV and 1 + 0 NT-PEMV treatments at 1% level and on the nymphs in 1 + 3 NT-PEMV treatment at 5% level in the 1-day AAP experiments. This indicated that an increased number of examined tissue samples needs to be taken to eliminate this variability in future research.

I found that the viral density in the midgut lumen in NT-PEMV infected aphids was higher than T-PEMV infected aphids (Table 3). However, the difference between these two variants was not statistically significant.

Both T- and NT-PEMV densities decreased with increasing acquisition access periods in adults (Table 3). This could be explained by the aging of the vector during the extended AAP's. The AAP treatment effect was not able to be determined in the nymphs due to incomplete data.

Differences of viral density in the vector's life stages were nonsignificant, except in 1 + 3 T-PEMV treatment. This phenomenon explains the results obtained in transmissibility tests which demonstrated that the difference of acquisition efficiency between the 1st instar nymphs and the adults was almost nonexistent after a 24-hr acquisition access period (Bath and Chapman, 1968). However, nymphs consistently contained higher viral density than the adults throughout all the experiments. That differences in body size probably results in a higher titer of virus acquired by the nymphs than the adults per unit time is a likely explanation for this phenomenon.

In the 1-day AAP T-PEMV experiments, a holding period effect was found to exist at the 1% level of significance. However, no significant holding period effect occurred in 1-day AAP NT-PEMV infected adults or 5-day AAP T- or NT-PEMV infected nymphs and adults (Table 4). It was hypothesized that fresh plant sap from broad beans flush virus material from the gut lumen. This was also true in the results between 1 + 0 and 1 + 3 T-PEMV treated adults.

Attempts were made to determine the holding period effects based on the vector's ages. Table 5 was derived from Table 3 concerning the viral density on daily basis. As shown in Table 5, 5-day old aphids

Table 5. Mean density of T- and NT-PEMV in aphid vectors based on the age of the vectors (days)

| Virus variants | Vector's age (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 5 (1+3 Ny)[a] | 6 (5+0 Ny) | 8 (1+0 Ad)[b] | 9 (5+3 Ny) | 11 (1+3 Ad) | 12 (5+0 Ad) | 15 (5+3 Ad) |
| T-PEMV | 1.660 ± 0.9732 | 1.103 ± 0.7668 | 1.666 ± 0.5871 | 2.337 ± 1.7433 | 0.642 ± 0.1933 | 0.715 ± 0.5019 | 0.901 ± 0.4256 |
| NT-PEMV | 1.957 ± 1.1677 | 1.134 ± 0.5126 | 2.685 ± 1.3591 | 2.734 ± 0.2570 | 1.387 ± 0.4318 | 0.520 ± 0.5597 | 1.388 ± 1.1814 |

[a] Ny = nymph.

[b] Ad = adult.

(1 + 3 nymphs) contained higher viral density than 6-day old aphids (5 + 0 nymphs) which had a longer acquisition access period in both T- and NT-PEMV experiments. This phenomenon also occurred in the comparisons between 8-day (1 + 0 adults) and 9-day (5 + 3 nymphs) old, 11-day (1 + 3 adults) and 12-day (5 + 0 adults) old, and between 12-day (5 + 0 adults) and 15-day (5 + 3 adults) old aphids with an exception in 11- and 12-day old aphids in NT-PEMV treatments.

Statistical analysis also indicated that no significant treatment effects existed between 6-day (5 + 0 nymphs) and 8 day (1 + 0 adults) old aphids. This suggested that the virus titer in the test aphids would reach a certain level within the first 24-hr AAP and thereafter tended to be kept at a constant level by reacquisition and excretion throughout the whole duration of the acquisition access periods. However, according to the comparisons between similar aged aphids with and without holding period treatments, it seemed that those which had 3-day holding period on broad beans also contained a virus titer similar to the nontreated ones, instead of reducing viral density by flushing. One possibility responsible for this phenomenon was thought to be the multiplication of PEMV in the aphids.

# DISCUSSION

The formation of electron-dense viroplasmlike areas and membrane-bound cytoplasmic structures in the cytoplasm of midgut cells is generally found in association with viral infections. However, the actual function of these structures is still unknown. It is presumed that midgut is the first contact site between the ingested virions and the absorptive tissue of the vector (Harris and Bath, 1972). Passage of the virions from gut lumen into hemocoel is likely to take place in the midgut. According to Harris (1971), these structures were found mostly at the apical part of the cell next to the microvilli and later in the basal portion of the cell near to the basal cell membrane. The sequence of the occurrence may indicate the transport of the ingested virions. Therefore, these structures could be presumed to form as a result of pinocytosis or phagocytosis. However, this presumption cannot provide an adequate explanation for the low frequency of their appearance in this study, particularly in the viruliferous nymphs which are believed to be more efficient than are the adults in the transmission of PEMV (Chapman and Bath, 1968).

Similar structures have been reported in rice dwarf or wound tumor viruses infected leafhopper vectors which acquired virions by feeding on source plants and/or by abdominal inoculation (Shikata _et_ _al_., 1969; Maramorosch _et_ _al_., 1969). These researchers suggested that the viroplasms were the sites where virus assembly and multiplication take place. Harris and Bath (1972) also implied that these viroplasmlike structures could be the loci for PEMV multiplication, due to the high content of virions and uneven distribution in midgut cells. However, the present study indicates that this is not true. Viroplasmlike or membrane-bound structures appeared most frequently in adult aphids and less frequently in the nymphal gut tissue which was suggested to be a suitable site for multiplication, if it occurs (Sylvester, 1969). The experimental results indicate that the formation of virus associated structures is more likely a cell response to viral invasion than an aggregation of virions. It has been reported that the gut permeability to virus decreases as the vector's age increases (Sinha, 1960, 1963; and Merril and Ten Broeck, 1965). One would presume that PEMV virions could penetrate the gut tissue rapidly in the viruliferous nymphs. However, with reducing permeability in the adults, PEMV virions may get trapped in the cell and consequently electron-dense or membrane-bound areas are formed by encapsulation or phagocytotic reaction of the cell. Gut permeability also may account for the different transmission efficiency of nymphs and adults.

The pea aphid, Acyrthosiphon pisum (Harris), has been reported to be a better transmitter of a New York PEMV isolate than a California isolate (Bath and Tsai, 1969). Since advanced comparative studies have not been done between these two isolates, it is uncertain whether the differences of transmissibilities are due to the number of virions acquired from the source plants or different compatibility of acquired virions in the aphid circulative system, or both. However, based on the formation of viroplasmlike structures in midgut epithelial cells, one probably can interpret the differences. Harris (1971) reported that viroplasmlike structures were detected in every New York isolate infected aphid that was examined. He also suggested that the invasion and the infection of midgut tissue was rapid because various stages of viroplasmlike structure formation appeared in the young instar nymphs which were allowed to have 1-day AAP's on source plants. However, in my study of California isolate, the occurrence of these structures was fairly uncommon and mainly found in the adults. It is likely that pea aphids acquire a titer of New York isolate virions relatively higher than the California ones and the formation of structures in the nymphs results from the invasion of high concentration of virions. By purification of PEMV from the infected peas, French (1973) reported that these two isolates could not be differentiated on the basis of nucleoprotein yield or component ratios. Differences in nucleic acid base sequence or protein capsids may provide an explanation for the various transmissibility by the same vector.

It has been reported that only low titers of the circulative-propagative wound tumor virus could be found scattered widely in one of the salivary gland lobes (Maramorosch _et al_., 1969). Also, in a less efficient leafhopper vector, accumulation of virions in the salivary gland was reported so low as to be inadequate for electron microscopical visualization (Granados _et al_., 1967). Wheat striate mosaic virus, another circulative-propagative leafhopper-borne virus, also could not be detected in the vector (Thottappilly and Sinha, 1974). The failure to locate PEMV virions in salivary gland of the vector which acquired virus by feeding also may indicate that only low titers of virions are attainable by this organ. Recently, Harris (personal communication) found PEMV virions in salivary glands following the injection of a high dosage of purified PEMV preparation into hemocoel. Presumably, it could be attributed to that high titer of virions injected or bypassing of gut enhanced chances of being transported to the salivary glands, or both.

Two types of particles (hexagonal and spherical) with various sizes were found in highly purified PEMV preparations examined in this study. T-PEMV preparation contained more hexagonal (24.4 ± 1.6 nm) than spherical (29.8 ± 2.1 nm) particles while NT-PEMV preparation mainly composed of spherical particles. The hexagonal particles (27.4 ± 1.7 nm) found in the NT-PEMV preparation is larger than that in the T-PEMV preparation. It is presumed that these three sized particles (24.4, 27.4 and 29.8 nm) may exist in different ratios in both PEMV variant preparations, because of particle swelling, which has been reported in the purified cowpea chlorotic, brome mosaic and broad bean mottle viruses (Incardona _et_ _al_., 1964; Bancroft _et_ _al_., 1967; and Bancroft, 1970), may occur in the intact hexagonal particles (24.4 nm) and result in a population of particles with various degrees of swelling.

Virions observed in aphid midgut lumen were of various electron densities; some were electron-dense, others less dense, and still others appeared almost electron-transparent. The plane of sectioning may provide one of the explanations. It is known that staining for electron microscopy results in the binding of uranyl acetate to the ribonucleoprotein. Therefore, variations in particle electron density also may reflect the differences in the composition and/or the structure of protein coats of the virions. Based on this assumption, virions of highest electron-density could be representatives of the intact hexagonal particles, those of electron-transparent could be the spherical particles and those of intermediate density could be the 27.4 nm hexagonal particles. Virions with various electron density also were observed in the infected plant tissues.

According to several researchers, purified PEMV preparations always contain two nucleoprotein components. Considering the sedimentation zones of virus in density gradient tubes when scanned with UV light, one would presume that the bottom (heavier) components would contain mostly the intact hexagonal particles and the top (lighter) components contain the spherical particles while both components are not free of the intermediate forms. This presumption is confirmed by the particle type ratio in purified preparations obtained in this study and also by the characterization study of nucleoprotein components of T- and NT-PEMV variants (French, 1973). Data collected from this present study show that the spherical/hexagonal particle ratio is 28:1 in NT-PEMV while it is 1:1.6 in T-PEMV preparation. French (1973) reported that the top/bottom component ratio data showed consistently NT-PEMV contained much higher ratios of top component than did T-PEMV.

Several attempts to locate PEMV in tissues other than gut tissue have met with limited success (Shikata et al., 1966; Harris, 1971; and

Harris and Bath, 1972). Presumably, the permeability of aphid midgut, as well as the salivary gland, is a barrier for viral transport. The concept of salivary gland selectivity (Rochow, 1969) also may be applied in the transmission of pea enation mosaic virus. The adsorptive complementarity between the virus capsid protein and the proten receptors on plasma membranes of gut or salivary gland may be the major limiting factor for transmissibility. It is assumed that the structure of the intact hexagonal viral capsid protein confers on T-PEMV a basis for entrance into gut and salivary gland. This also provides a possible explanation for the vector transmissibility between T- and NT-PEMV. It was found that both variants can be acquired by feeding aphids, but that only certain hexagonal particles are able to get into gut or salivary gland tissue in sufficient amounts by pinocytosis. However, incorporation with other types of particles may occur in a few cases. These particles are then inoculated to plants during subsequent feeding.

Virions, which are inoculated to the susceptible host by the vector, propagate in the plant tissue. Presumably, both 24.4 and 27.4 nm hexagonal particles are formed in viral synthesis. However, the 27.4 nm particles tend to convert into spheroids as soon as they are formed. This gives an explanation for the presence of both intact and spherical particles in T-PEMV highly purified preparation.

Loss of vector transmissibility by continuous maintenance in host plants has been demonstrated in strains of potato yellow dwarf (Black, 1953) and wound tumor (Black *et al.*, 1958) viruses. A similar phenomenon also has been shown for PEMV. NT-PEMV variant is originated from T-PEMV culture (Tsai and Bath, unpublished). Long-time mechanical transmission may eliminate the chances of synthesizing intact hexagonal particles. Destruction of 27.4 nm particles may occur and thus build up a large population of spherical particles in NT-PEMV preparation.

The multiplication of several plant viruses in their insect vectors has been demonstrated in leafhopper-borne viruses such as wound tumor, rice dwarf, rice stripe and potato yellow dwarf viruses and some aphid-borne viruses such as potato leafroll, sowthistle yellow vein and lettuce necrotic viruses. Most of these viruses undergo a long latent period in the vector and their transmission is independent of the quantity of virus ingested. The long latent period is assumed to be the time required for the ingested virions to multiply to a sufficiently high titer to enable transmission to host plants. However, strawberry crinkle virus exhibits a long latent period in its aphid vector but no reported evidence for multiplication exists (Prentice, 1952; Frazier, 1968; and Richardson _et al_., 1972), while the propagative potato leafroll virus has a latent period ranged from 4 to 20 hr depending on the virus titer in the source plant (Day, 1955). Therefore, a short latent period in most aphid-borne viruses would neither prove nor disprove the existence of multiplication unless the speed of multiplication in the vector was known. The virus titer in host plants in relation to the concentration of virus required for the insects to transmit may be the factor to determine whether the virus propagates in the insect vector.

Experimental results have been shown both for and against the hypothesis that PEMV is a propgative aphid-borne virus. Although no direct evidence is reported, limited multiplication of PEMV in the vector is believed to be possible. The declining transmission efficiency of the vector following virus acquisition is one of the arguments against this hypothesis. However, Sylvester (1967) reported that the decline in transmission efficiency occurred with a similar decline in the rates of reproduction and excretion, and presumably reflected a general lessening in the feeding activity of aging vectors. This conclusion was supported later by Harrewijn _et al_. (1970) who reported that the imaginal aphid had a lower feeding ratio than the nymph and presumably the excretion of saliva was directly related to the uptake of food. In addition, gut permeability to virions decreases with increasing of vector age (Watson and Sinha, 1959; Sinha, 1960, 1963). It becomes more difficult for ingested virions to penetrate through the gut wall and be transmitted to plants by the adults. These physiological changes occur during the process of serial transmission and result in the decline of the transmission rate before the onset of significant vector mortality. This phenomenon has also been reported in the propagative western X-disease (Whitcomb _et al_., 1966).

That vectors with declining transmission efficiency can recharge virus by additional acquisition feeding is another argument against the hypothesis of multiplication. Aging factor is considered to be the reason. Sylvester (1969) implied that the multiplication of PEMV, if it occurs, is evidently rapid and most likely in the nymphal gut tissue. The loss of inoculativity also has been reported associated with the final molt when aphids become adults (Ehrhardt and Schmutterer, 1965). The decline in the rate of larviposition was found concurrent with the gradual loss of inoculativity, as if a common pool of metabolic reserves was being used in both the transmission and the reproductive process (Sylvester, 1966). According to Sylvester and Richardson (1966), recharging occurring in the adults could result from temporary excess of transmissible virus being independently moved from the gut to the salivary glands and does not exclude multiplication of the virus in its vector.

Failure in serial passage of hemolymph from aphid to aphid is a strong argument against multiplication. PEMV virions were rarely located in the hemocytes of pea aphids (Harris, 1971; Harris and Bath, 1972; and the present study). This indicates the hemocytes are not suitable carriers of virions. In comparison with the aphid body volume, only a minute amount of hemolymph collected from donor aphids is injected into recipients and consequently diluted by the hemolymph. In addition, hemocytes may not be the site of propagation (Clarke and Bath, 1973). These may give an explanation why maintenance of virus within a vector population by serial passage could not be achieved with PEMV.

Another argument is that the vector inoculativity is a function of the dose of inoculum. This phenomenon also has occurred in the potato leafroll virus-vector relationship (Kirkpatrick _et_ _al_., 1952; Heinze, 1959). It has been suggested that limited multiplication or localized infection could account for the dose-related transmission efficiency (Black, 1953).

Experimental results such as transstadial passage, existence of latent period, retention of inoculativity for a relatively long period of time, and the effect of ambient temperature on the retention of inoculativity and the duration of latent period are evidences favorable to the hypothesis of multiplication (Sylvester, 1969). Recently, electron microscopic study detected high concentration of PEMV virions in the mid- and hindgut lumen (Harris and Bath, 1972). Harris and Bath (1972) suggested that it resulted from the releasing of PEMV virions, which were produced in the midgut epithelial cells, into the lumen and suggested that PEMV multiplication might occur in the pea aphid.

Although cytological and metabolical changes in the viruliferous vectors may not be necessarily related to the activities associated with the multiplication of virus in the vectors, the changes have been reported more frequently in the vectors of propagative plant-pathogenic viruses such as potato leafroll (Schmidt, 1960; Ehrhardt, 1960; and Ponsen, 1969) and rice dwarf (Yoshii, 1959; Fukushi _et al_., 1962; and Nasu, 1963), and mycoplasmalike organisms such as rice yellows (Takahashi _et al_., 1962), rice stripe (Okuyama, 1962; and Maramorosch _et al_., 1970), and aster yellows (Maramorosch _et al_., 1963) than the circulative viruses. Enlargement of cell nuclei, changes in the integrity of cytoplasm, degeneration of membrane structure in cytoplasmic organelles and reduction of the ultrastructural contrast have been noticed in the PEMV infected vector (Schmidt, 1960; and Harris, 1971). The canals of the vacuolar system in the infected stomach cell also has been reported to be enlarged (Seryczyńska, 1972). These cytological abnormalities in aphid vector may indicate an indirect relation to the limited virus multiplication in the tissue.

Flushing of the virus contaminated material by ingestion of healthy plant sap has been demonstrated in lettuce necrotic yellow virus (LNYV). O'Loughlin and Chambers (1967) reported that virions of LNYV could not be found in the lumen of alimentary canal, when the vector aphids were allowed a 7-day AAP on healthy test plants prior to fixation. By injection of honeydew excreted from PEMV infected aphids, Richardson and Sylvester (1965) also reported that the alimentary tract can be cleared of detectable active viruses after feeding the test aphids on healthy plants for a day or so. However, my study does show detectable PEMV virions exist in the midgut lumen after a 3-day feeding on non-infected broad beans. Different techniques applied such as honeydew injection and electron microscopy may result in the various conclusions. Richardson and Sylvester (1965) found that honeydew lost its infectivity within 15 min after collection and that probably only the most recently excreted honeydew droplets collected were responsible for the infectivity tests. This indicates that the infectivity tests of honeydew may not be a sufficiently sensitive technique to detect virions in the aphid gut lumen. Although an electron microscopic study is not a way to test the infectivity of virions in the gut lumen, it did show the existence of PEMV virions after a 3-day holding period.

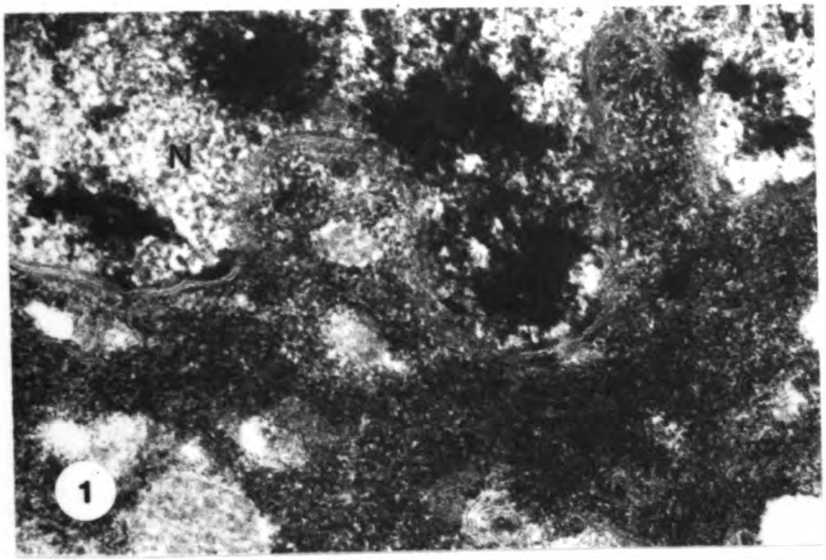
Richardson and Sylvester (1965) also reported that only one single droplet of honeydew from an adult was sufficient to inoculate several aphids and that the minimum access period after which an infectious honeydew droplet was excreted was approximately 1.5 hr. This indicates that honeydew contains a high virus titer and the metabolic rate of an aphid is rapid. Presumably, the alimentary tract would be cleared of virion after feeding on healthy plants for 3 days. However, by the quantitative study in this research, similar viral densities were obtained from these aphids as were from those fixed immediately after leaving source plants. A supplement of extra virions is assumed to occur during the holding period. Additional transmission trials indicated that re-acquisition of PEMV virions from the broad beans seemed to be impossible. Therefore, it is likely that at least limited multiplication occurs in the pea aphid vector. Further studies such as isotopic labeling or electron microscopic autoradiography should provide more evidences for this conclusion.

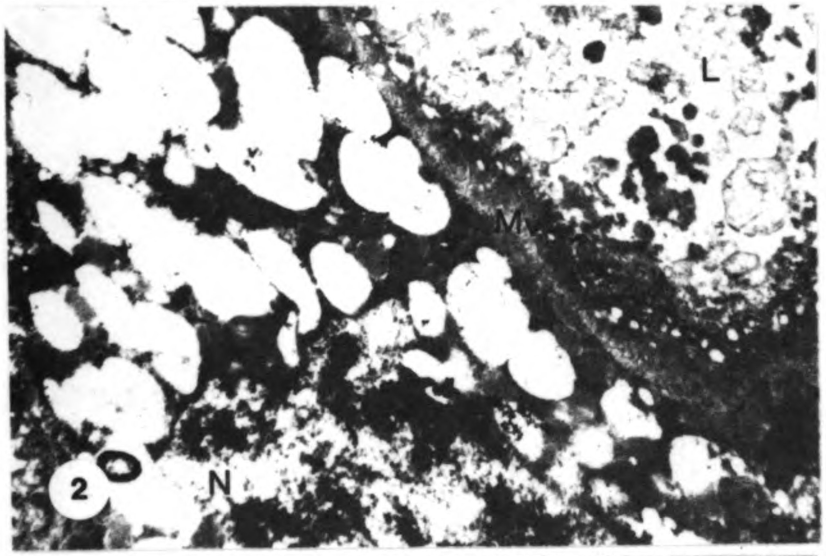
# FIGURES

Fig. 1. A midgut epithelial cell of a pressure treated aphid. The cytoplasmic background appeared darkly stained and unclear. N, Nucleus. Magnification: X32,000.

Fig. 2. A pressure-treated gut epithelial cell. Note numerous air bubbles formed in the cell. L, Lumen. Mv, Microvilli. N, Nucleus. Magnification: X6,400.

Fig. 3. Closed gut lumen obtained after pressure treatment. Magnification: X16,200.


N
1


L
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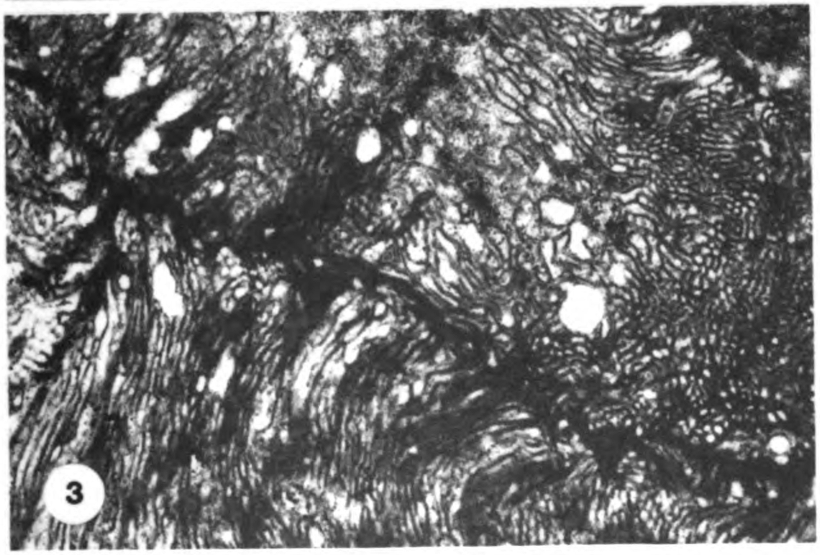
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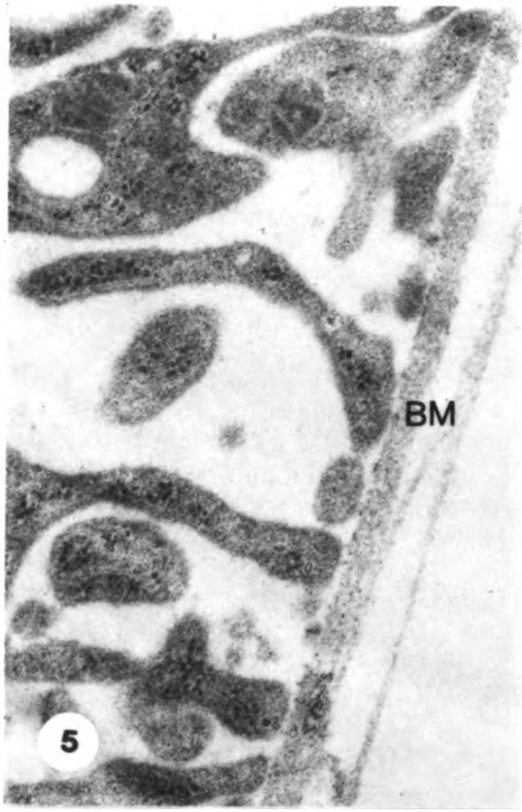
Fig. 4. A cross section of aphid midgut treated with colloidal iron hydroxide. Iron granules attached to the basal cell membrane (BM) of the midgut. Magnification: X31,200.

Fig. 5. An untreated basal cell membrane (BM) of the midgut. Note it was free of iron granules. BM, Basal cell membrane. Magnification: X31,200.

Fig. 6. A relatively clear cytoplasmic background induced by the dissociation of ribosomes in a gut epithelial cell treated with ethylene dinitrilo tetraacetic acid (EDTA). M, Mitochondria. Mv, Microvilli. R, Ribosome. Magnification: X31,200.

Fig. 7. An untreated gut epithelial cell. It contained a high concentration of ribosomes (R). M, Mitochondria. N, Nucleus. Magnificantion: X40,800.


BM
4


BM
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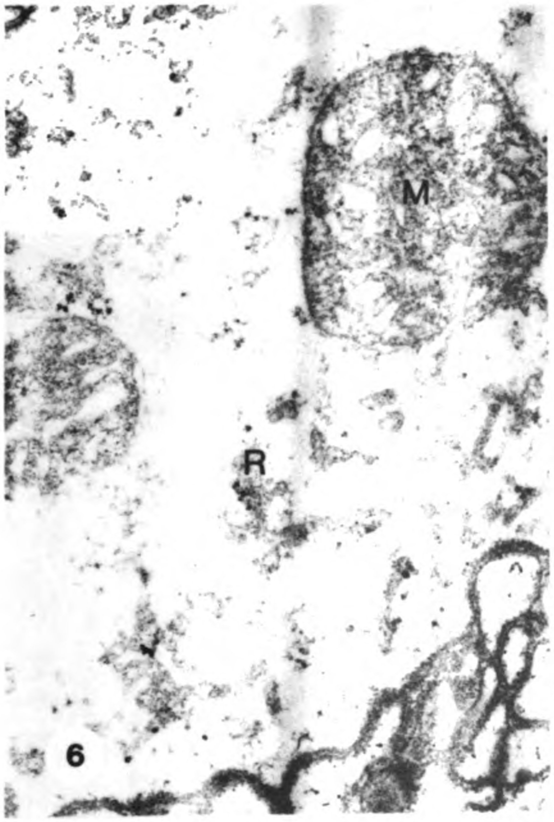
M
R
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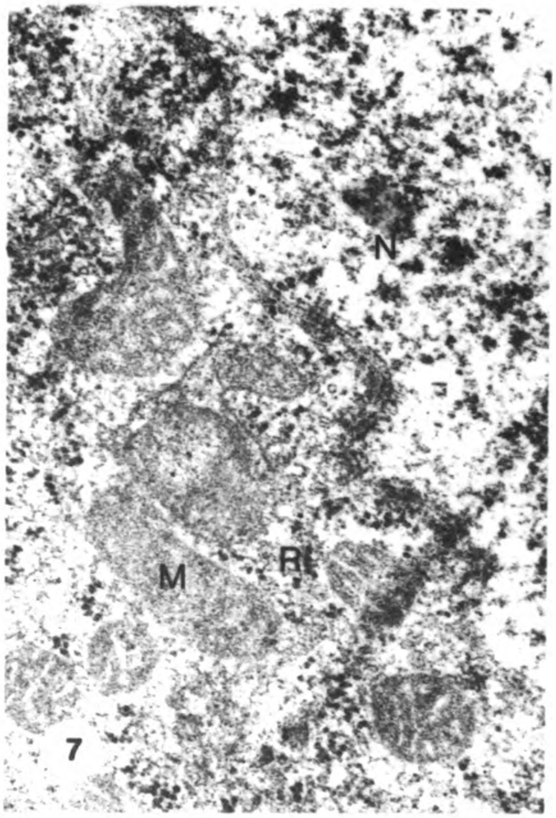
N
M
R
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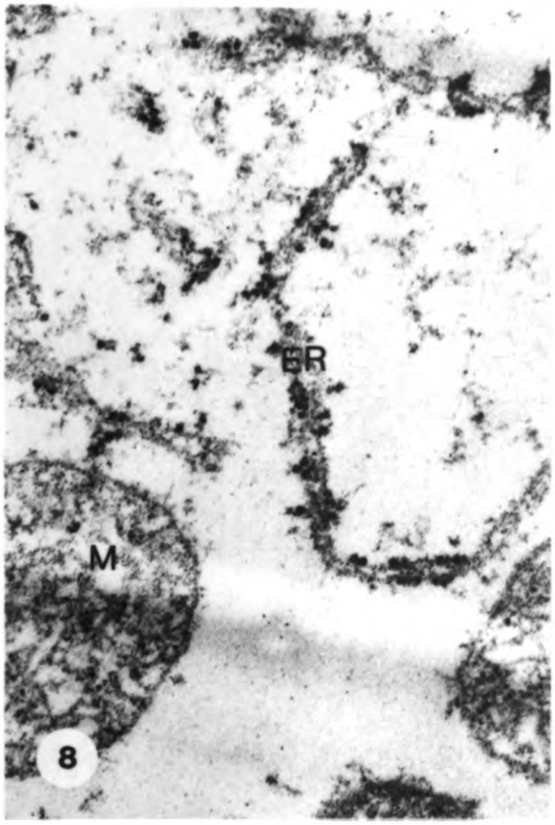
Fig. 8. An EDTA-treated midgut epithelial cell. A few ribosomes attached to the endoplasmic reticulum (ER). Note the cytoplasmic background was relatively clear of ribosomes. M, Mitochondria. Magnification: X53,550.

Fig. 9. Chromatin material in the nucleus (N) became less dense in the EDTA-treated gut cell than in the untreated one (Fig. 7). Magnification: X25,500.

Figs. 10 and 11. Large numbers of PEMV virions (V) in association with ingested food material (IF) appeared in untreated (Fig. 10) and EDTA-treated (Fig. 11) specimens. Magnification: X43,200 and X31,200, respectively.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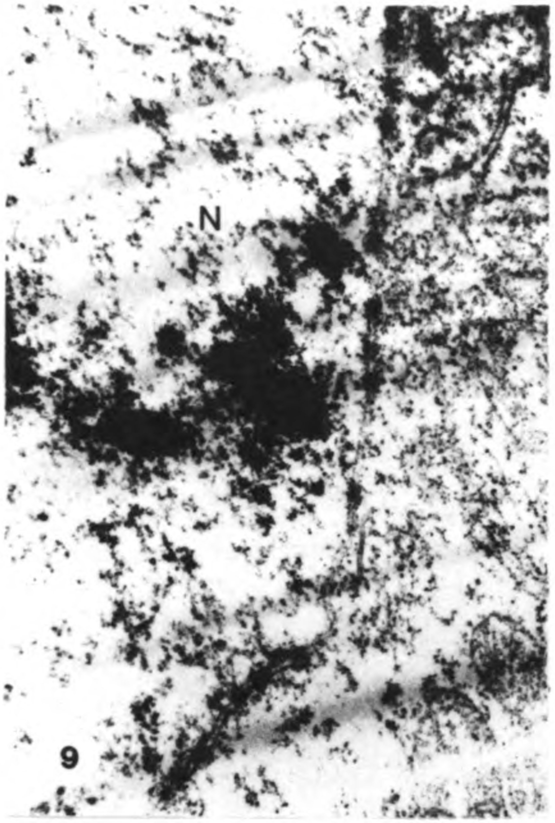
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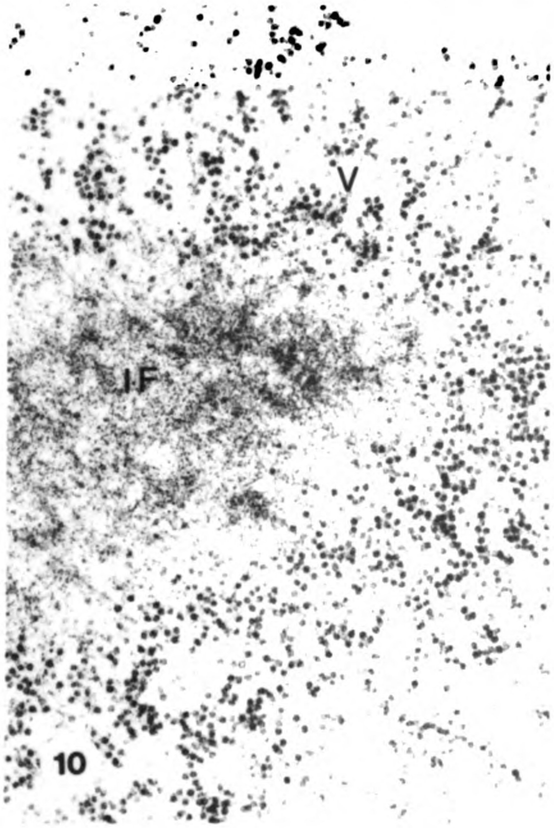
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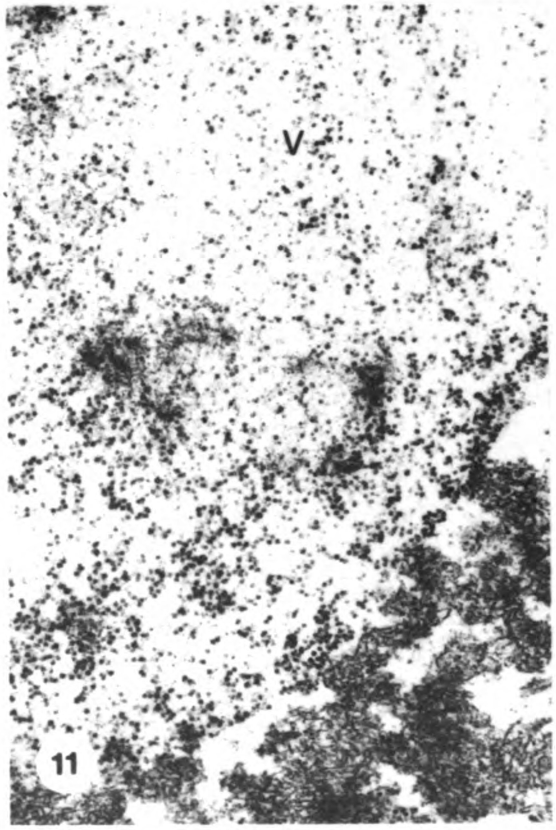
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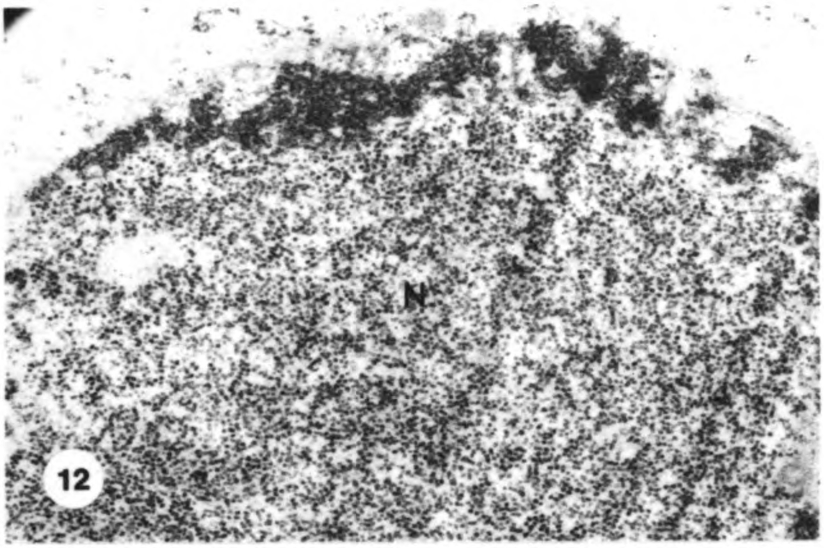
Fig. 12. A nucleus (N) in a NT-PEMV infected mesophyll cell. High concentration of virions were found in the nucleus. Note the virions occupied all the nucleus and the chromatin material appeared at the periphery of the nucleus. Magnification: X31,200.

Fig. 13. A nucleus (N) in a mesophyll cell infected with T-PEMV. Note the T-PEMV virions scattered loosely. Magnification: X31,200.

Fig. 14. A NT-PEMV infected nucleus (N) in a mesophyll cell. Virus particles aggregated and formed bands. Magnification: X51,200.



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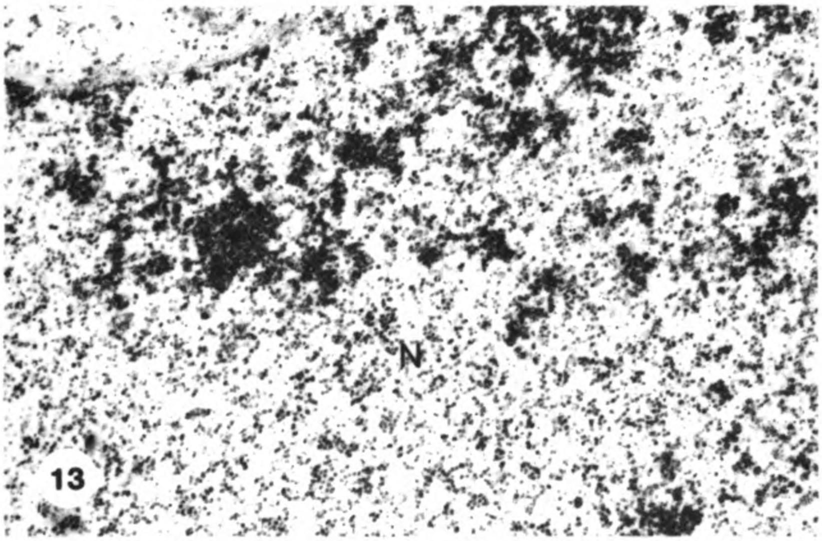

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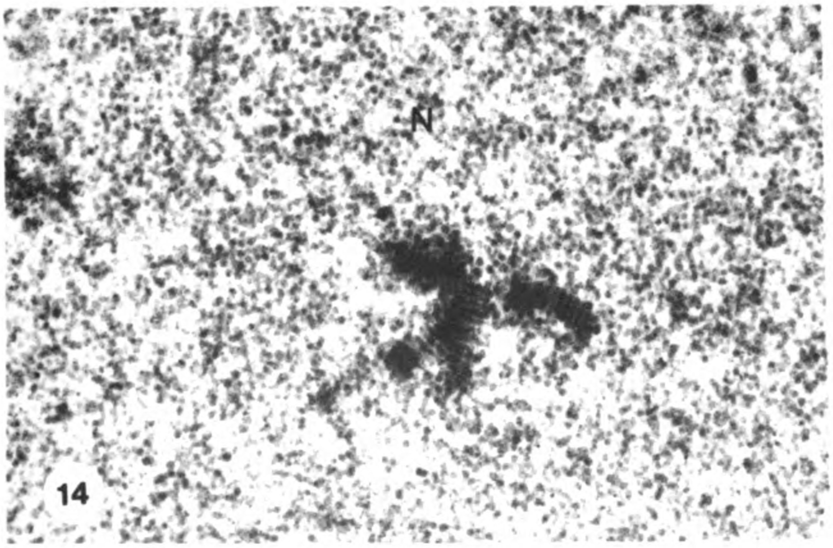

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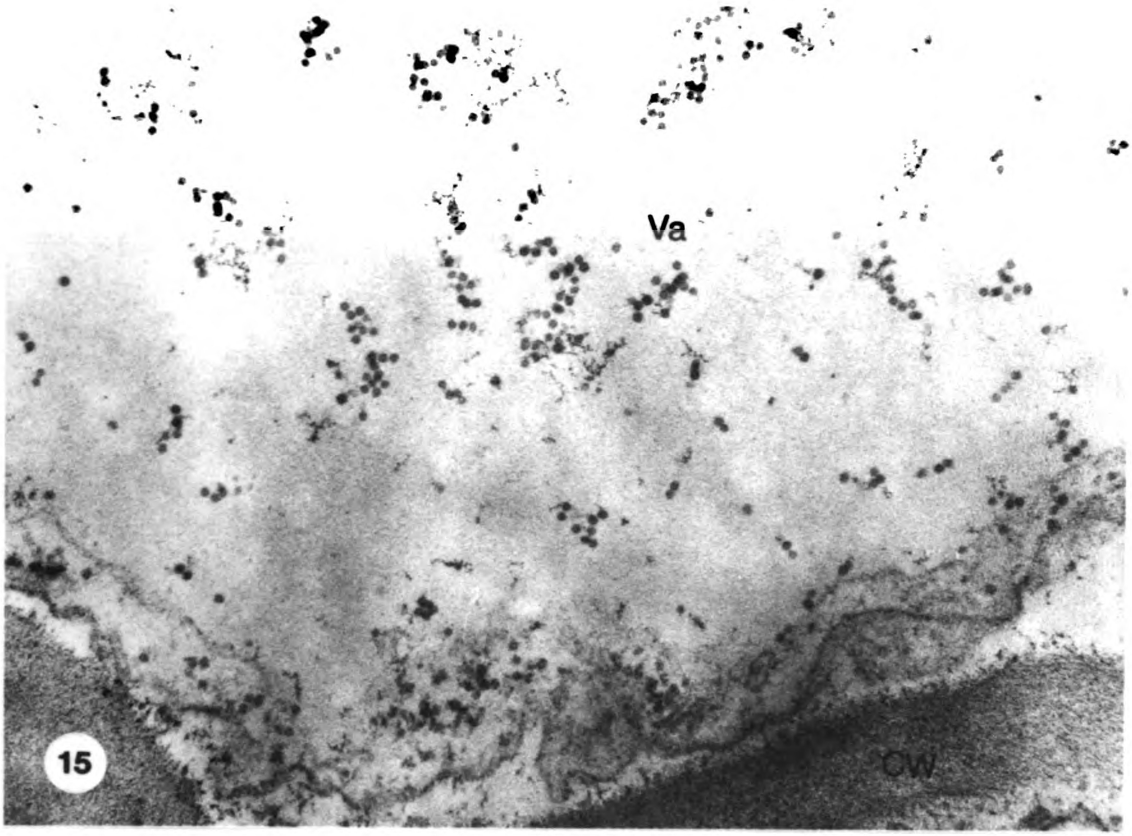
Fig. 15. Central vacuole (Va) in a PEMV infected mesophyll cell. Note PEMV virions in the vacuole showed different electron densities. CW, Cell wall. Magnification: X62,500.

Fig. 16. A NT-PEMV infected mesophyll cell. Virion aggregates (VA) formed in the vacuole (Va). Ch, Chloroplast. ER, Endoplasmic reticulum. N, Nucleus. Magnification: X34,400.


Va
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CW

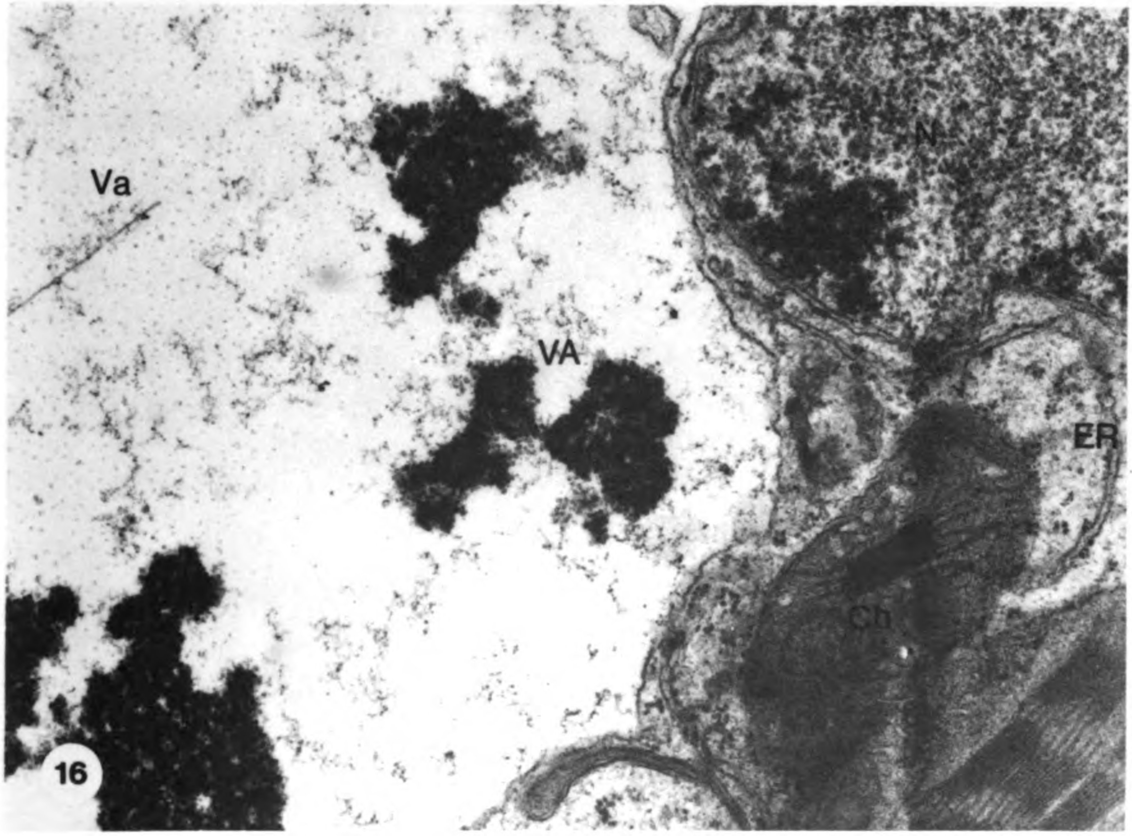
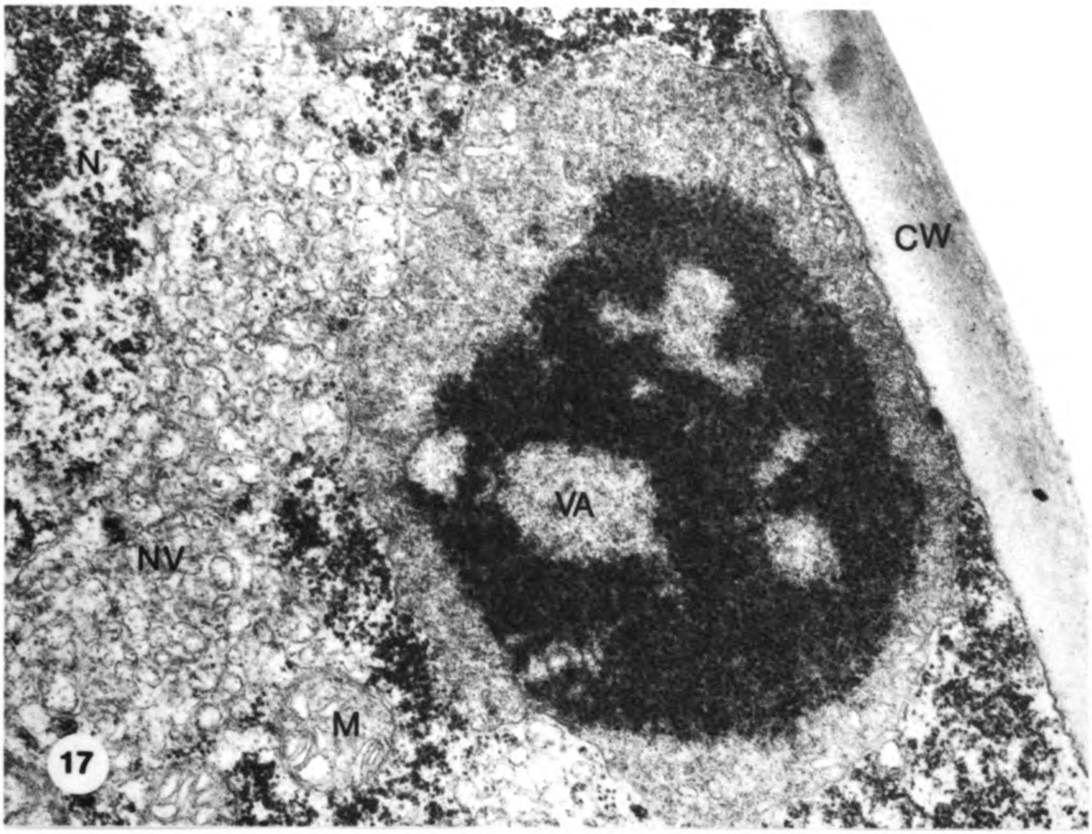

Va
N
VA
ER
Ch
16

Fig. 17. A cross section of an infected mesophyll cell. Accumulation of NT-PEMV virions (VA) formed in the cytoplasm. It was bounded by a membranous layer. CW, Cell wall. M, Mitochondria. N, Nucleus. NV, Nuclear vesiculation. Magnification: X28,800.

Fig. 18. Cytoplasm of a NT-PEMV infected mesophyll cell. Virions formed bands which were arranged in a subparallel manner through the cytoplasm. VA, Viral aggregation. Microtubulelike structures, which were referred to as X-tubules (X), were either in a special arrangement or scattered in the cytoplasm. Magnification: X61,600.



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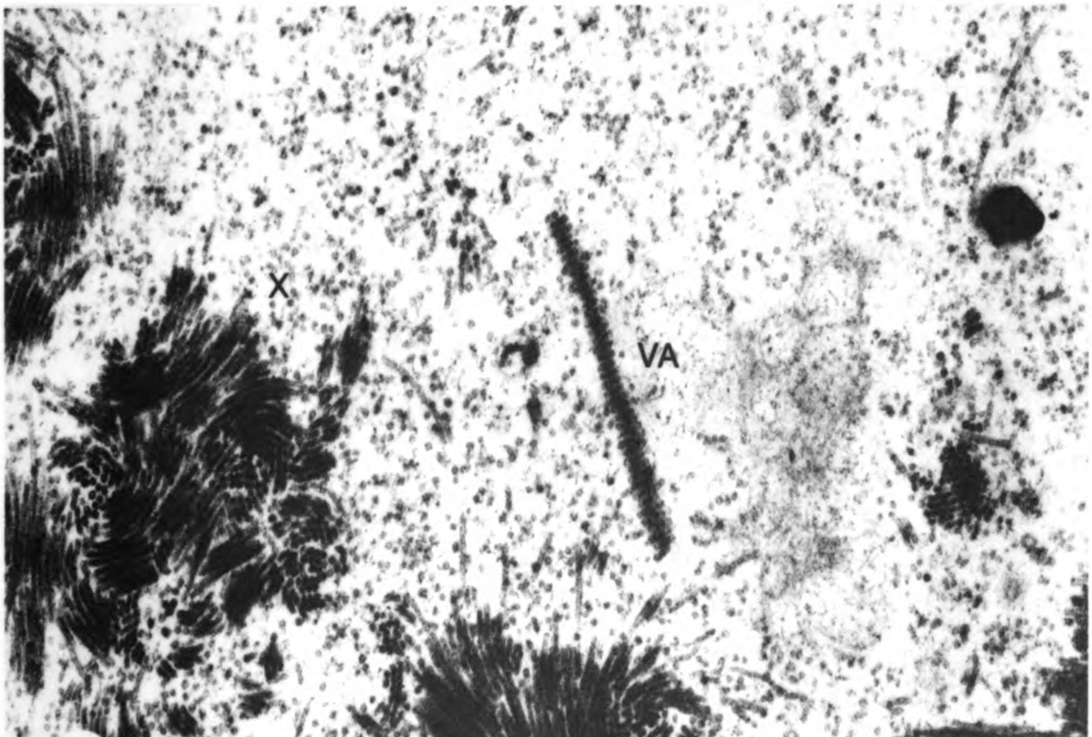

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Fig. 19. A vacuole structure in the central vacuole of NT-PEMV infected mesophyll cell. Magnification: X52,500.

Fig. 20. Various sizes and shapes of vacuole structures in a T-PEMV infected leaf mesophyll cell. Note virions aligned along the vacuoles and also scattered or aggregated inside the vacuoles. Magnification: X19,500.

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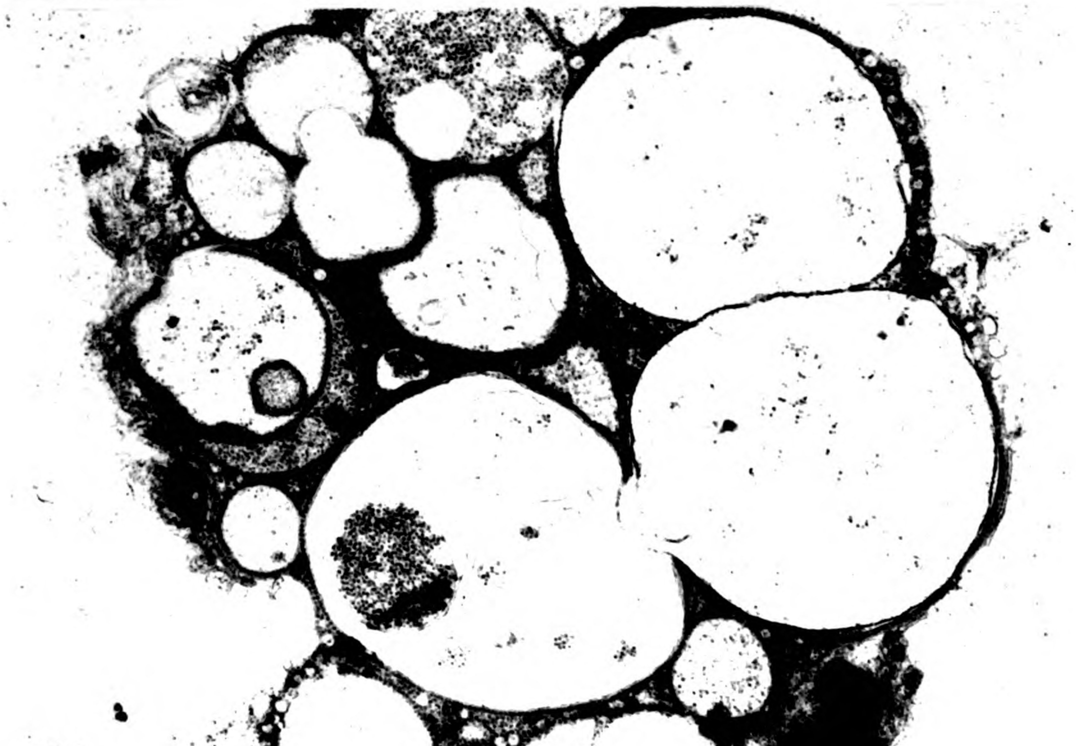

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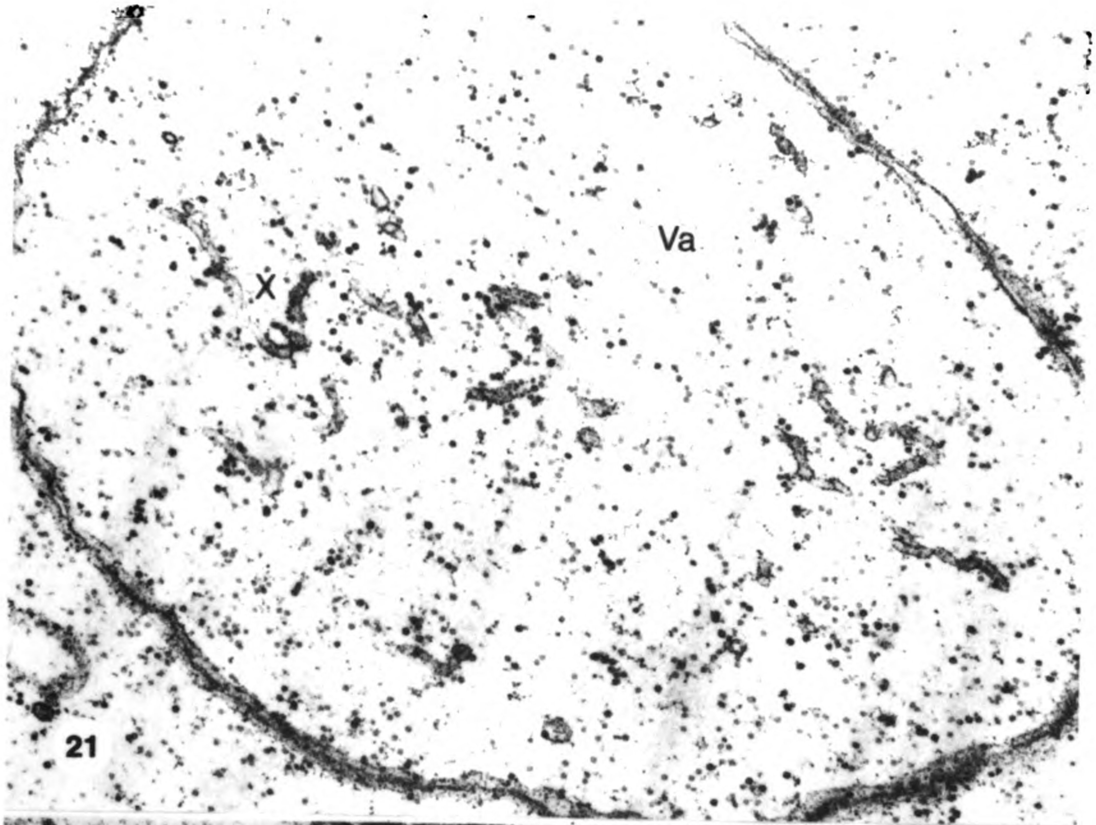
Figs. 21 and 22. Microtubulelike structures (X-tubules) formed in NT-PEMV infected mesophyll cells. Figure 21 shows one type of tubule associated with PEMV virions scattered in a vacuole whereas Figure 22 shows the other type of X-tubule in the cytoplasm. Ch, Chloroplast. Va, Vacuole. VA, Viral aggregate. X, X-tubules. Magnification: X42,000 and X34,400, respectively.


Va
X
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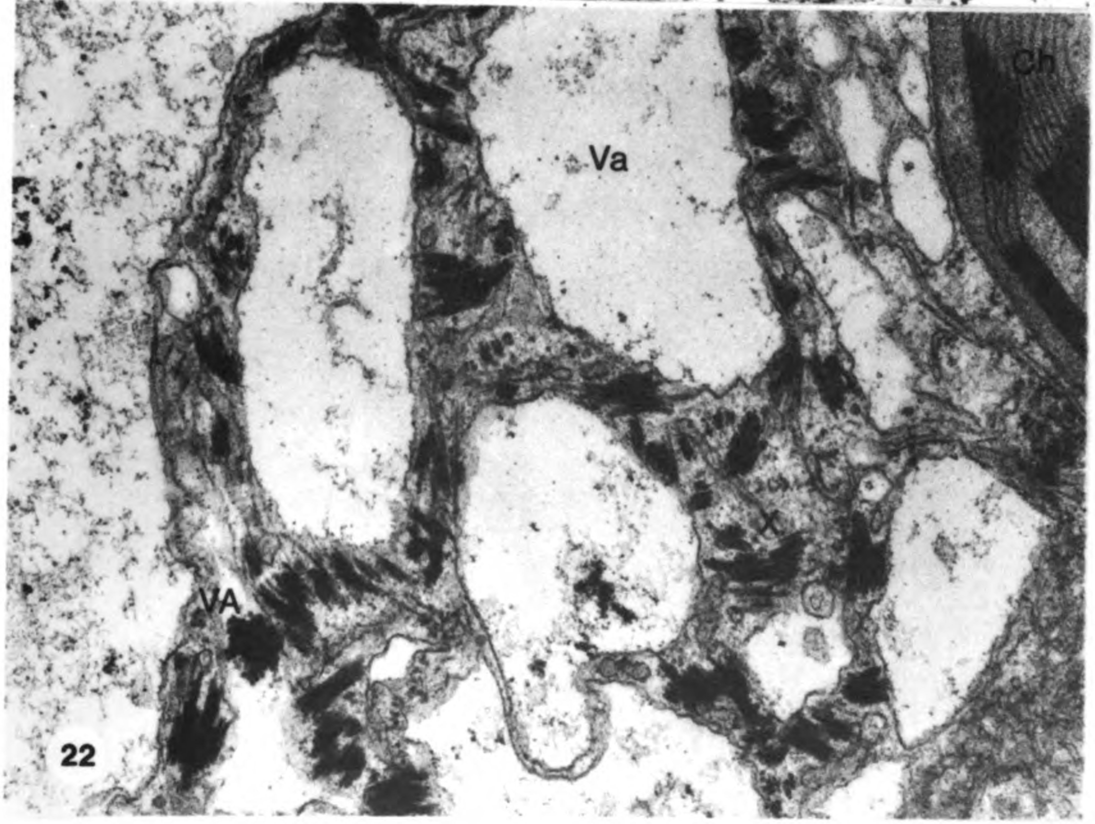
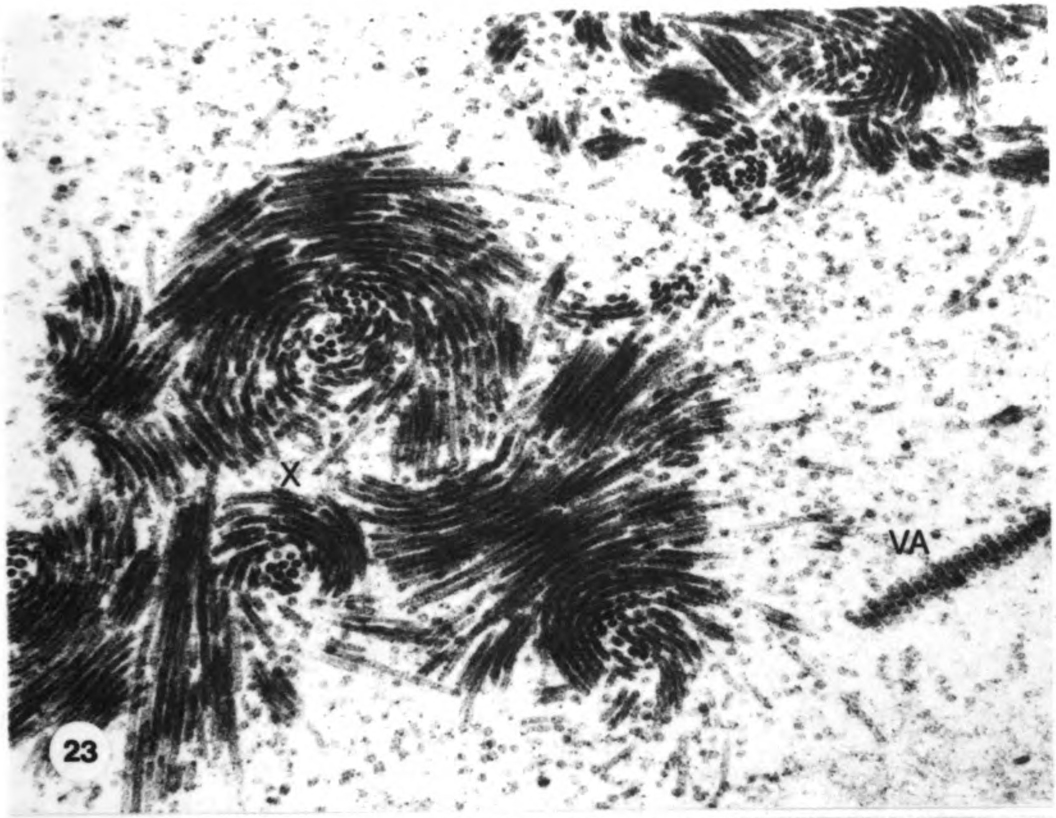

Ch
Va
X
VA
22

Fig. 23. Cytoplasm of NT-PEMV infected mesophyll cell. Aggregates of X-tubules were in an unusual arrangement. Transverse sections of the tubules were situated in the center of the aggregates or scattered in the cytoplasm nearby. VA, Viral aggregation. X, X-tubules. Magnification: X64,000.

Fig. 24. Cytoplasm of NT-PEMV infected mesophyll cell shows some flexuously, irregularly scattered X-tubules (X). Note the ruptured chloroplasts (Ch) associated with virus infection. Magnification: X50,000.



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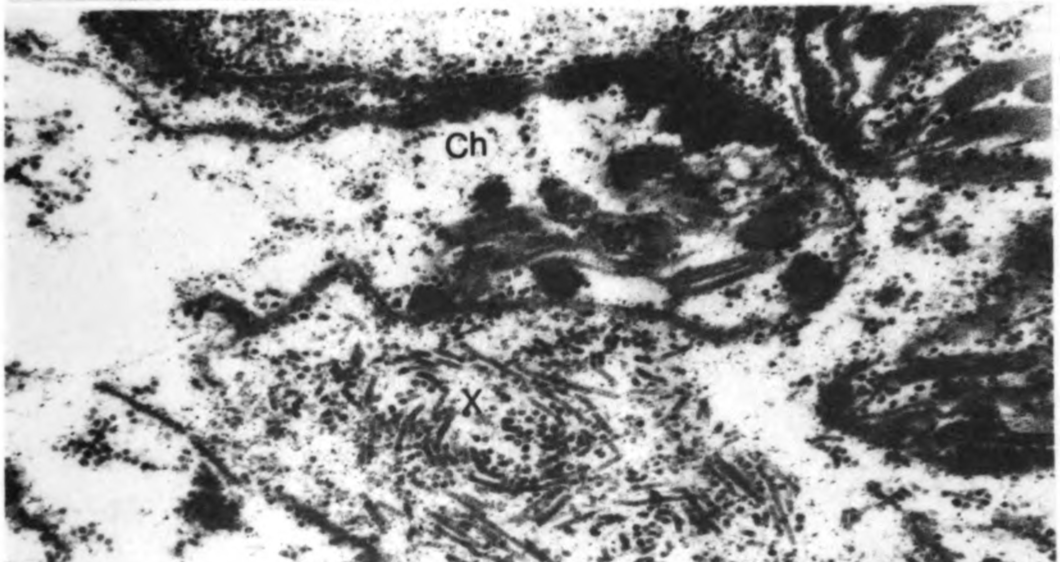
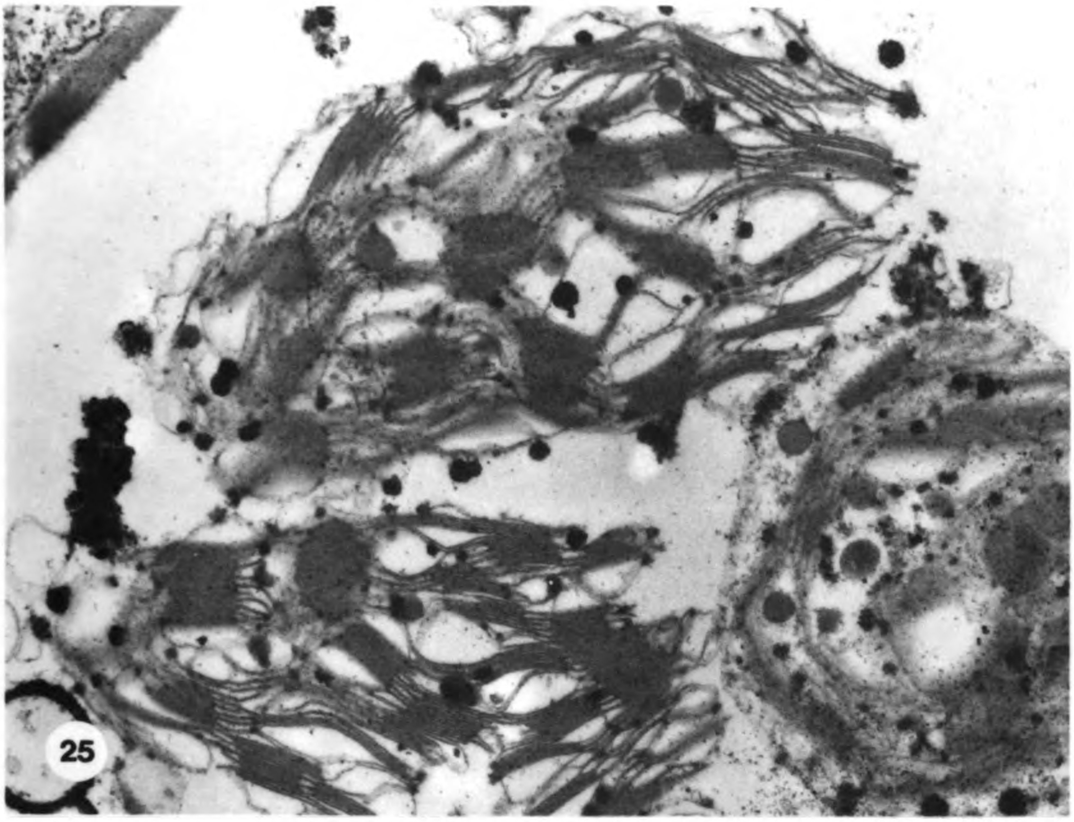


24

Fig. 25. Ruptured chloroplasts caused by PEMV infection appeared in an infected mesophyll cell. Note the unit membranes broke off and the osmiophilic globules dispersed at the periphery. Magnification X26,400.

Fig. 26. Malformed chloroplasts (Ch) associated with highly concentrated virus aggregates (VA) in a NT-PEMV severely infected mesophyll cell. Magnification: X35,200.



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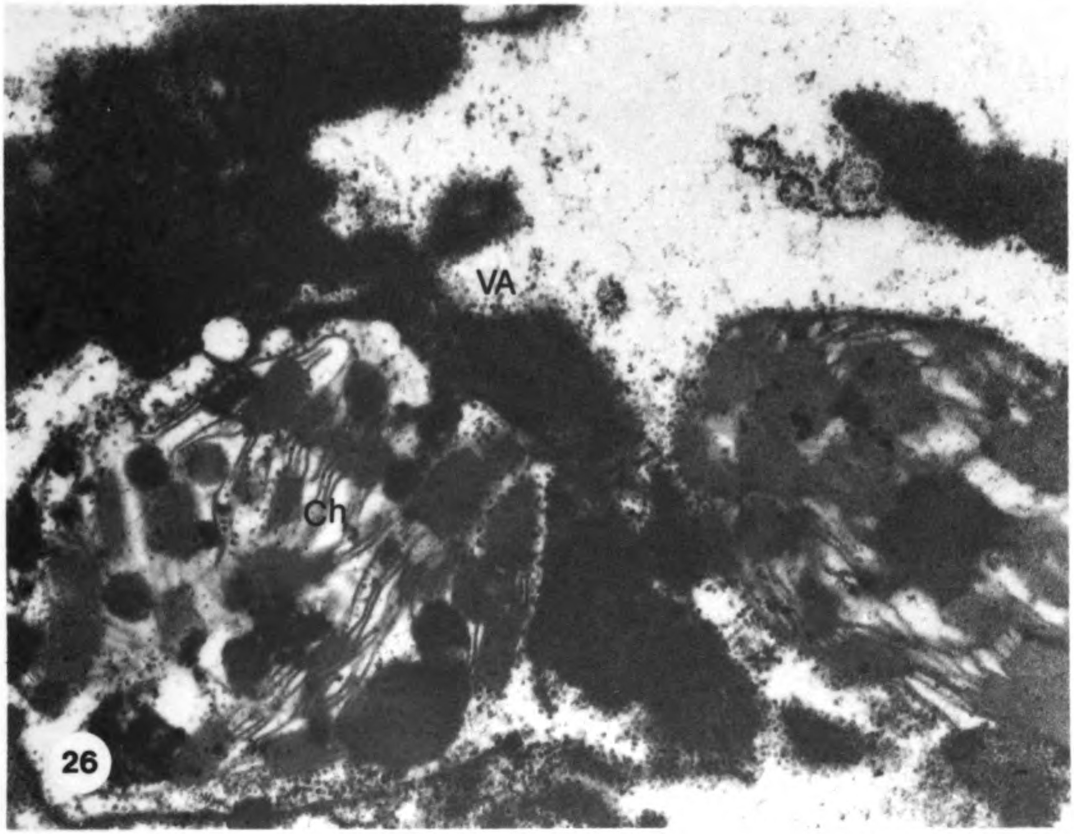
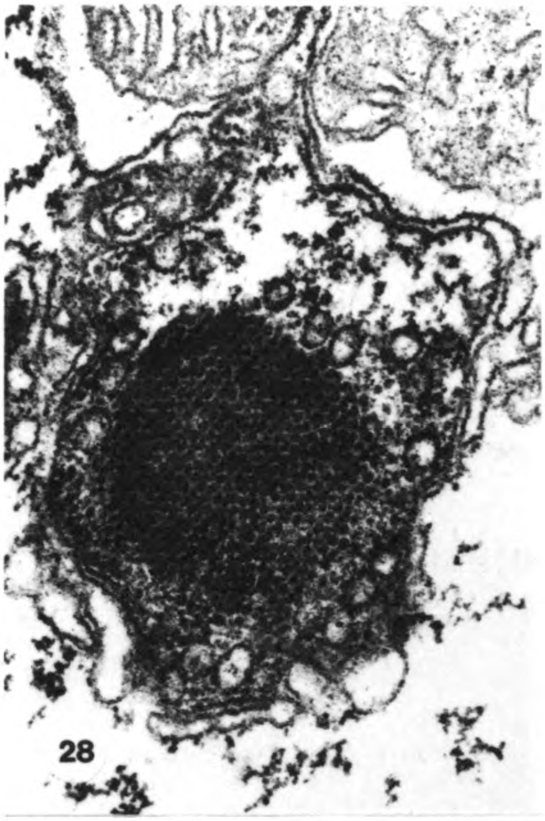


26

Figs. 27 and 28. PEMV microcrystals in vascular bundle sheath cells of PEMV heavily infected pea leaves. Magnification: X65,100.

Fig. 29. Cytoplasm of PEMV infected mesophyll cell. Nuclear vesiculation (NV) was visualized continuously in the infected tissues. Virions can be detected both in the nucleus (N) and among the vesicles. Magnification: X43,200.

27



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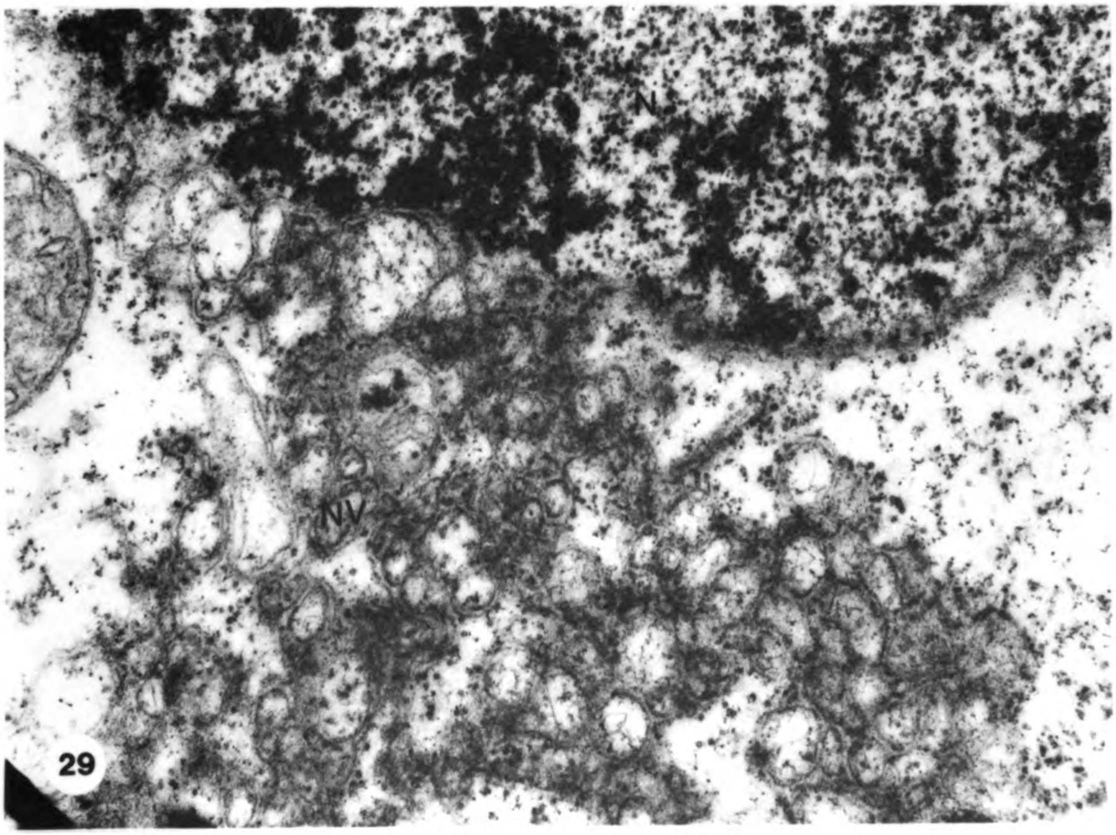

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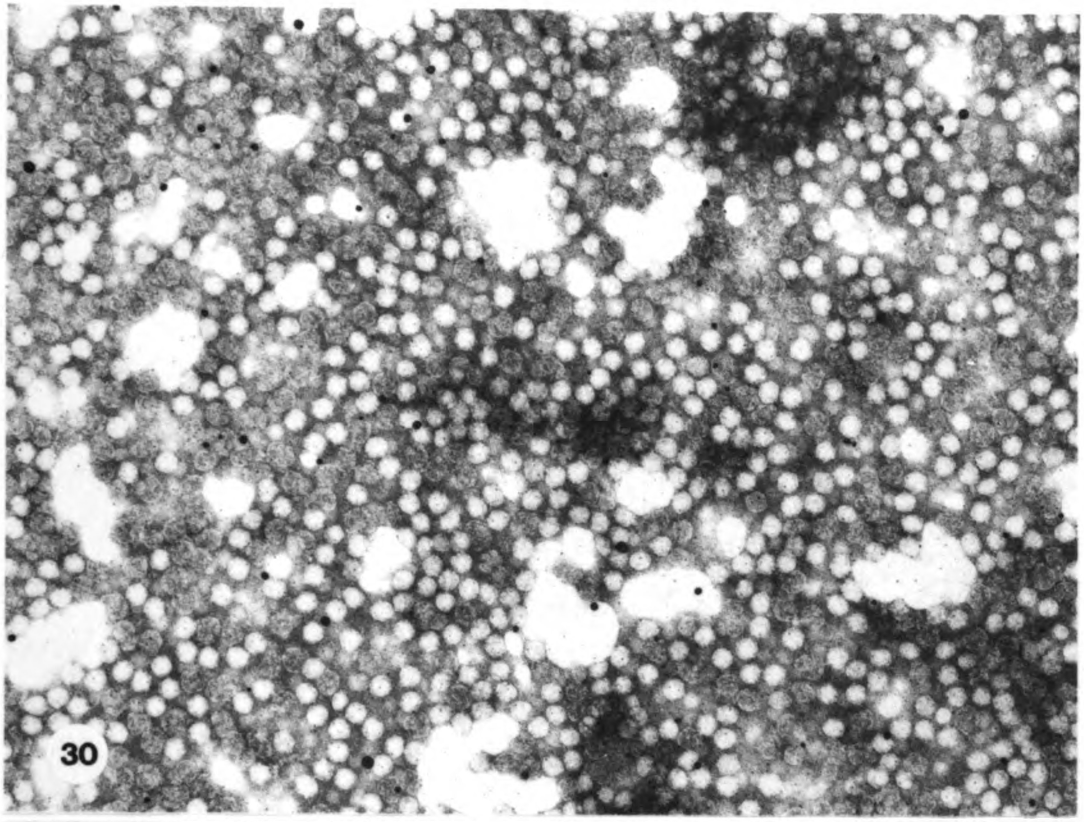
Fig. 30. Highly purified T-PEMV preparation contained distinctly two types of virus particles: one had clear hexagonal outlines, the other was relatively electron opaque and spherical in shape. Magnification: X108,000.

Fig. 31. Highly purified NT-PEMV preparation contained mainly the spherical particles. A few hexagonal particles (arrows) also were present. Magnification: X108,000.



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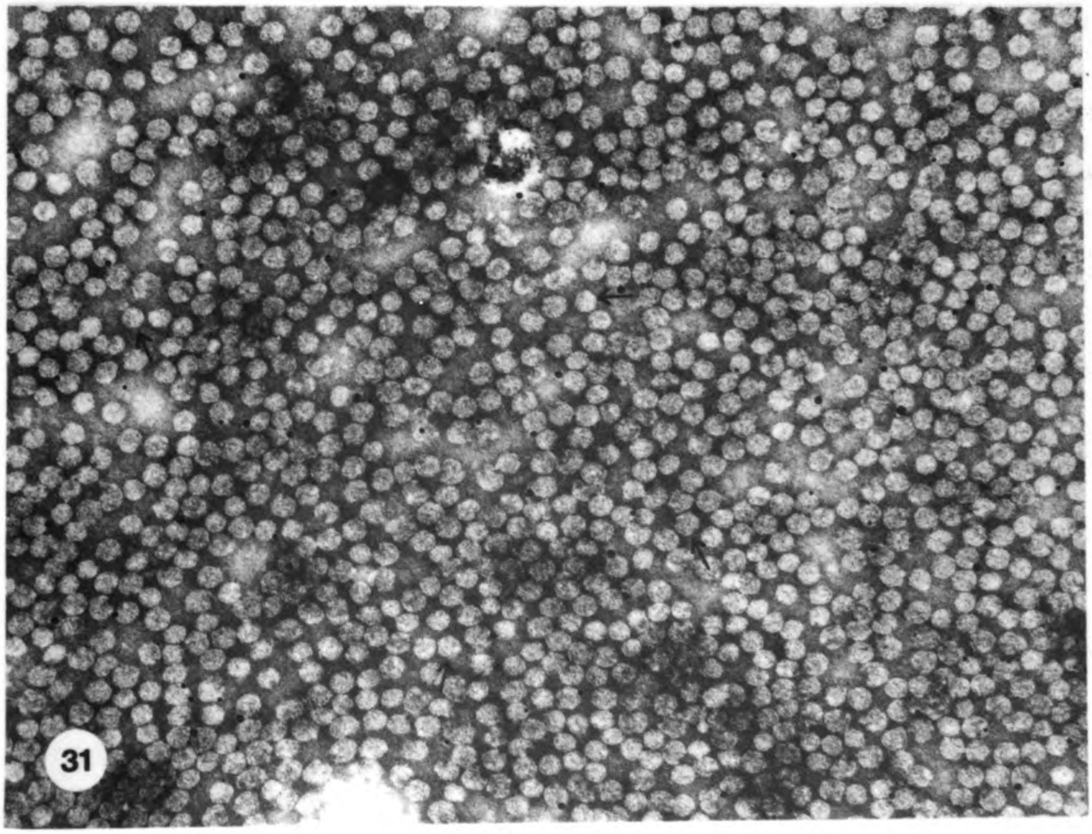
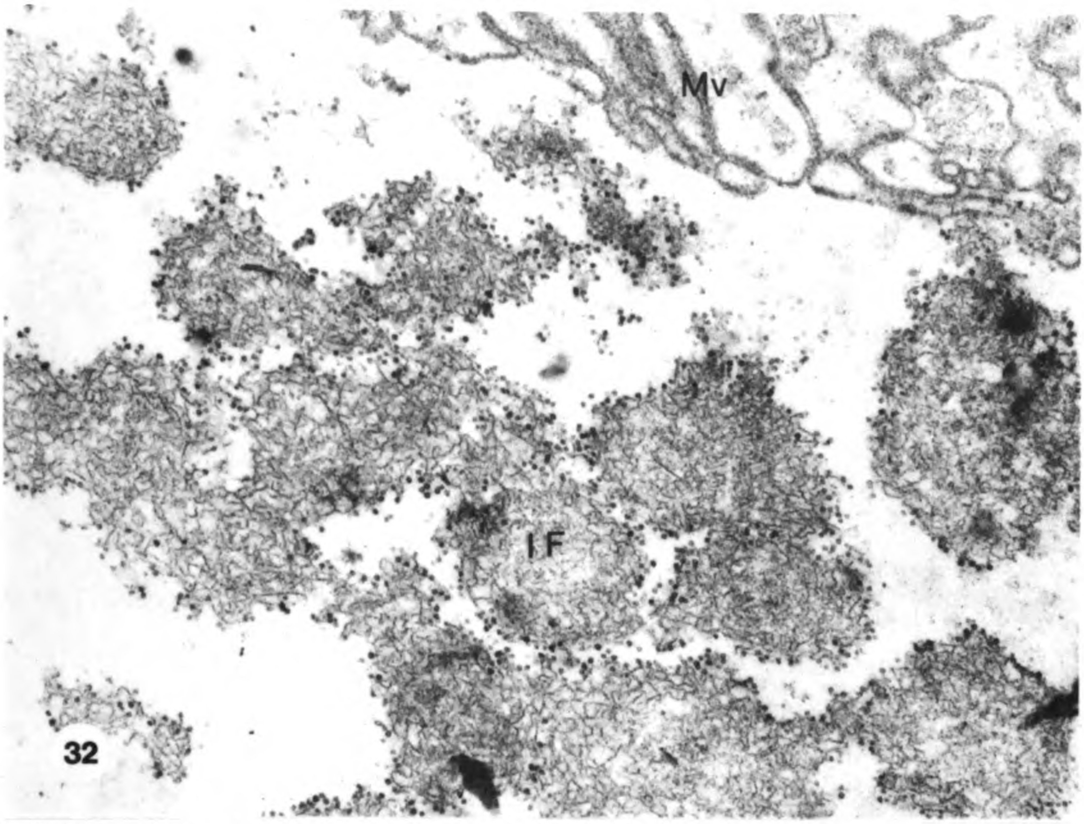


31

Figs. 32 and 33. PEMV virions in the gut lumen of viruliferous aphids. Virions scattered either along the margin (Fig. 32) or within (Fig. 33) the ingested food material (IF). Note the virions show different electron densities. Also, Figure 32 shows a number of small food masses whereas figure 33 shows a large food ball. Magnification: X42,000 and 50,000, respectively.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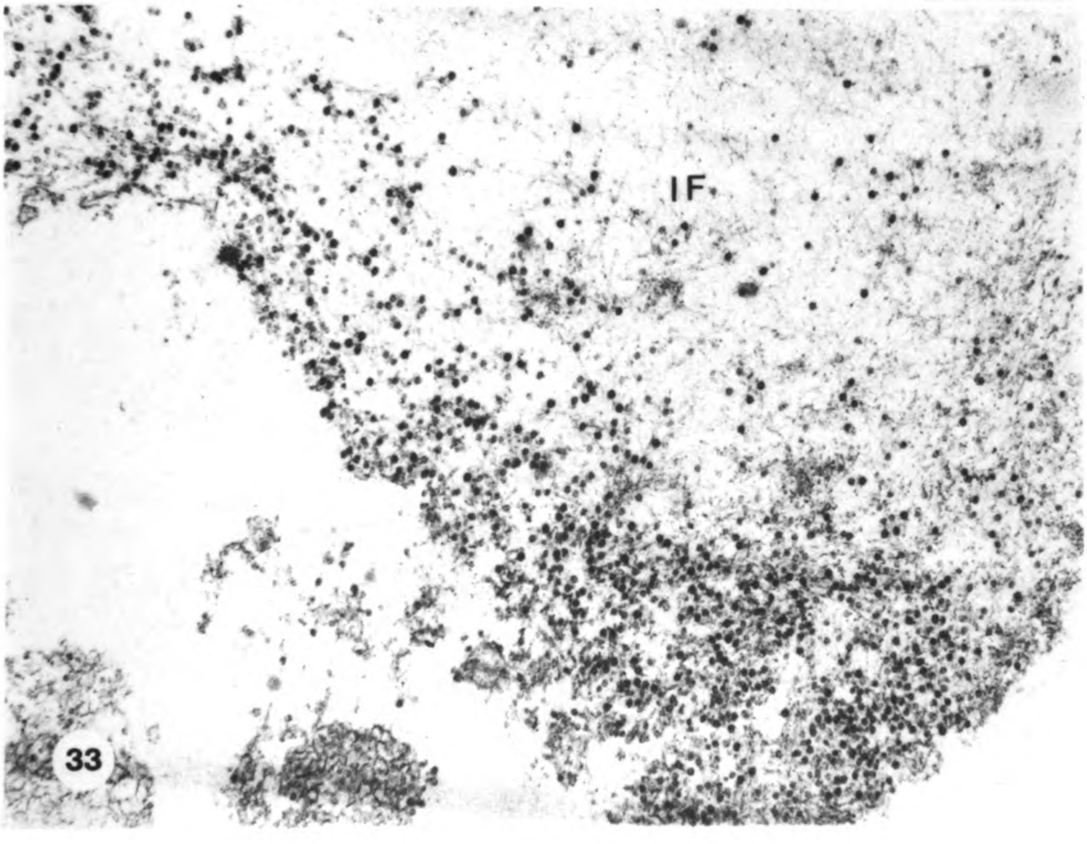
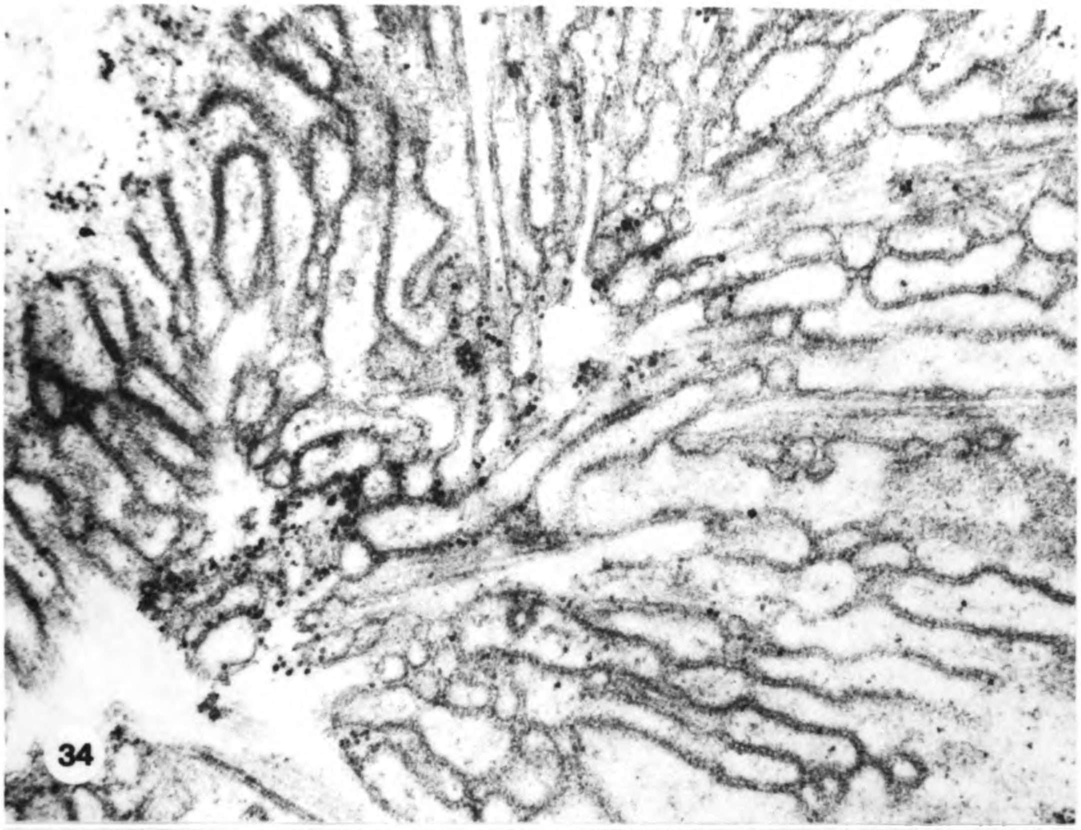

33

Fig. 34. Microvillous borders in association with PEMV virions in the gut of a viruliferous aphid. Magnification: X41,250.

Fig. 35. Viroplasmlike structures in a midgut epithelial cell of a T-PEMV infected aphid. The virions located at the periphery or in the central region of electron-dense area. Magnification: X48,750.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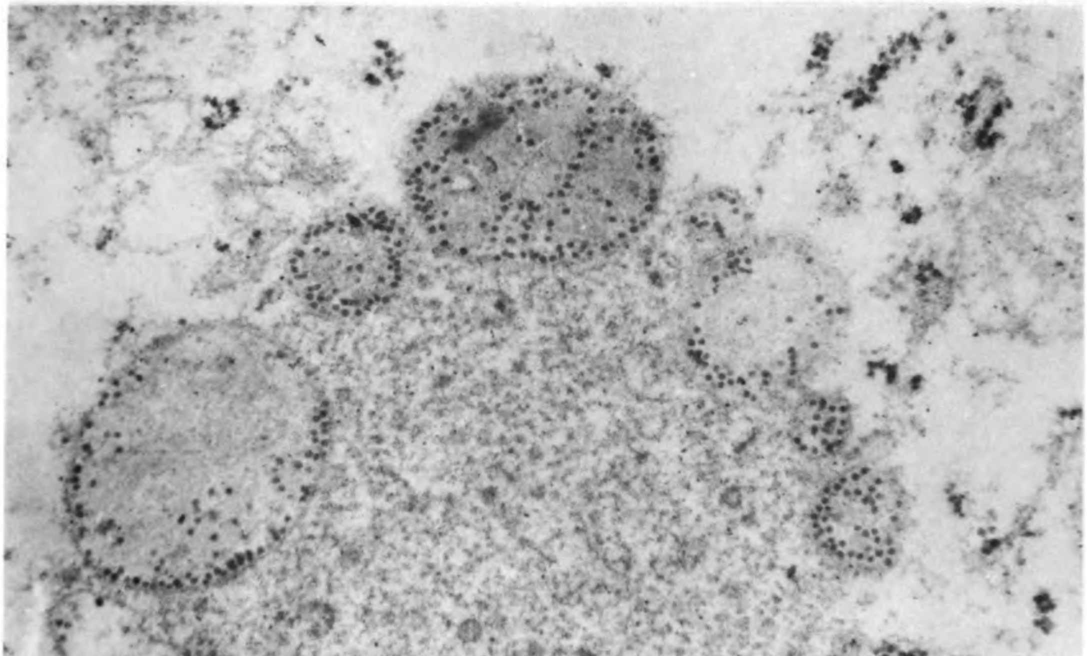
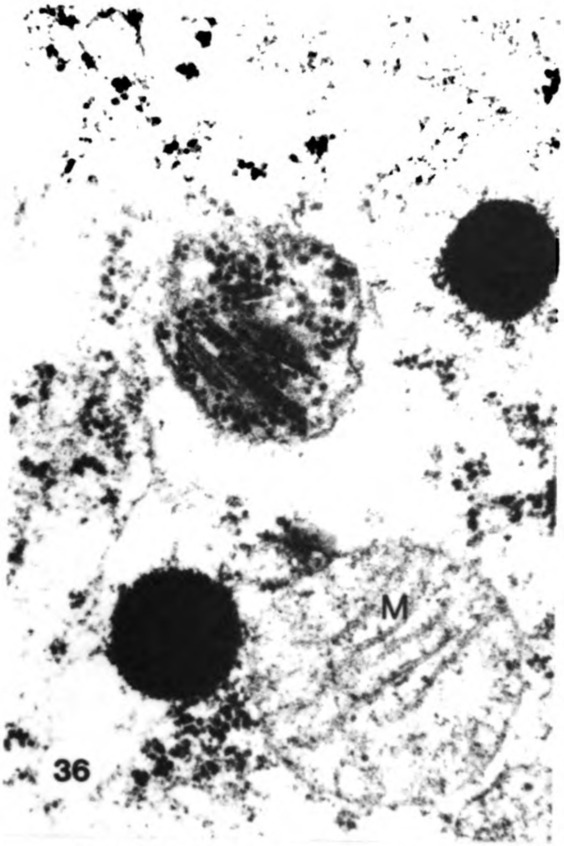

35

Figs. 36 and 37. Some defined structures in the midgut epithelial cell of viruliferous aphids. Note numerous T-PEMV virions aggregated in these structures. M, Mitochondria. Magnification: X50,000 and X58,750, respectively.

Figs. 38 and 39. Membrane-bound virions in the T-PEMV infected epithelial cells. Magnification: X40,950 and X64,000, respectively. .



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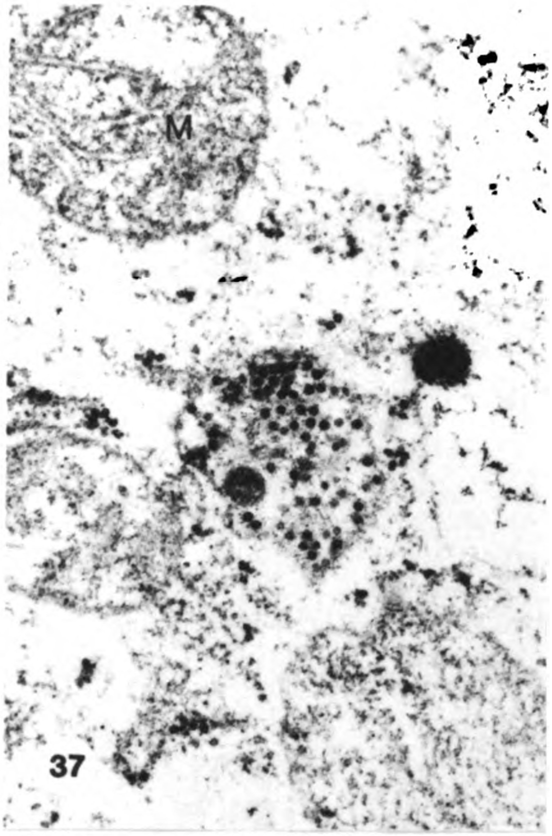

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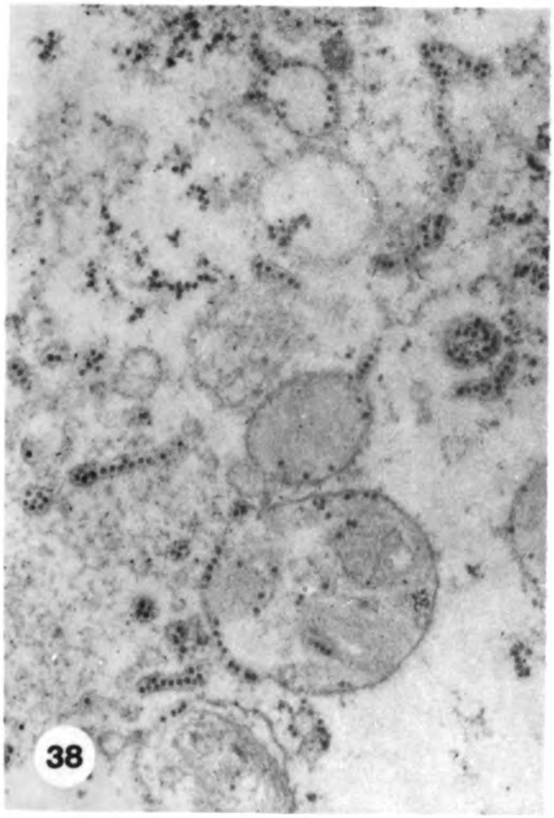

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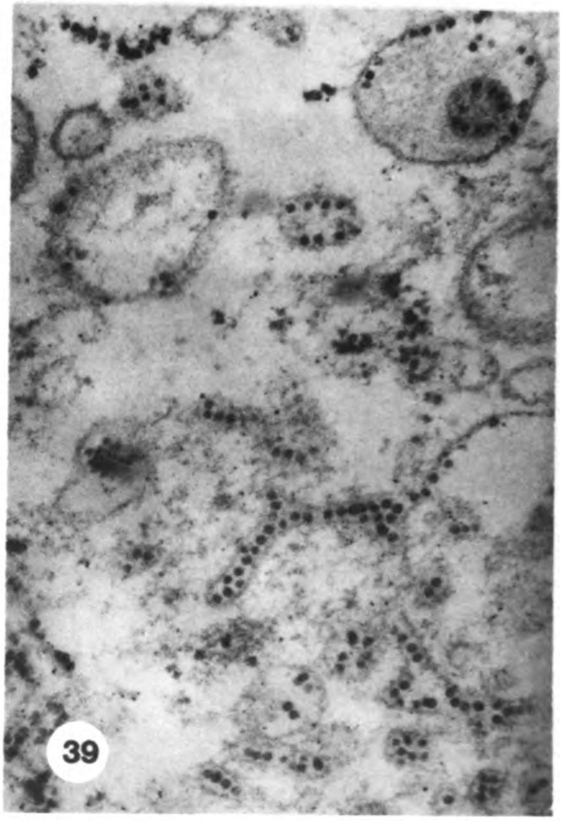
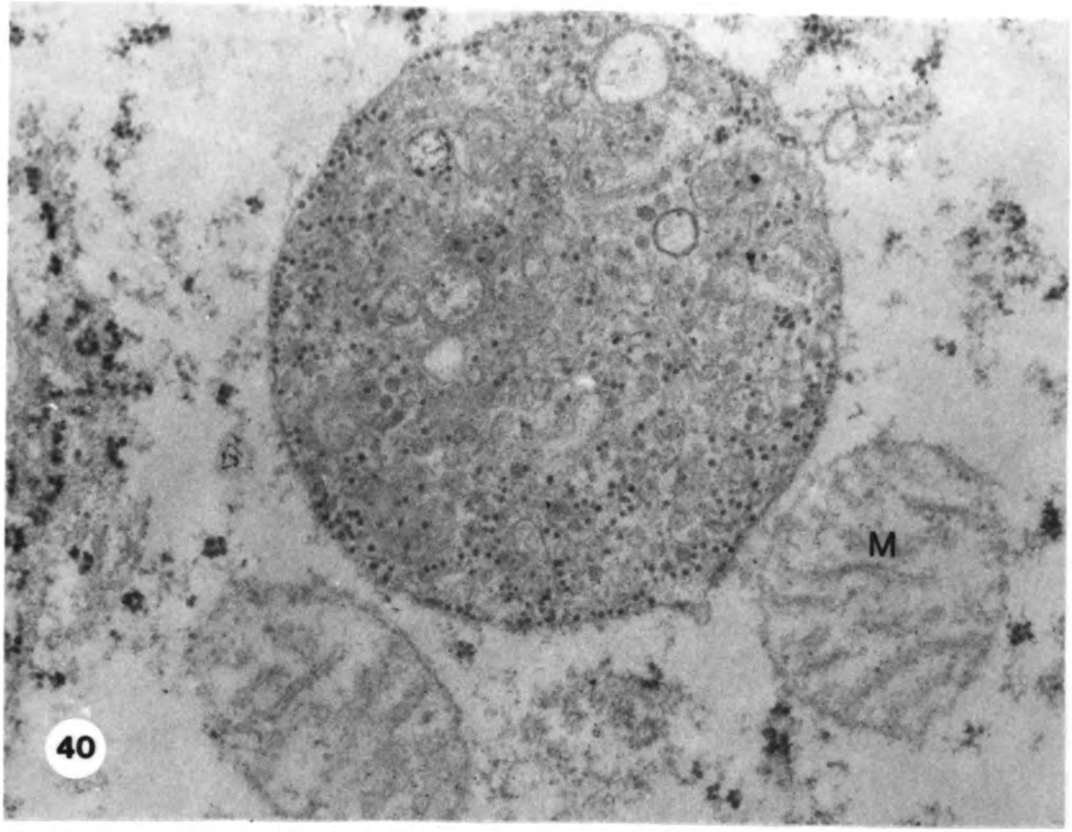


39

Fig. 40. T-PEMV virions in an unidentified structure in an infected epithelial cell. M, Mitochondria. Magnification: X49,350.

Fig. 41. A viroplasmlike structure with NT-PEMV virions (arrows) in a midgut epithelial cell. Magnification: X58,750.



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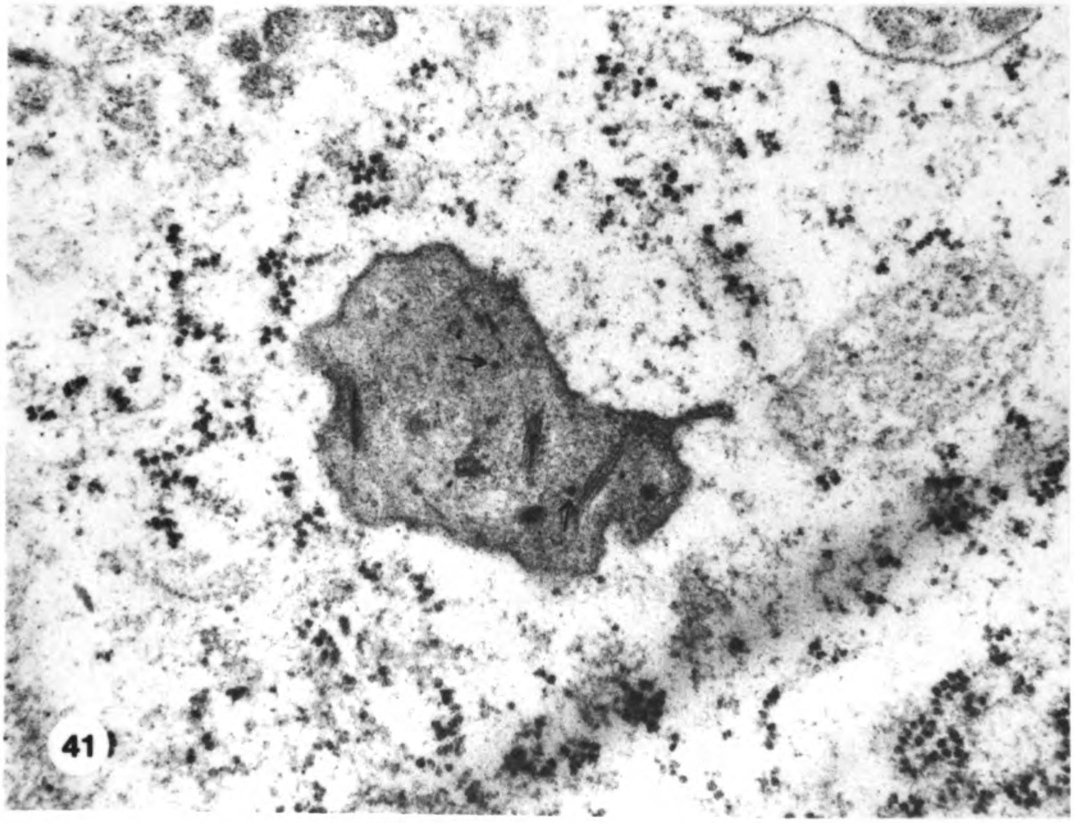
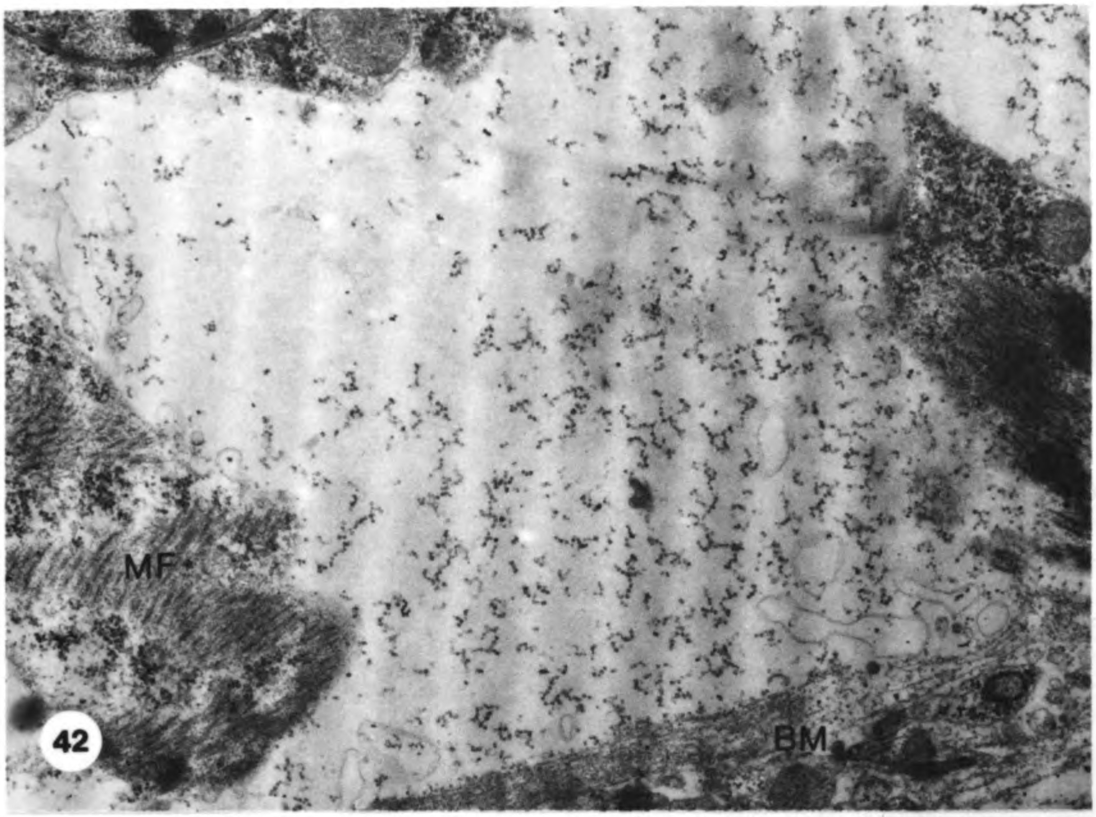


41

Fig. 42. A ruptured midgut muscle fiber cell (MF). T-PEMV viruslike particles scattered in the cell. Similar particles also can be seen in the basement membrane (BM) of midgut. Magnification: X23,500.

Fig. 43. A hemocyte adjacent to the midgut epithelial cell (E). Large numbers of T-PEMV viruslike particles appeared in the nucleus (N). Note an aggregation (Ag) was formed in the nucleus. These particles also were observed in the pinocytic vesicles (PV). Magnification: X26,875.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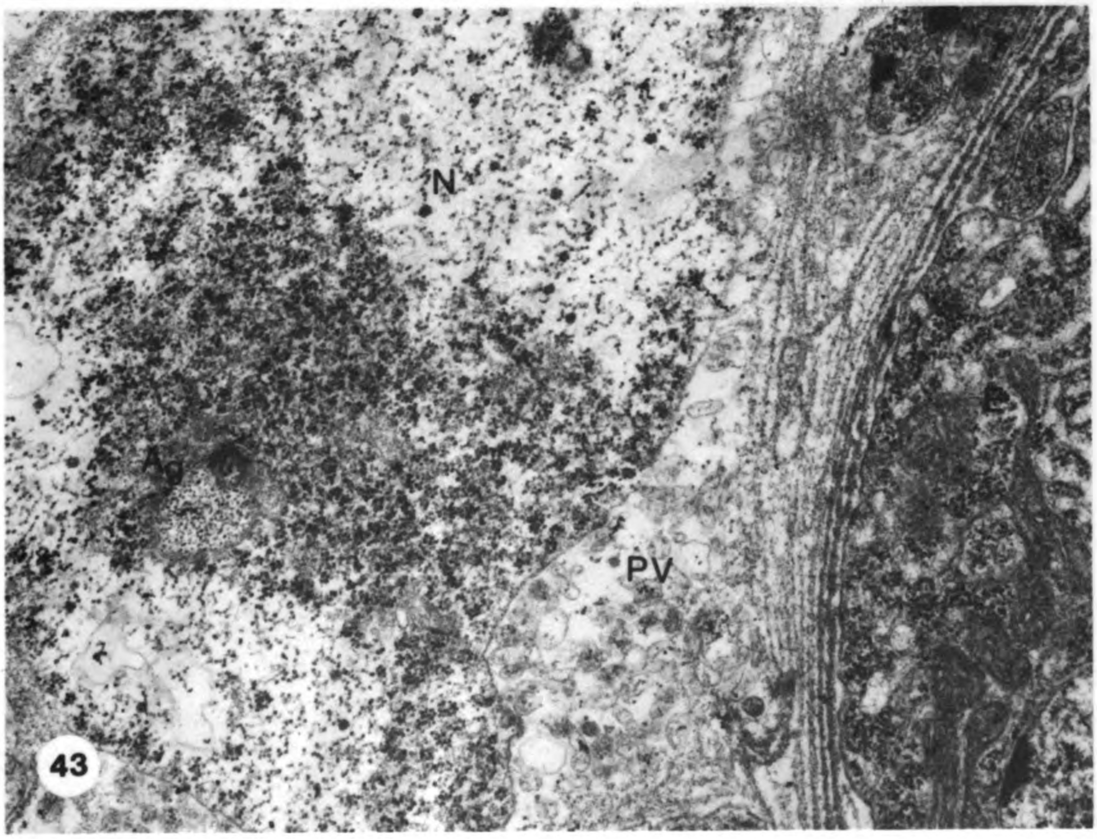
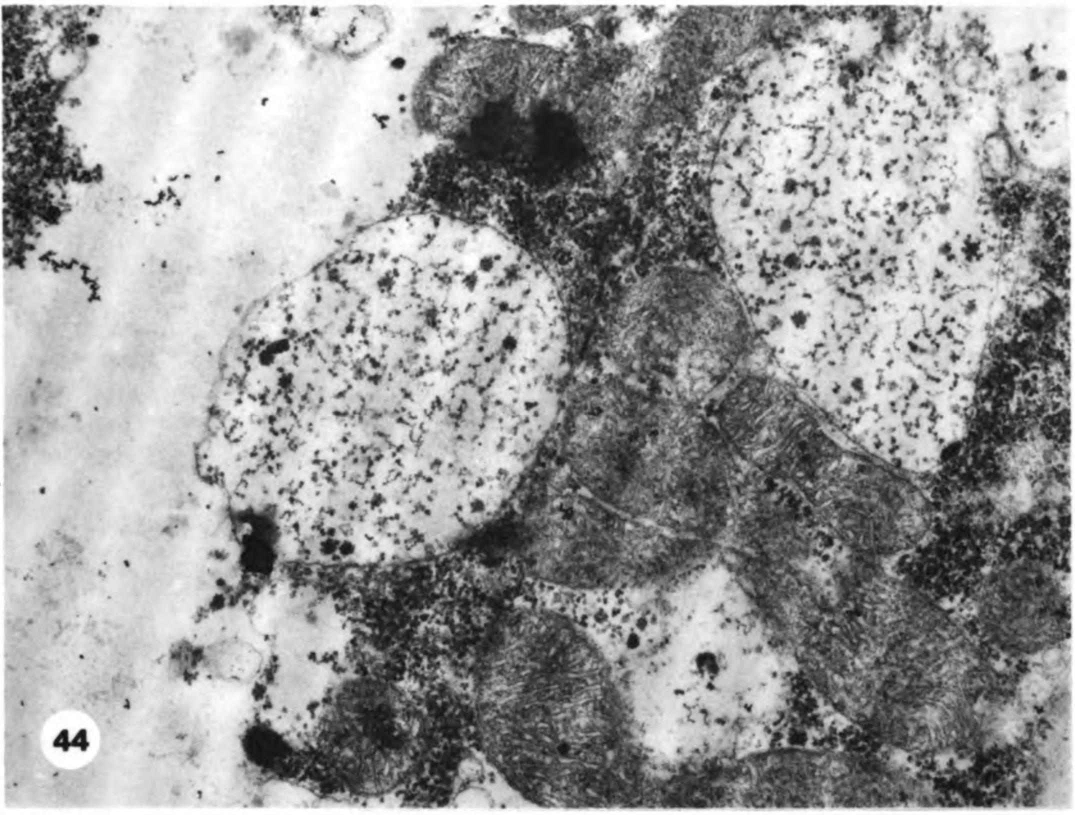

43

Fig. 44. Degenerated fat body cell containing viruslike particles in the hemocoel of T-PEMV infected aphid. Magnification: X28,800.

Fig. 45. A vacuole containing viruslike particles free in the hemocoel. It seemed to be released from degenerated fat body. Magnification: X36,000.



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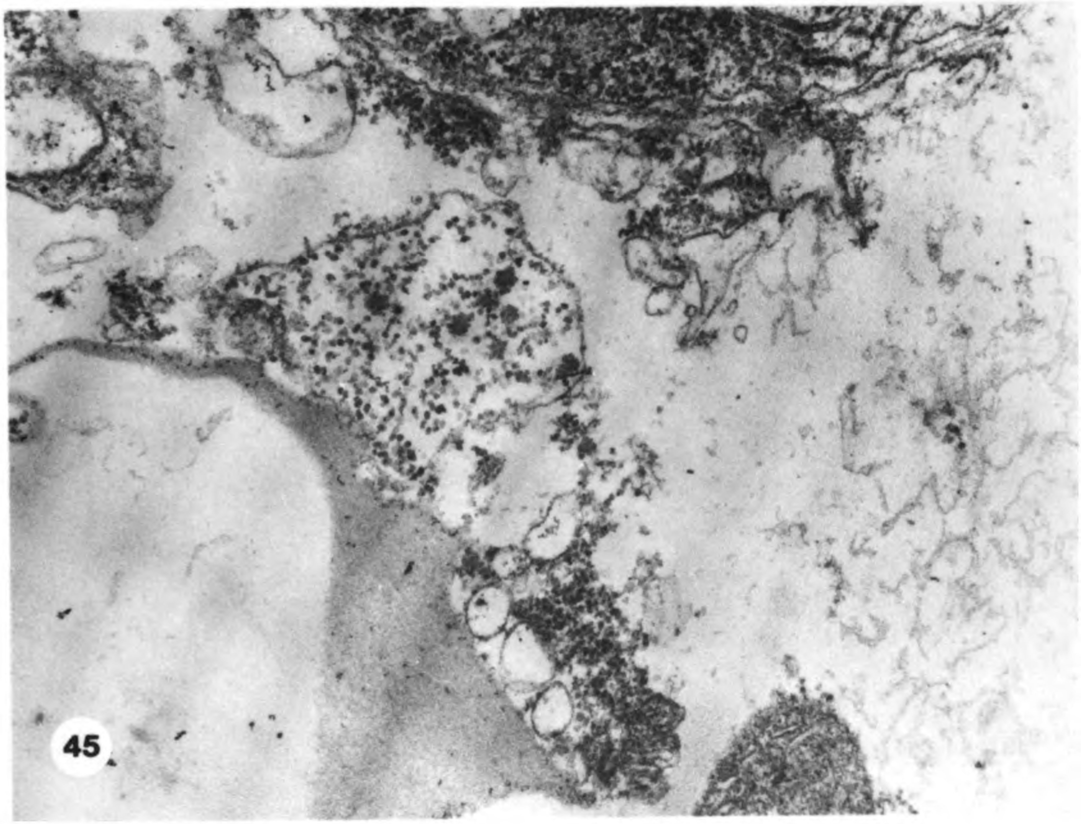

45

# LIST OF REFERENCES

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