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Double membrane-bound particles associated with eriophyid mite-borne plant diseases of unknown etiology : a potentially new group of plant viruses?

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ABSTRACT

Unique virus-like particles were associated with five eriophyid mite-borne plant diseases of unknown etiology; fig mosaic, redbud yellow ringspot, rose rosette, thistle mosaic, and high plains disease of corn and wheat. Quasi-spherical, double membrane-bound particles (DMPs), 120 - 200 nm in diameter, were observed in the cytoplasm of all cell types in symptomatic leaves of infected plants. No DMPs were observed in symptomless plants. The DMPs in symptomatic thistles were associated with two types of inclusions, electron-dense amorphous material and tubular aggregates. Similar amorphous inclusions were also found in corn and wheat with high plains disease, while tubular inclusions were observed in figs with mosaic symptoms. The particles and inclusions were similar in some aspects to immature particles associated with viroplasms of animal and insect poxviruses and also to the double-enveloped particles of tomato spotted wilt virus associated with viroplasms during early stages of infection, but were unique and unlike any known plant viruses. The DMPs and associated viroplasm-like inclusions in the high plains disease were specifically immunogold labeled *in situ* with the disease-specific antiserum. Thread-like structures, similar to tenuivirus particles, present in the partially purified virus preparations were also immunogold labeled with the antiserum. It is suggested that the thread-like structures are derived from the DMP. In many cells of symptomatic corn and wheat samples, DMPs occurred together with flexuous rod-shaped particles and cylindrical inclusions of wheat streak mosaic potyvirus (WSMV), suggesting that the disease is caused by a mixed infection of WSMV and the agent represented by the DMPs. Based on cytopathology, symptomatology and mite and/or graft-transmissibility, the five diseases described in this paper are potentially caused by virus(es) and the DMPs associated with these diseases may represent virus particles. If the DMPs are indeed viral in nature, they would comprise a new group of plant viruses.

INTRODUCTION

A number of eriophyid mites, that are very small phytophagous arthropods, have been known to transmit a number of plant pathogenic viruses and virus-like agents (Slykhuis 1980; Matthews 1991). Of the eriophyid mite-vectored viral or virus-like agents only a few potyviruses have been characterized (Oldfield 1970; Slykhuis 1980). These potyviruses are agropyron mosaic virus, ryegrass mosaic virus and wheat streak mosaic virus, which were recently classified as members of the genus rymovirus of Potyviridae (Barnett 1991; Ward & Shukla 1991). Other disease agents naturally transmitted by eriophyid mites have not been characterized. Black currant reversion, fig mosaic, peach mosaic, pigeon pea sterility, and wheat spot mosaic diseases are members in this group and a viral etiology has been suggested for each of them based on symptomatology and transmissibility by grafting and/or mite vectors (Gibbs 1969; Oldfield 1970; Slykhuis 1980).

With two of these diseases, fig mosaic and wheat spot mosaic, an association of unusually large, ovoid, double membrane-bound bodies of 100-200 nm in diameter has been demonstrated (Bradfute & Nault 1969; Bratfute et al. 1970; Nault et al. 1970; Plavsic & Milicic 1980; Appiano 1982; Hiruki 1989). The nature of these bodies is, however, still unknown, probably because of the difficulty in purifying them from diseased plants for further characterization. Three additional diseases that are associated with double membrane-bound particles (DMPs), redbud yellow ringspot (Kim & Martin 1978), rose rosette (Gergerich & Kim 1983) and thistle mosaic (Ahn et al. 1993), have been discovered. The particles, which were structurally indistinguishable from those associated with fig mosaic and wheat spot mosaic diseases, were unique and unlike any known plant viruses. In addition, the thistle mosaic disease exhibited striking cytopathic effects including viroplasmic inclusions that are similar, in certain aspects, to those of some plant and animal viruses (Ahn et al. 1993; Kim et al. 1995).

Recently, a new virus-like disease affecting corn was identified in several locations in the high plains region of the central and western United States including the Texas panhandle, western Kansas, northern Colorado and central Idaho (Jardine et al. 1994; Jensen & Lane 1994). The disease was also observed to affect wheat, most varieties of which seemed to be highly susceptible. Under field conditions, this disease, named as high plains disease (HPD) of corn and wheat, was more severer on wheat than on corn (Jensen 1994; Jensen & Lane 1995). The agent(s) of HPD was not mechanically transmitted, but demonstrated to be transmitted by wheat curl eriophyid mite, *Aceria tosichella* Keifer (Jensen et al., 1996), which is the well-known vector of wheat streak mosaic potyvirus (WSMV) (Brakke 1971). Initial attempts to identify the disease agent as a possible virus through transmission and serological tests revealed the presence of WSMV in most field-collected samples, however, an additional viral agent was suspected in this disease because typical HPD symptoms were much severer than those caused by WSMV

alone (Jensen & Lane 1995; Jensen et al. 1996). Polyacrylamide gel electrophoresis of proteins in the partially purified virus preparations from symptomatic corn leaf tissues revealed a distinct band of 32 kilodaltons (kd) protein, that is in the size range of coat proteins of tenuiviruses, in addition to a 44 kd coat protein of WSMV. Moreover, thread-like structures resembling tenuivirus particles were observed in the 32 kd protein-containing fraction of sucrose gradients loaded with partially purified virus preparations and, therefore, it was suggested that the HPD is caused by a new tenuivirus in addition to WSMV (Jensen & Lane 1994; - 1995). Electron microscopic examination of infected corn and wheat leaf specimens, however, revealed the presence of DMPs and associated inclusions that are similar to those reported in the diseases mentioned above (Ahn et al. 1995; - 1996).

It appears that diseases associated with DMPs have become more common since the first report by Bradfute and Nault in 1969. These diseases affect a variety of plants, ranging from grasses to trees, some of which are economically important. This paper compares the ultrastructural aspects of DMPs and inclusions associated with the diseases, and reports the immunogold labeling of DMPs in the high plains disease using the disease-specific antiserum. Cytopathological evidence that the DMPs are viral in nature and the possibility that they may represent a new group of plant viruses are discussed.

MATERIALS AND METHODS

Plant sources. Redbud (*Cercis canadensis* L.), multiflora rose (*Rosa multiflora* Thumb.), and field thistle (*Cirsium discolor* (Mull) Spreng) showing yellow ringspot, rosette, and mosaic symptoms, respectively, and symptomless, presumably healthy plants were collected in northwest Arkansas, USA. Seeds from symptomless redbud and thistle were germinated to produce healthy plants under greenhouse conditions. Fig (*Ficus* spp.) with mosaic symptoms was obtained from California, USA, and symptomatic figs were propagated vegetatively from stem cuttings and maintained in the greenhouse. Corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.) expressing symptoms of high plains disease were collected from several locations in the high plains of central and western USA. Symptomatic corn and wheat plants were also obtained by exposing healthy seedlings to a number of wheat curl eriophyid mites (*Aceria tosichella* Keifer), that had been collected from wheat with high plains disease. Leaf symptoms of the diseases are shown in figure 1.

Partial purification of HPD agent and production of antiserum. Based on the initial hypothesis that the additional disease agent might be a new tenuivirus (Jensen & Lane 1994), the purification method for maize stripe tenuivirus (Falk & Tsai 1984) was used in early

attempts to purify the disease agent from symptomatic corn leaf tissues. After DMPs were found in leaf cells of HPD-affected plants, the purification method was modified by omitting Triton X-100 treatment to preserve the particle membranes and obtain intact DMPs. The partially purified virus preparatrions concentrated from the 32 kd protein-containing fraction of sucrose gradients were used to produce an antiserum. A total of 3 injections, 1 mg each, of the virus preparation were done intramuscularly into rabbits on a biweekly schedule. Blood was collected one week after the final booster injection, and the resulting serum was treated as the HPD-specific antiserum.

Electron microscopy. Small pieces of leaf tissue (1-2 mm^2) taken from symptomatic and healthy plants were fixed in a modified Karnovsky's fixative (Kim & Fulton 1984) for 2 h at room temperature (RT) under a low vacuum. The specimens were postfixed in 1% osmiun tetroxide for 2 h, *en bloc* stained overnight in 0.5% aqueous uranyl acetate at 4℃ and dehydrated in an ethanol series before they were embedded in Spurr's low viscosity medium (Spurr 1969). Embedded tissues were sectioned with a diamond knife and double stained with 2% aqueous uranyl acetate for 5 min and lead citrate for 2 min before examination under a JEOL 100 CX electron microscope.

Negative staining of the partially purified HPD agent was done as described (Hayat & Miller 1990). A carbon-enforced Formvar (Ladd Research Industries)-coated grid was floated for 10 min on a drop of the preparation. The grid was washed four times with distilled water for 1 min each and stained with 2% aqueous uranyl acetate for 5 min.

Immunogold labeling. Immunogold labeling of thin sections was performed as described (Herman 1989; Hoffman et al. 1987) with modification. Leaf pieces of symptomatic and symptomless plants were fixed in a modified Karnovsky's fixative, *en bloc* stained with uranyl acetate, and dehydrated in an ethanol series up to 70% before they were embedded in LR White medium (London Resin Co.). Thin sections mounted on uncoated 300- or 400-mesh nickel grids were floated for 10 min at RT on a drop of blocking buffer, consisting of 5% (w/v) non fat dry milk in Tris-buffered saline (TBS: 150 mM Tris, 50 mM NaCl, pH 7.4) containing 0.1% (v/v) Tween-20 (TBS-T). The grid was then transfered to 10 μl of the HPD-specific antiserum diluted 1:50 in blocking buffer for 1 h at RT in a moisture chamber. After washing with TBS-T, the grid was incubated with 10 μl of goat anti-rabbit IgG antibody conjugated with 10 nm colloidal gold (Sigma Chemical Co., St. Louis) diluted 1:10 in TBS-T for 1 h at RT. The grid was washed with TBS and distilled water, and stained with uranyl acetate and lead citrate.

Immunogold labeling of the partally purified HPD agent was done as described (Hay et al. 1994; Hayat & Miller 1990) with modification. A Formvar-coated nickel grid was floated for 10

min on a drop of the preparation, and blocked for 10 min with blocking buffer. The grid was incubated with 10 µl of the HPD-specific antiserum diluted 1:10 in blocking buffer for 1 h at RT and then washed five times in drops of blocking buffer. The grid was then floated on 10 µl of goat anti-rabbit IgG antibody conjugated with 10 nm gold particles diluted 1:20 in blocking buffer for 1 h at RT. The grid was washed four times with TBS and twice with distilled water, and stained with uranyl acetate.

RESULTS

Cytopathology. Thin-section electron microscopy revealed the presence of virus-like particles of unique size and morphology in the symptomatic leaves of all host plants examined. Quasi-spherical, double membrane-bound particles (DMPs) of 120-200 nm in diameter occurred in the cytoplasm of all leaf cell types including epidermal, mesophyll, and vascular parenchyma cells. The bounding double membranes of each DMP were separated by a thin electron-lucent space and were parallel to each other throughout the entire circumference of the particle. Although the DMPs were consistently observed in many cells of symptomatic leaves, no paracrystalline arrays of the particles were encountered.

The DMPs associated with redbud yellow ringspot and rose rosette diseases were usually scattered randomly in the cytoplasm, but the aggregation of several or more particles was common (Fig. 2, 3). The DMPs in redbud and rose were, however, not associated with any type of virus-induced inclusion bodies that are common to infections of many groups of plant viruses (Martelli & Russo 1984; Francki et al. 1985; Edwardson et al. 1993).

The DMPs in symptomatic thistle were more numerous throughout the cytoplasm than in redbud yellow ringspot or rose rosette, and also associated with characteristic inclusions resembling those induced by many groups of plant viruses and interpreted as viroplasms (Martelli & Russo 1984; Francki et al. 1985; Edwardson et al. 1993). Two types of inclusions were associated with the DMPs in thistle. One type was an accumulation of electron-dense, amorphous material. The amorphous inclusions in cells of younger leaves were often mixed with membranous vesicles that appeared to be derived from nearby Golgi bodies and/or rough endoplasmic reticulum (Fig. 4). In some other cells, the amorphous inclusions occurred as a great number of electron-dense patches about the size of DMPs often together with large masses (Fig. 5). At a higher magnification, these patches were mixed with partially double membrane-bound or fully double membrane-bound particles, and the patches and particles were directly associated with proliferated smooth membranes which appeared as irregularly shaped tubules or vesicles. And, at some points, the smooth membranes were continuous with rough endoplasmic reticulum (Fig. 6). In cells of older leaves, the amorphous inclusions often occurred

as large masses associated with proliferated rough endoplasmic reticulum and DMPs. Small portions of amorphous material partially enclosed by double membranes, which appeared to be DMPs in the process of assembly, were also encountered (Fig. 7). The other type of inclusion in thistle consisted of thin, intertwined, tubular structures appearing as tubular aggregates (Fig. 8). Inclusions of the two types, the amorphous and the tubular, usually occurred independently although both types were associated with and surrounded by DMPs. In some cells, however, both types of inclusions were closely associated at the same sites. Often a large amorphous inclusion contained a compact aggregate of tubular inclusions in its center (Fig. 8).

The DMPs in fig were less numerous in a given cell than in thistle, although they were consistently present. Inclusions similar to the tubular aggregates of thistle mosaic were also found in close association with the DMPs in some fig cells. Figure 9 shows a large mass of tubular inclusion surrounded by DMPs.

The DMPs found in corn and wheat were as numerous as in thistle and occurred with inclusions of the amorphous type in many cells (Fig. 10). The inclusions were usually dispersed in the cytoplasm closely associated with DMPs. In many wheat cells, cylindrical inclusions, characteristic of potyvirus infection, were also observed with DMPs (Fig. 11), which indicates an additional infection of a potyvirus in these cells. The cylindrical inclusions were also observed frequently in many cells of severely symptomatic corn leaves but rarely in leaves with mild and moderate symptoms.

Immunogold labeling of DMPs and inclusions. When thin sections of symptomatic corn and wheat leaves were immunogold labeled with the HPD-specific antiserum, gold particles were specifically localized on the DMPs and associated amorphous inclusions (Fig. 12). No gold particles were associated with other structures such as cell organelles and WSMV inclusions.

Ultrastructure and immunogold labeling of partially purified HPD agent. The intact DMPs were not successfully purified with the modified procedure, however, the final preparations contained more preserved thread-like structures, which were often circular and twisted (Fig. 13). When these structures were immunogold labeled, gold particles were specifically associated with them (Fig. 14). This suggested that these thread-like structures contained the HPD-specific 32 kd protein that was also demonstrated to be parts of the DMPs *in situ*.

DISCUSSION

DMPs were first reported to be associated with wheat spot mosaic and fig mosaic diseases a quarter century ago (Bradfute & Nault 1969; Bradfute et al. 1970; Nault et al. 1970). Since

then, the presence of DMPs has been reported repeatedly not only in those diseases (Plavsic & Milicic 1980; Appiano 1982; Hiruki 1989; Zaychuk & Hiruki 1991) but in four additional diseases (Kim & Martin 1978; Gergerich & Kim 1983; Ahn et al. 1993; - 1996; Jensen et al. 1996). The further characterization of DMPs has been unsuccessful from any of the diseases, however, probably because they are doubly membrane-bound, which makes it difficult to separate them from membrane-bound host cell organelles during purification processes.

Plant viruses induce the formation of intracellular inclusions that are indicators of virus infection. Most of these virus-induced inclusions including viroplasms are specific to particular viruses, especially at the virus group level, and have been used as valuable tools in virus identification and taxonomy (Edwardson & Christie 1978; Martelli & Russo 1984; Francki et al. 1985; Edwardson et al. 1993). In the case of redbud yellow ringspot and rose rosette, DMPs occurred randomly in the cytoplasm and were not associated with any particular inclusions. In thistle, however, not only were the number of DMPs greater than in redbud or rose but they were associated with viroplasmic structures such as proliferated rough endoplasmic reticulum, electron-dense amorphous inclusions and tubular aggregates, which are common constituents in viroplasms of many plant and animal viruses. Amorphous inclusions in thistle were very similar to those induced by tospo-, caulimo-, and phytoreoviruses (Francki et al. 1985; Kitajima et al. 1992) and, furthermore, the DMPs and associated inclusions shown in Figure 4 and 7 closely resemble immature particles and viroplasms of animal and insect poxviruses (Granados 1973; Stern et al. 1977). In addition, structures shown in Figure 6 and 7 suggest possible assembly intermediates of DMPs and these structures are also similar to double-enveloped particles of tomato spotted wilt virus reported to occur in viroplasms at early stages of infection (Francki et al. 1985; Kitajima et al. 1992).

The amorphous inclusions associated with DMPs in thistle (Fig. 5, 7, 8) and corn (Fig. 10) appear to be similar in appearance, and the tubular inclusions in thistle (Fig. 8) and fig (Fig. 9) are morphologically indistinguishable. These observations suggest that the DMPs associated with the diseases could be related. The tubular inclusions observed in figs in this study were also identical to those described by Appiano (1982) in fig mosaic disease occurring in Italy.

From most specimens of corn and wheat affected with high plains disease including field samples and those experimentally infected through eriophyid mite vectors, DMPs and cylindrical inclusions were consistently present (Fig. 11). The presence of both potyvirus-characteristic cylindrical inclusions and DMPs in the HPD specimens supports the hypothesis that the HPD is a result of a mixed infection with WSMV and an agent represented by DMPs. Higher population of DMPs in cells that contain cylindrical inclusions may be a reflection of such a synergism (Fig. 11).

Specific immunogold labeling of the DMPs, associated amorphous inclusions, and the thread-like structures in partially purified preparation (Fig. 12, 13, 14), using the HPD-specific

antiserum, indicates that these structures are made up, at least in part, of the disease-specific 32 kd protein. It is possible that the thread-like structures may represent tightly packed structural components, such as nucleocapsids, released from ruptured DMPs during the purification process.

The DMPs associated with the five plant diseases are unique in morphology and some of them occur with cytopathological inclusions which have been considered to be the signs of virus infection. Based on these findings and other common characteristics of viral diseases such as symptomatology and graft- and mite vector- transmissibility, it is believed that the DMPs are viral in nature and are the causal agents of the diseases described here.

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FIGURE LEGENDS

Figure 1 Symptoms of the diseases associated with virus-like DMPs. (*a*) A redbud leaf showing severe ringspot symptoms. The spots are yellow when they first appear and later become necrotic. (*b*) Rose rosette symptoms on multiflora rose. Two lateral shoots with reddish green, stunted leaves arise from a cane that shows mild witches' broom symptoms. A cluster of green normal leaves and stunted shoots with small red leaves is also shown. (*c*) A mature leaf of thistle with moderate mosaic symptoms. (*d*) A fig leaf with mosaic symptoms. (*e*) High plains disease symptoms on two corn leaves. (*f*) High plains disease symptoms on wheat leaves. The severity of chlorotic spots and streaking increases from left to right in the figure.

Figure 2 DMPs associated with redbud yellow ringspot. Four DMPs (arrowheads) are shown close to a large microbody (M) in a mesophyll cell of redbud leaf infected with yellow ringspot disease. x 68,000.

Figure 3 DMPs in rose rosette. DMPs (arrowheads) are shown in a mesophyll cell cytoplasm of a multiflora rose leaf infected with rose rosette. x 60,000.

Figure 4 DMPs in thistle mosaic. In an epidermal cell of a thistle leaf infected with thistle mosaic, DMPs (arrowheads) are shown in association with viroplasm-like amorphous inclusion (AI). The amorphous materials are mixed with a number of membranous vesicles (V). N: nucleus; G: Golgi body. x 52,000.

Figure 5 Amorphous inclusions associated with DMPs in thistle mosaic. An epidermal cell showing amorphous inclusions (AI) which are scattered as small round patches (arrows) throughout the cytoplasm. DMPs (arrowheads) and greatly proliferated membranous vesicles and tubules (Me) are associated with the inclusion patches. W: cell wall; Mi: mitochondrion; Ch: chloroplast. x 13,000.

Figure 6 Amorphous inclusions in thistle mosaic. The small patches of amorphous inclusion (arrows), DMPs (arrowheads), partially double membrane bound-particles (X) and associated smooth membranes (Me) are shown in detail. A segment of rough endoplasmic reticulum (ER) is continuous with a tubular membrane (double arrowheads). x 50,000.

Figure 7 DMPs and inclusion in thistle mosaic. A DMP (arrow) appears to be in the process of maturation or budding as shown by segments of double membranes partially enclosing amorphous material (AI). x 75,000.

Figure 8 Two types of inclusions associated with DMPs (arrowheads) in thistle mosaic. A large amorphous inclusion (AI) is encircling a tubular inclusion (TI) in its central area. x 60,000.

Figure 9 DMPs and tubular inclusion associated with fig mosaic. A large mass of tubular inclusion (TI) surrounded by DMPs (arrowheads) in a fig mosaic-infected leaf cell. x 61,000.

Figure 10 DMPs and amorphous inclusions in high plains disease of corn. DMPs (arrowheads) scattered throughout the cytoplasm of a corn leaf cell infected with high plains disease. Amorphous inclusions (AI) are closely associated with the DMPs and proliferated membranous tubules (Me). M: microbody; W: cell wall. x 20,000.

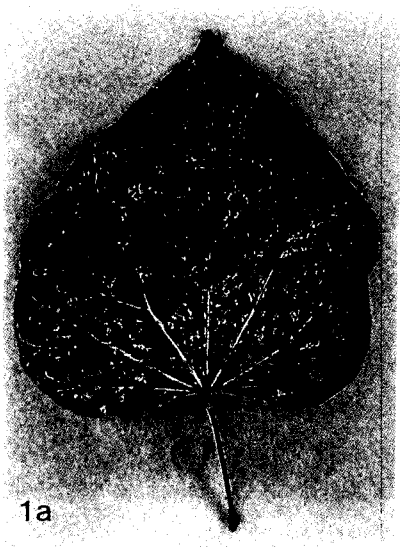
Figure 11 DMPs and WSMV inclusions in an HPD-affected wheat cell. A great number of DMPs (arrowheads) are present throughout the cytoplasm together with WSMV cylindrical

inclusions, which appeared as pinwheels (P) and bundles (B). The number of DMPs in cells with WSMV inclusions was usually greater than in cells without the inclusions. Mt : mitochondrion. x 28,000.

Figure 12 Immunogold labeling of DMPs *in situ*. When the DMPs (arrowheads) in the cytoplasm of a wheat leaf cell affected with HPD are immunogold labeled with the disease-specific antiserum, gold particles were specifically associated not only with DMPs but also with electron-dense amorphous inclusions (arrows) dispersed in the cytoplasm. x 75,000

Figure 13 Ultrastructure of the partially purified preparation of the HPD agent. Thread-like structures (arrows), which were often circular and twisted, were present in the partially purified preparations. The threads were negatively stained with 2% aqueous uranyl acetate. x 160,000.

Figure 14 Immunogold labeling of the partially purified HPD agent. When the thread-like structures were immunogold labeled with the disease-specific antiserum, gold particles reacted specifically with these structures (arrows). x 140,000.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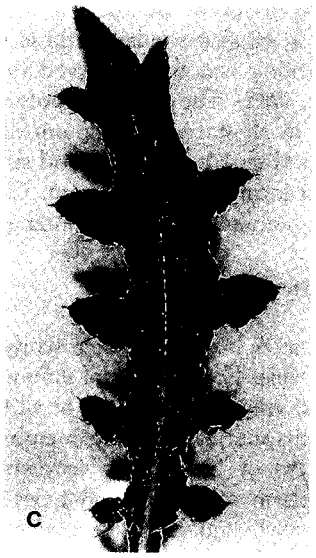
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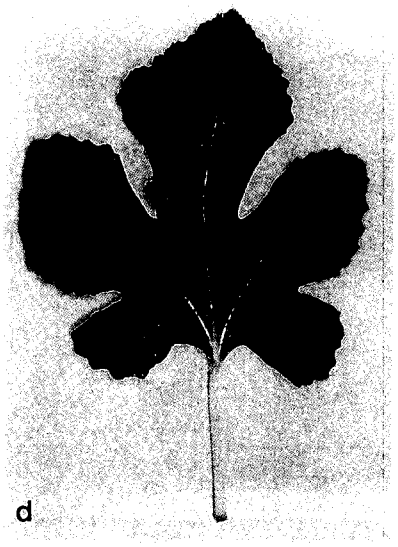
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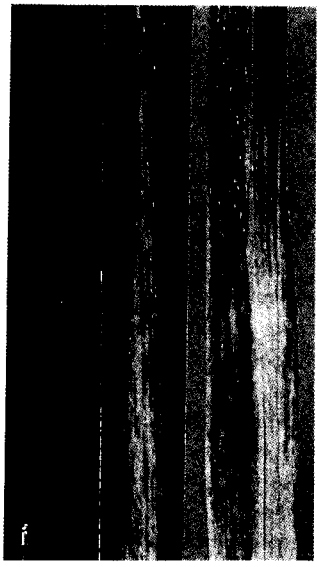
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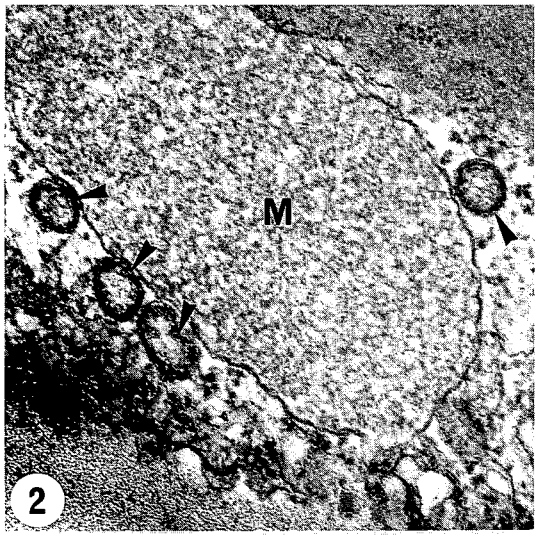
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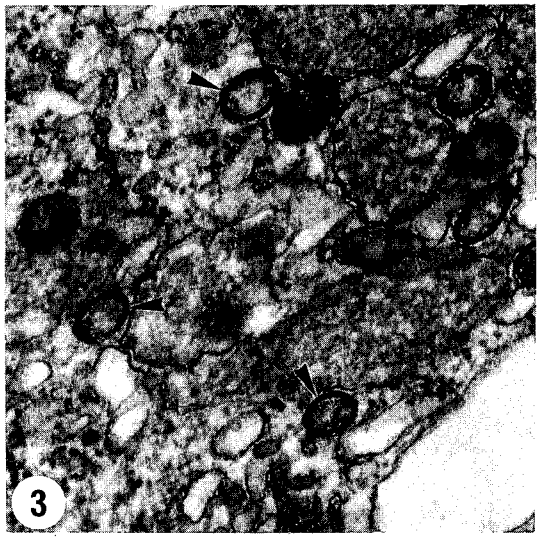
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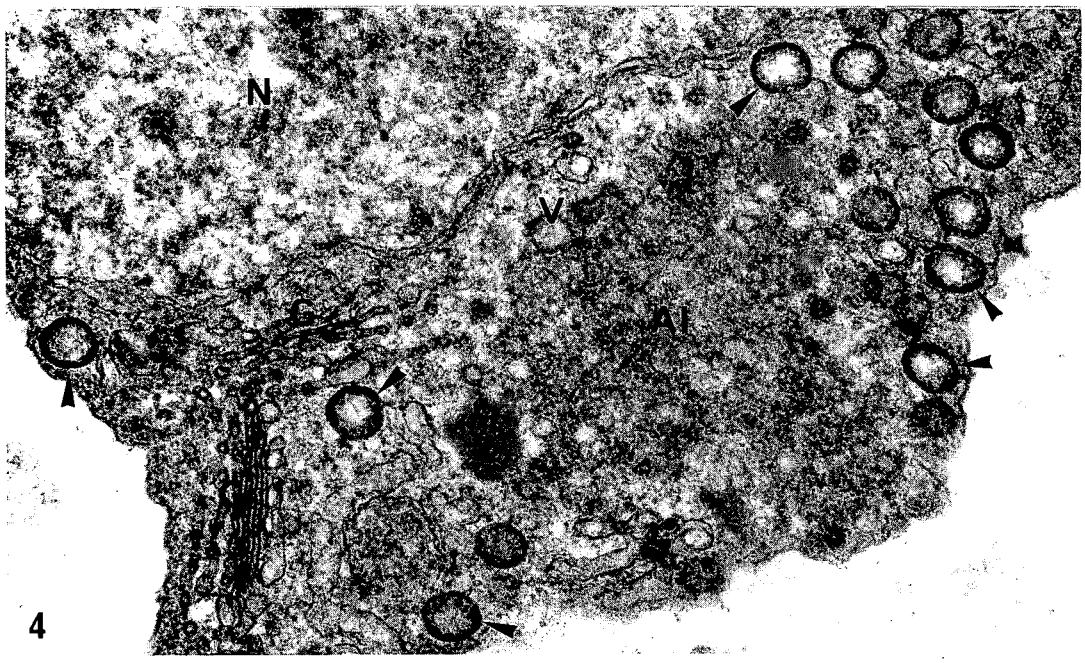
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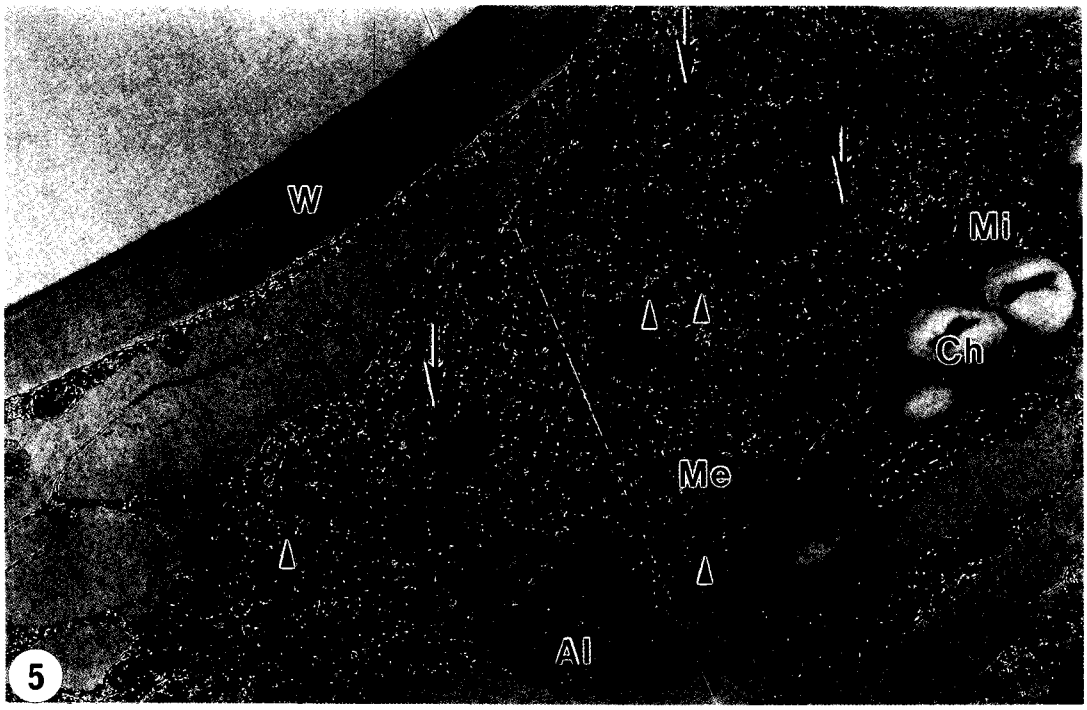
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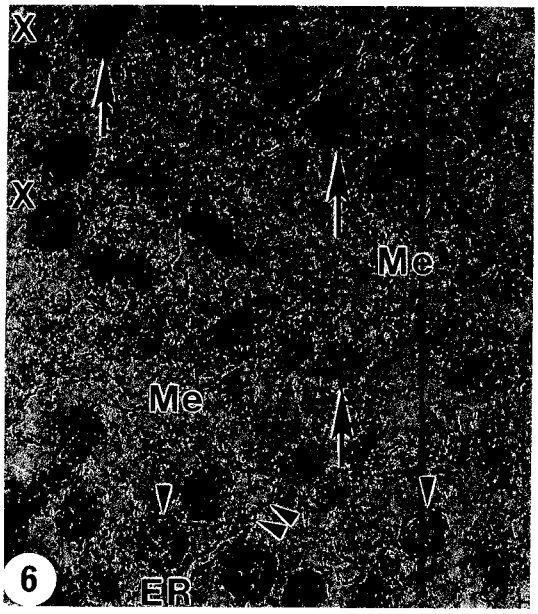
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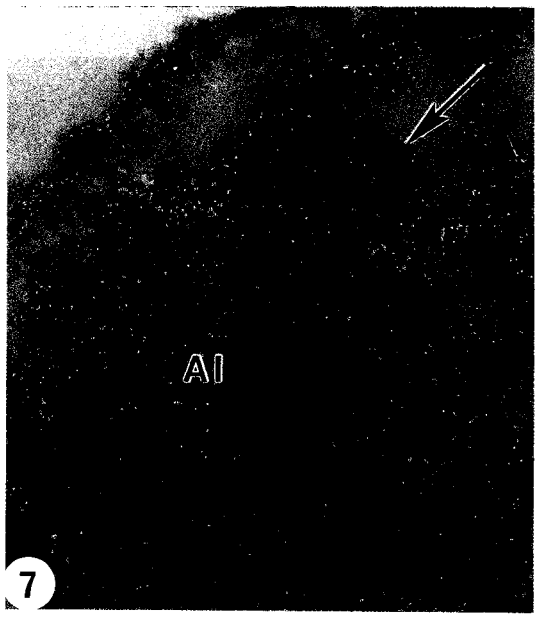
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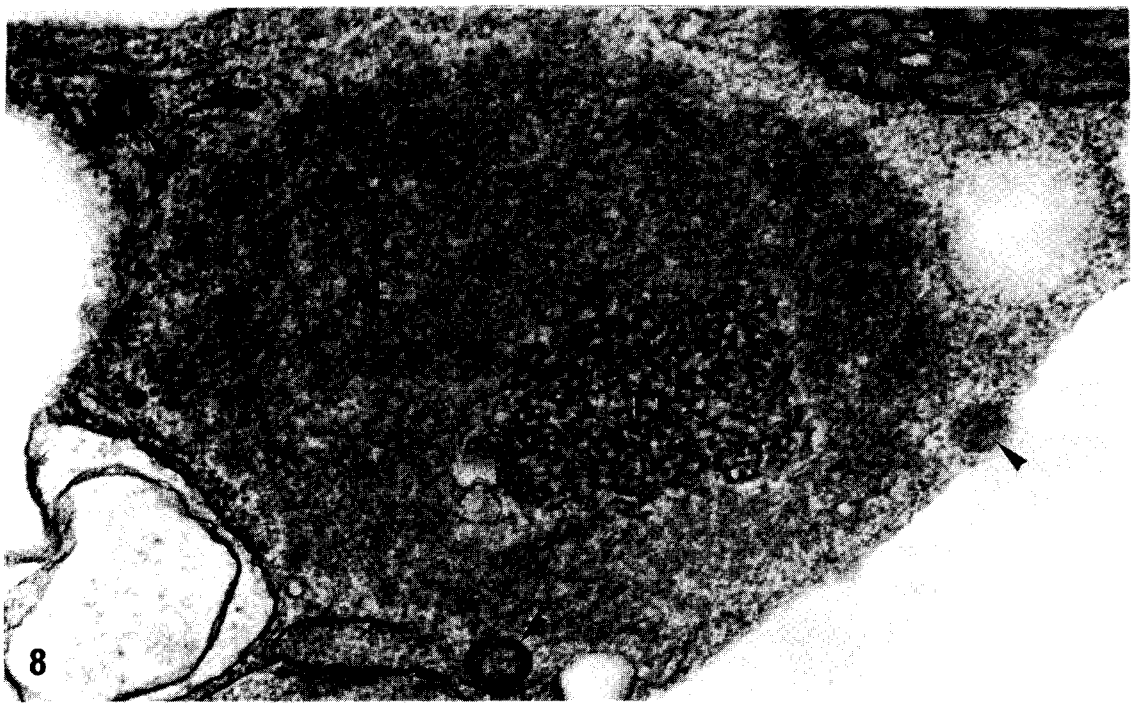
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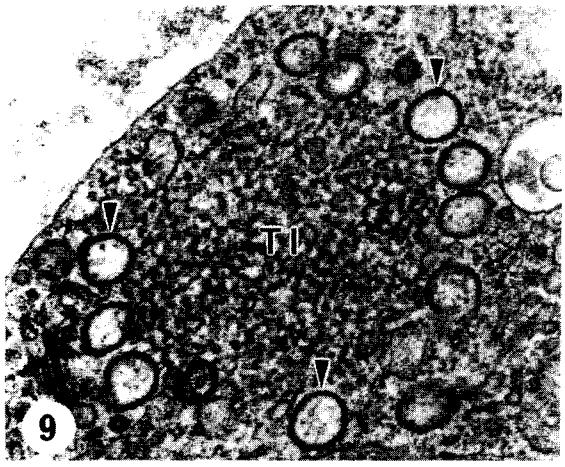
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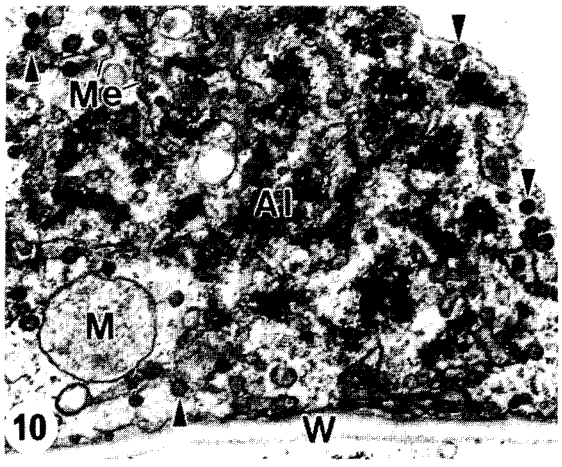
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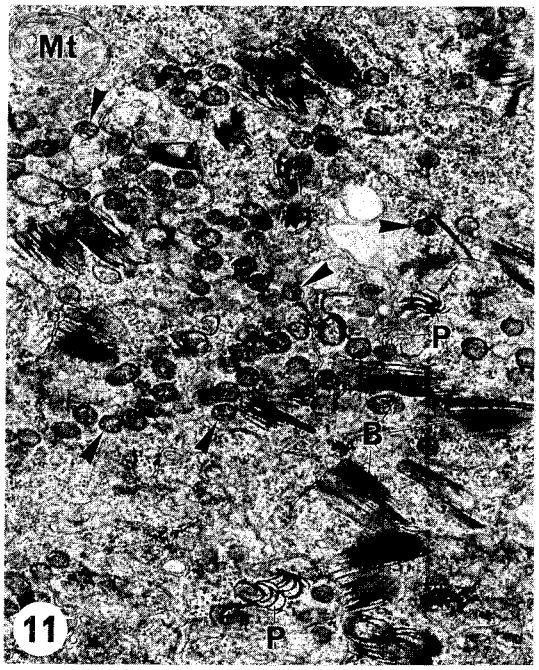

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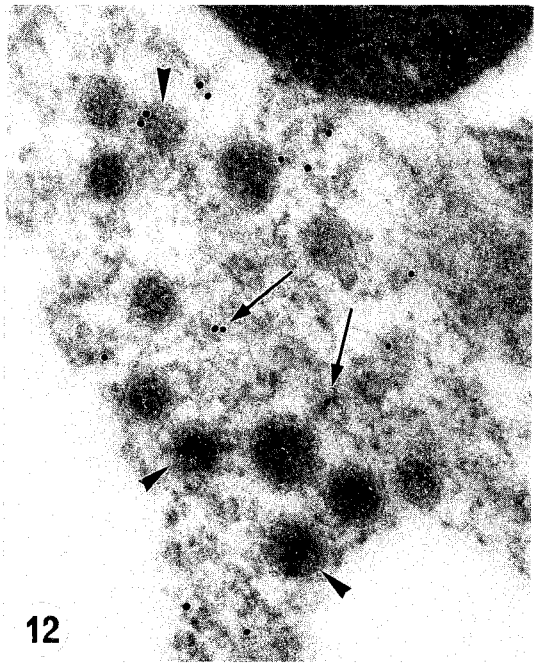

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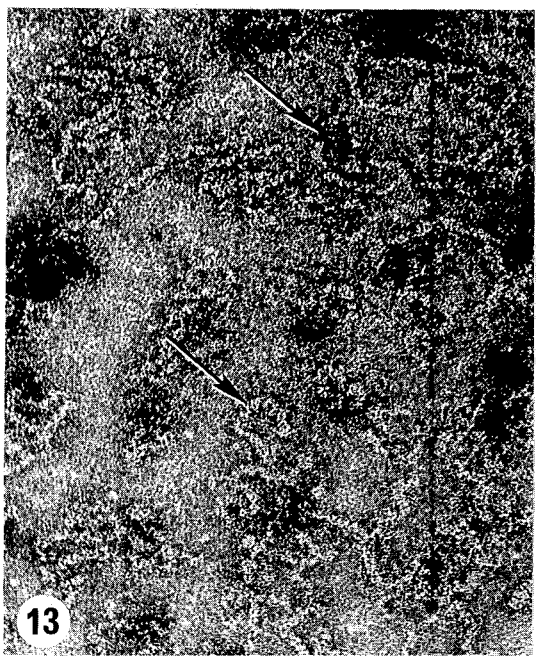

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