Fate of Pea Enation Mosaic Virus in PEMV-Injected Pea Aphids

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Ultrathin sections were prepared for electron microscopy from excised organs and tissues of viruliferous pea aphids, *Acyrthosiphon pisum* (Harris). Aphids were rendered viruliferous by injecting pea enation mosaic virus (PEMV) directly into the hemocoele. Virions of PEMV were observed in situ in the salivary systems, connective tissue cells, and fat body of injected aphids. In the salivary system, virions occurred in the basal laminae of both primary and accessory glands and in the plasma-membrane cisternae of accessory gland cells, thus suggesting membrane flow as a means by which virions are transported from the hemocoele to the salivary duct lumina. Virions also occurred in the salivary systems of aphids fed on PEMV-infected plants; however, none were observed in the glands of aphids exposed either per os or by injection to a nonaphid-transmissible variant of PEMV. PEMV is the first isometric, aphid-borne virus to be localized in the salivary glands of its vector. It is hypothesized that virions engulfed by connective tissue cells enter into the phagosome-lysosome system of these cells and, like engulfed secondary symbionts, are destined for eventual lysosomal digestion. Virions were localized in the fat body tissue in “basophilic” mesodermal cells. Virions occurred in relatively high concentrations in the stomach lumens of all test aphids examined.

INTRODUCTION

Pea enation mosaic virus (PEMV) is the first circulative virus to be localized by electron microscopy both in plants and aphids in situ; Shikata *et al.* (1966) observed PEMV virions in the gut lumen and in the fat body of viruliferous pea aphids, *Acyrthosiphon pisum* (Harris). De Zoeten *et al.* (1972) found virions in the gut contents (lumen) of viruliferous pea aphids but could not identify PEMV with certainty in any other part of the aphids. Seryczyńska and Węgorek (1972) were unable to find PEMV in the alimentary canal (lumen or epithelial lining) of viruliferous *A. pisum*, but they did report pathogenic changes in the ultrastructure of the stomach epithelial cells of these aphids. Harris and Bath (1972) reported that PEMV was highly concentrated in the stomach and hindgut lumen in viruliferous aphids and that virions could invade stomach epithelial cells and hemocytes in the insect.

We undertook the present research to obtain additional information about the vector–virus relationships of PEMV at both the cellular and subcellular level. The role of the electron-dense, sometimes viroplasm-like, viral inclusions observed in aphids fed on PEMV-infected plants (Harris and Bath, 1972), and why researchers have thus far been unable to detect virions in the salivary systems of viruliferous aphids were among the many questions it was hoped this research might answer. The latter question is particularly intriguing since PEMV is generally believed to be transmitted in a circulative manner by its aphid vector and, therefore, presumably inoculated into plants in the form of virus-laden saliva secreted during feeding.

To date, only the tissues of aphids rendered infective by exposure to PEMV-infected plants have been examined by electron microscopy. In this study, the alimentary canal was bypassed by inject-
ing aqueous suspensions of partially purified PEMV directly into the insects' hemocoels. If PEMV is indeed removed from the hemocoel in the form of complete particles or virions, we reasoned that by thus overloading the vector's system with virions, the chances of seeing PEMV in the glands would be greatly enhanced. A preliminary report on this investigation has been presented (Harris, 1974).

MATERIALS AND METHODS

The virus-vector combination consisted of a highly aphid-transmissible virus isolate derived by successive aphid transmission of a California isolate of PEMV (Tsai, unpublished) and an East Lansing biotype of the pea aphid, A. pismum, previously shown to be a highly efficient vector of PEMV (Tsai et al., 1972). The technique used to prepare partially purified PEMV from young infected garden pea plants, Pisum sativum L. cv. "Midfreezer," was as described by Thottappilly et al. (1972). Seedlings of the same garden pea variety served as test plants for transmission and as host plants for aphids after inoculation.

Injection of aphids. Frozen aqueous suspensions of partially purified PEMV were thawed, diluted with distilled water to an absorbance of 30 at 260 nm in a cell with a 1 cm light path, and held in an ice bath to serve as inoculum during the injection process. Needles were drawn from 1.6 to 1.8 x 100 mm glass capillaries using a Narishige (Tokyo) pulling machine. Chipping the needle tips under a dissecting scope produced syringes with sharp jagged points. A syringe was filled with inoculum by touching the tip to the surface of the PEMV suspension \( (A_{260 \text{nm}} = 30) \) and allowing it to fill by capillarity. Syringes prepared and filled in this way hold a volume of inoculum ranging from 0.005 to 0.01 \( \mu l \) (Clarke and Bath, 1973). Virus-free, first instar viviparae borne by apterous, parthenogenetic females left overnight on broad bean, Vicia faba L., were each subjected to a total of three injections during a 5-day period.

Injections were performed on the stage of a dissecting microscope at room temperature. At the first injection, a number was assigned to each of 20 insects to identify it, as well as the test plants on which it was to feed, for the remainder of the experiment. Aphids, starved for several hours to reduce hemocoel pressure, were immobilized with \( \text{CO}_2 \) and steadied for injection with their dorsal sides uppermost on the microscope stage using a camel's hair brush. A new, freshly filled syringe was used for each injection. The syringe was inserted dorsolaterally in an anteroposterior direction into the hemocoel at a point in the abdomen approximately equidistant between the siphunculi and the thorax of the recipient larva. An attempt was made to pierce the integument through the conjunctiva of an intersegmental fold since wounds made directly through the cuticle more often result in the loss of hemolymph (Mueller and Rochow, 1961). The entire inoculum was then blown into the hemocoel via a polyethylene tube attached at one end to the syringe and held at the other end in the experimenter's mouth. If, as occasionally happened, additional pressure was needed to drive the inoculum into the hemocoel, the polyethylene tubing could be connected to a laboratory air supply in a manner similar to that described by Richardson and Sylvester (1965). Control insects consisted of aphids injected in the same manner but with syringes containing distilled water only.

After injection and recovery in Petri dishes, aphids were placed on individual healthy pea seedlings for a 1.5 day feeding period before being subjected to the next injection in the series or, as was the case following the third and last injection, before being processed for electron microscopy. Once aphids were removed from their respective test plants, the plants were sprayed with Malathion and placed in an insect proof greenhouse to complete the incubation period.

Preparation of aphids for microscopy. The procedure for fixing, dehydrating, and embedding specimens for electron microscopy was similar to that used in a previous study of PEMV in its vector (Harris and Bath, 1972). Aphids were dissected in cold 6% glutaraldehyde in 0.1 M phosphate buffer using a stereomicroscope. Excised organs and tissues, as well as whole abdo-
mens and whole aphids, were held in glutaraldehyde for 90 min, given three 15-min washings in buffer, postfixed in 1% osmic acid overnight in the refrigerator at 4°C, dehydrated in increasing concentrations of ethanol in distilled water with three final 15-min washings in 100% ethanol, and embedded in standard viscosity Spurr’s (1969) epoxy resin embedding medium. Ultrathin sections were cut with a diamond knife using the Porter-Blum MT2 ultramicrotome, “stained” with uranyl acetate and lead citrate (Venable and Coggeshall, 1965), and examined and micrographed in a Philips EM 300 electron microscope (EM) at 60 kV. The microscope was calibrated with a diffraction grating replica having 54,864 lines per inch.

Organs dissected and excised from a few aphids in phosphate-buffered glutaraldehyde were dipped in 1% osmic acid for 5 sec, washed in phosphate buffer, placed in a drop of buffer in the depressed area of a glass microscope slide, and photographed through a stereomicroscope using transmitted light from a tungsten point source and Kodak Panatomic-X film.

RESULTS

Figures 1 and 2 are light micrographs. All other figures are electron micrographs and, unless otherwise stated in the figure legend, pertain to ultrathin sections of organs and tissues from aphids injected with an aphid-transmissible isolate of PEMV.

Each of the 20 test aphids (PEMV-injected) transmitted PEMV to the three individual test plants on which it had fed during the injection schedule. Organs and tissues that were examined in the EM included the salivary glands (whole systems or individually excised and embedded primary and accessory glands), central nervous system (brain and subesophageal ganglionic mass), reproductive system, mycetome (primary and secondary), fat body, epidermis, tracheal system, anterior midgut (stomach), musculature, eye tissues, and mesodermal cells. PEMV virions were observed in the salivary glands, mesodermal connective tissue cells (“hemocytes”), fat body, and stomachs of test aphids but never in any organs or tissues from control aphids (water-injected).

Salivary system. Figure 1 shows the salivary glands in relation to other organs of the pea aphid. The salivary system (Fig. 2) consists of paired accessory glands (Ag) and bilobed primary glands (Sg), and ducts from each which join to form lateral ducts leading to a common salivary duct (Sd). The myoepithelioid cell at the distal end of the primary glands received special attention in this study since Ponsen (1972) implicated it as the avenue by which virus-laden hemolymph could be channeled directly into the internal salivary ducts of the primary glands. The cell is bordered by gland cells and intercellular secretory canaliculi (Isc), and the bulk of its cytoplasm is occupied by myofibrils arranged in an interwoven pattern (Fig. 3). Thus far, virions have not been seen in this cell or in any part of the primary gland other than the basal lamina that surrounds this bilobed organ (Fig. 4).

Virions were relatively more concentrated in the basal laminae of the accessory glands (Fig. 5), and they also occurred in the labyrinth of cisternae formed by the extensive infolding and anastomosing of the plasma membrane of accessory gland cells (Figs. 6 and 7). No PEMV virions or PEMV-like particles were observed in any of the salivary systems from ten control aphids. In both test and control aphids, the

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**Fig. 1.** Light micrograph of the salivary system in relation to other aphid organs. Ag, accessory gland; Br, brain; Lb, labium; Seg, subesophageal ganglion; Sg, primary salivary gland; St, stomach.

**Fig. 2.** Light micrograph of an excised aphid salivary system. Ag, accessory gland; Sd, common salivary duct; Sg, primary salivary gland; asterisks, myoepithelioid cells.

**Fig. 3.** Myoepithelioid cell (My) at the distal end of the bilobed primary salivary gland. The cell is bordered by gland cells (Gc) and intercellular secretory canaliculi (Isc), and the bulk of its cytoplasm is occupied by myofibrils arranged in an interwoven pattern.

**Fig. 4.** PEMV virions (V) in the basal lamina (B) of a primary salivary gland. The virions are shown at higher magnification in the inset. Sg, secretory granule.
basal laminae of other organs such as the neurilemma of the central nervous system and the basement membrane of the alimentary canal were also apparently free of PEMV virions.

To further characterize the role of the aphid's salivary system in the PEMV transmission cycle, the electron microscopy of PEMV in the salivary glands of aphids exposed either per os or by injection to the highly aphid-transmissible (T) isolate of PEMV was compared with that of aphids exposed to PEMV in a similar fashion, but to a nonaphid-transmissible (NT) variant. The NT variant was derived by successive mechanical transmission of the California isolate of PEMV until aphid transmissibility was lost (Tsai, unpublished).

PEMV virions were observed in the salivary systems (Fig. 8) of each of ten aphids which were previously fed for 5 days on pea plants infected with the T isolate and held for 1 day on individual healthy pea seedlings before being processed for electron microscopy. No virions could be found in the salivary glands of 20 control aphids, half of which had been held for 5 days on healthy pea plants and half on pea plants infected with the NT variant of PEMV. All ten of the test seedlings on which T isolate-exposed aphids had fed became infected with PEMV; whereas, those fed on by control aphids remained uninfected.

PEMV virions could not be found in any of the salivary systems from ten aphids injected with the NT variant of PEMV (Figs. 9 and 10). Each aphid was subjected to a total of three injections with aqueous suspensions ($A_{260} = 30$) of partially purified NT variant. The infections were performed as described earlier for the T isolate. All of the test seedlings on which the insects were held between injections and before processing for electron microscopy remained uninfected. Virions were observed in the salivary glands of ten additional aphids injected with the T isolate.

**Mesodermal cells.** Connective tissue cells, “basophilic” mesodermal cells, and fat cells all fall under the general heading of mesodermal cells. According to Ponsen (1972), these three cell types are derived from embryonic mesodermal cells and, over the years, they have been mistakenly identified in various aphid species and by numerous authors as blood cells, phagocytes, hemocytes, and lymphocytes.

The viral inclusion structures in Figs. 11–20 occurred in the cytoplasm of mesodermal cells previously described as “blood cells” or “hemocytes” (Harris and Bath, 1972). Hinde (1971a), referring to the same cell type in *Brevicoryne brassicae* L., *Myzus persicae* Sulz., and *Macrosiphum rosae* L., demonstrated electron microscopically that these “phagocytic hemocytes” engulf and digest by the action of lysosomes secondary symbiotes released into the hemolymph from mycetocytes, a process also described by Griffiths and Beck (1973) in *A. pisum*. Ponson (1972), however, has reported that *M. persicae*, and presumably other aphid species, are characterized by the absence of circulating hemocytes, and he noted that these “hemocytes” are, in fact, mesodermal connective tissue cells.

PEMV virions were localized in defined, electron-dense structures, presumably lysosomes (De Duve and Wattiaux, 1966), and in vacuoles in the cytoplasm of connective tissue cells. These cells were often characterized by the presence of secondary symbiotes in their cytoplasm (Fig. 11). It

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**Fig. 5.** Accumulation of PEMV virions in the basal lamina (B1) of an accessory salivary gland. Note that three particles (arrows) have moved into the labyrinth of cisternae (C) formed by extensive infolding and anastomosing of the basal plasma membrane of the gland cell (Gc).

**Fig. 6.** Virus particles in the basal lamina (B1) and in the plasma-membrane cisternae (arrows) of an accessory salivary gland. The three virions near the center of the micrograph are shown in higher magnification in the inset.

**Fig. 7.** A group of six virions (arrow and inset) in a plasma-membrane cisterna in the more central cytoplasm of an accessory gland cell.

**Fig. 8.** Virions in the basal lamina (B1) of an accessory gland of an aphid rendered viruliferous by feeding on a pea plant infected with an aphid-transmissible isolate of PEMV.
was not unusual to observe symbiotes and PEMV virions in the same electron-dense lysosomal structures (Figs. 12 and 13). The symbiotes were undergoing lysosomal breakdown as indicated by their “darkly stained” cytoplasm and shriveled cell walls (Hinde, 1971a). In the electron-dense structures, virions often occurred with or surrounded by the myelin figures typically associated with secondary or later stages of lysosomal activity (De Duve and Wattiaux, 1966; Hinde, 1970, 1971a). Two such myelinated lysosomes can be seen in the cytoplasm of a connective tissue cell in Fig. 14. Secondary lysosomes containing virions but no myelin figures were sometimes “viroplasm-like” in appearance (Fig. 15).

Virus particles were localized in vacuoles (heterophagosomes) in both the peripheral and more central cytoplasm of connective tissue cells. Occasionally, virions occurred in small electron-dense areas, presumably primary or early secondary lysosomes, surrounding the vacuolar membranes of vacuoles that themselves contained virus (Fig. 16). Secondary lysosomes containing both virions and myelin figures were also observed in close association with vacuoles (Fig. 17).

Whorled membranous structures containing PEMV, as well as lysed secondary symbiotes and other electron-dense materials, sometimes occurred in a single membrane-bounded vacuole occupying most of the cytoplasmic area of a connective tissue cell (Fig. 18). Griffiths and Beck (1973) described similar structures called cytolyosomes or autophagous vacuoles in the secondary mycetome and “hemocytes” of A. pisum as sometimes containing a dozen or more secondary symbiotes in various stages of breakdown. However, the structure in Fig. 18 is more properly referred to as a telolysosome (De Duve and Wattiaux, 1966) since it also contains heterophagous materials such as virions and symbiotes. The cell in Figs. 19 and 20 contains PEMV in a myelin figure (Mf) as well as several large aggregates of virions in a multilamellar telolysosome (TL). The telolysosome is surrounded by cytoplasm containing numerous dark staining residual bodies (Rb), presumably the remains of lysed secondary symbiotes (De Duve and Wattiaux, 1966; Hinde, 1971a; Griffiths and Beck, 1973).

Fat body. One of the most commonly encountered tissues in ultrathin sections of aphids is the fat body. PEMV virions were observed in the fat body but in cells identified as “basophilic” mesodermal cells (Fig. 21), rather than as fat cells, by their location in the fat body, by their cisternal endoplasmic reticulum, and by the presence of ingested secondary symbiotes in their cytoplasm (Ponsen, 1972). Virions are contained in a myelin figure in the cytoplasm of the “basophilic” mesodermal cell in Fig. 21. PEMV virions were never observed in the mesodermal cells of control aphids.

Stomach. Relatively large concentrations of PEMV were observed in the lumens of stomachs taken from each of ten test aphids. The virus particles were localized in food boli and concentrated along the periphery of ingested materials (Fig. 22). The virions in Fig. 23 border a portion of the intima that extends into the stomach from the foregut in the region of the esophageal valve. Virions were not observed in the stomach epithelial cells of aphids in
this research. No virions or PEMV-like particles were seen in the stomach lumens of any of eight control (water-injected) aphids.

**DISCUSSION**

The average diameter of virions in aphid tissues or of embedded and sectioned isolated particles obtained by sucrose, density-gradient centrifugation (Fig. 20, inset) was calculated to be $20.7 \pm 1.3$ nm. This size estimate compares favorably with the 22–24 nm diam assigned to isolated virions that were fixed in formalin prior to being placed on grids, stained with phosphotungstic acid and observed in the EM (Farro and Vanderveken, 1969; Farro and Rassel, 1971). Shrinkage of the embedding medium during polymerization, failure of the protein coat of virions in thin section to ”stain” adequately, and probable flattening of virions in purified preparations during drying on EM grids, are all possible contributors to the smaller size of embedded, isolated virions or of virions in situ.

The localization of virions in the stomach was somewhat surprising since gut permeability to virus is generally thought to occur in a gut to hemocoele direction only. Virions also occur in the stomachs of PEMV-injected aphids that are fed through parafilm membranes on virus-free feeding solutions between injections and before processing for electron microscopy (Harris, unpublished). The virions in the stomach (Figs. 22 and 23) could represent progeny particles ingested with plant sap from test seedlings or virus particles ingested as virus-laden saliva secreted during feeding. But in light of recent evidence that aphids can regurgitate previously ingested materials (Harris and Bath, 1973; Garrett, 1973), the possibility of virus movement from hemocoele to gut lumen to plant by regurgitation should not be overlooked. Regurgitation appears to play a major role in nonpersistent virus transmission, and it could be important in some semipersistent and persistent transmission as well (Harris and Bath, 1973).

The presence of virions in the salivary system strengthens the hypothesis that PEMV and other small, isometric, aphid-borne viruses are transmitted in a circulative manner. The greater concentrations of virions in the basal laminae of accessory glands (Figs. 5 and 6) and the inclusion of particles in the plasma-membrane cisternae of accessory gland cells (Figs. 6 and 7) suggest membrane flow as the process by which virions are transported from the hemocoele to salivary duct lumina and, finally, to plants during feeding (Bennett, 1956; Pease, 1956; Wohlfarth-Bottermann and Moericke, 1960). It is interesting to note that the watery saliva originates from the accessory glands (Miles, 1969). The apparent ability of the aphid’s salivary system to distinguish between PEMV virions of the aphid-transmissible isolate and its nonaphid-transmissible variant suggests that the glands contribute to the vector-virus and virus-vector specificity phenomena associated with PEMV transmission by aphids.

The electron-dense, sometimes myelinated, sometimes viroplasm-like areas observed in midgut epithelial cells (Harris and Bath, 1972), connective tissue cells, and “basophilic” mesodermal cells (Figs. 11–21) of viruliferous aphids are considered to be part of the phagosome-lysosome or vacuolar apparatus (De Duve and Wattiaux, 1966). The following hypothetical sequential account of the fate of virions in connective tissue cells is presented as a

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**Fig. 14.** Two secondary lysosomes containing virions and myelin figures (Mf) in the cytoplasm of a connective tissue cell.

**Fig. 15.** PEMV virions in a “viroplasm-like” secondary lysosome in the cytoplasm of a connective tissue cell.

**Fig. 16.** PEMV (V) in a vacuolated area of the cytoplasm of a connective tissue cell. Virions also occur in small electron-dense bodies, presumably early secondary lysosomes, surrounding the vacuole (Va). A single unit membrane (arrow) is discernible around some of the bodies.

**Fig. 17.** Virions (V) in a myelinated secondary lysosome adjacent to a vacuole (Va) in the cytoplasm of a connective tissue cell.
logical explanation for the thousands of static images of events observed in the same cell or in different cells of the same aphid or in cells of more than a hundred different aphids over more than a 4-yr period. This explanation is further supported by the fact that, in these cells, ingested secondary symbiotes (Figs. 11-13) and virions apparently share similar fates (Hinde, 1970, 1971a, 1971b; Griffiths and Beck, 1973). The terms used to refer to various structures are according to De Duve and Wattiaux (1966).

Virions enter connective tissue cells in endocytic vacuoles and these (hetero)phagosomes later combine with primary or secondary lysosomes (Figs. 16 and 17), which may themselves already contain virions, to form phago- or secondary lysosomes. In the secondary lysosome stage (Figs. 12 and 13), close contact is established between ingested materials and the vacuolar membrane of the old heterophagosome (Hinde, 1971a). Secondary lysosomes may appear as “viroplasm-like” areas (Fig. 15) or contain myelin figures (Fig. 14) depending on the area sectioned and on the amount of undigested lipids contained in their matrices (De Duve and Wattiaux, 1966). Lysosomes of both heterophagous and autophagous lines can combine to form large membrane-bounded “vacuoles” or telolysosomes (Figs. 18 and 20), or they can individually progress to the dark staining residual body and finally to the whorled myelin figure stage (Fig. 19). Griffiths and Beck (1973) observed similar lysosomal structures, but without virions (Griffiths, personal communication), in their thorough study of the fate of symbiotes in A. pisum. Lysosomal digestion of PEMV virions in the mesodermal cells of vectors could account in part for the observed drop in the transmission efficiency of aphids removed from a continuous source of virus.

Lack of multiplication in the aphid as well as invasion of only a limited number of vector tissues by virions probably explain why the small, isometric, aphid-borne viruses, such as PEMV, barley yellow dwarf (Paliwal and Sinha, 1970) and potato leafroll (Moericke, 1963; Peters, 1971; Ponson, 1972) viruses have proved far less amenable to study in situ by electron microscopy than the multiplicative, bacilliform viruses of sowthistle yellow vein (Richardson and Sylvester, 1968; Sylvester and Richardson, 1970) and lettuce necrotic yellows (O'Loughlin and Chambers, 1967). There is no compelling electron microsopical evidence for multiplication of PEMV in its vector. Concentrations of virions in the stomach lumen, virions in the nucleus of one cell and in “viroplasm-like” areas in the cytoplasm of other stomach epithelial cells, were previously cited as being suggestive of PEMV multiplication in aphid gut epithelium (Harris and Bath, 1972). As noted, both the myelinated and “viroplasm-like” viral inclusions previously observed in cells lining the midgut (Harris and Bath, 1972) are now considered to be lysosomes which become noticeably numerous in reaction to high levels of foreign materials (virions) in the diet. PEMV ingested with plant sap is quickly concentrated in the stomach lumen, presumably by the removal of water (Moericke and Mittler, 1965; Treherne, 1967). Virions very rarely observed “in the nuclei of midgut cells” (Harris and Bath, 1972) likely represent ingested particles, not progeny particles, in cells where the distinction between cytoplasm and nucleoplasm is no longer discernible.

Negative evidence from electron microscopy, when added to negative transmission

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**Fig. 18.** Large membrane-bounded vacuole or telolysosome (Tl) occupying much of the cytoplasmic area of a connective tissue cell. The telolysosome contains virions in whorled myelin figures (arrow and inset), lysed secondary symbiotes and other electron-dense materials. L, tracheal lumen; N, tracheoblast nucleus.

**Fig. 19.** PEMV virions in a myelin figure (Mf) and in a telolysosome (Tl) in a connective tissue cell. Rb, residual body.

**Fig. 20.** High magnification of the telolysosome (Tl) in Fig. 19. Cer, cisternal endoplasmic reticulum; V, virions. Inset (same magnification), PEMV virions isolated by sucrose density-gradient centrifugation, picked up in 4% agar and processed for ultrathin sectioning and electron microscopy.
data (Sylvester, 1969), perhaps tilts the scale in favor of nonpropagation of PEMV in the aphid. The tracing of “hot” or “cold” labeled virions or viral RNA precursors in aphid cells using autoradiography and electron microscopy should prove useful in arriving at a still more complete understanding of the fate of this virus in its vector.

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