Transcellular Transport of Barley Yellow Dwarf Virus Into the Hemocoel of the Aphid Vector, *Rhopalosiphum padi*

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ABSTRACT

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The ultrastructure of *Rhopalosiphum padi* reared on healthy oats or oats infected with the RPV isolate of barley yellow dwarf virus (BYDV) was studied to determine the site and cellular mechanisms involved in BYDV transport from the gut lumen into the hemocoel of aphid vectors. Virus particles were consistently associated only with cell membranes of the hindgut in 37 of 40 *R. padi* reared on RPV-infected oats. Virions were not associated with membranes of the stomach or intestinal regions of the midgut. Virions were not observed in midgut or hindgut of 19 aphids reared on healthy oats. The observed virions were immunologically labeled in vivo and identified as RPV. The mechanism for BYDV uptake into hindgut cells by endocytosis was supported by observation of virions adsorbed to the hindgut apical plasmalemma, in coated pits and in coated vesicles. Virions were observed to accumulate in tubular vesicles and lysosomes. Release of the virus into the hemocoel by fusion of virus-containing tubular vesicles with the basal plasmalemma was indicated. A site of luteovirus ingress into the vector at the hindgut is demonstrated. This specific site could be involved in regulating virus uptake.

Luteoviruses are small (25 nm) nonenveloped isometric RNA viruses that are persistently transmitted by aphids (15). To be transmitted, luteoviruses must penetrate aphid cell membranes both at the alimentary canal and salivary gland, be transported through these cells, and released from them in an infectious form. This route implies an intimate association of these viruses with several insect cell types. Although luteoviruses apparently do not replicate in aphid cells (3,19), they show a remarkable degree of vector-specificity for transmission. Only certain aphid species transmit specific luteoviruses (14,15). The mechanisms for virus penetration and transport are not well understood, but aphid cell regulation of these processes is probably involved.

The route of luteoviruses out of aphid vectors, resulting in virus transmission, has been described (4,5). The site and mechanism for luteovirus penetration into the hemocoel from the lumen of the alimentary canal, following virus acquisition from infected plants, are unknown. Transport of virus across cells of the alimentary tract of aphids may involve specific mechanisms and occur in a selective fashion. Gut barriers to virus penetration have been shown to regulate transport of maize streak virus into the hemocoel of the leafhopper, *Cicadulina mbila* (18). Mesenteronal barriers preventing penetration or escape of virus from gut epithelial cells of mosquitoes are thought to play a significant role in regulating transmission of western equine encephalomyelitis virus (7).

The purpose of this study was to locate the site of luteovirus transport from the gut lumen into the hemocoel of aphid vectors and to determine the cellular mechanisms involved in virus uptake and release from the cells.

MATERIALS AND METHODS

Virus-free colonies of a New York clone of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) were maintained on caged plants of cultivar Briggs barley (*Hordeum vulgare* L.) in growth chambers at 15 C with a 24-hr photoperiod at 80 $\mu E \cdot s^{-1} \cdot m^{-2}$. Virus acquisition and transmission experiments utilized cultivar California Red oats (*Avena byzantina* C. Koch) infected with the previously characterized (13) New York RPV isolate (RPV-NY) of BYDV, the type member of the luteoviruses. Oats used as virus source plants were inoculated as 7-day-old seedlings at the one-leaf stage and used for acquisition feedings 3 or 4 wk later. Aphids were allowed to feed on healthy or RPV-infected oats for 7–14 days in the growth chamber.

Aphids were fixed and prepared for ultrastructural examination as previously described (4,5). Micrographs were made with a JEOL 100 CX or Phillips 300 transmission electron microscope operating at 80 kV. Most micrographs were made at 3.3 or 5×10^4 magnification.

To immunologically label virions in the hemocoel, aphids were fed for 10 days on RPV-infected oats or on healthy oats as controls, anesthetized with carbon dioxide gas, and injected with the IgG fraction of rabbit antiserum made against the serologically distinct RPV or MAV isolates of BYDV (13) or *R. padi* virus (RhPV), an aphid virus (1). These aphids were allowed to recover and to feed an additional 24 hr and then were anesthetized and injected with ferritin-conjugated goat antirabbit IgG (Miles Laboratories Inc., Elkhart, IN) diluted to 0.3 mg/ml in 0.01 M phosphate buffer (pH 7). The aphids were allowed to recover and feed overnight on healthy oats before being fixed for electron microscopy. Injections were done with glass needles as previously described (4,9).

RESULTS

The alimentary canal of *R. padi* (Fig. 1) was very similar to that described for *Myzus persicae* (Sulzer) (12) and for *Sitobion* (= *Macrosiphum*) *avenae* F. (11). It consisted of a narrow chitin-lined foregut (not shown), an enlarged anterior region of the midgut (MG), the stomach, and a narrow posterior region of midgut consisting of a lengthy convoluted intestine. Following the midgut was the almost transparent hindgut (HG) which extended the length of the abdomen. In transverse section (Fig. 2) of an aphid abdomen, the thin-walled nature of the hindgut, consisting of stretched squamous epithelial cells, can be compared to thick-walled cells of the midgut, which was coiled through the abdomen adjacent to the hindgut. Hindgut epithelial cells (Fig. 3) had few microvilli, however, numerous microtubulelike structures (mt),

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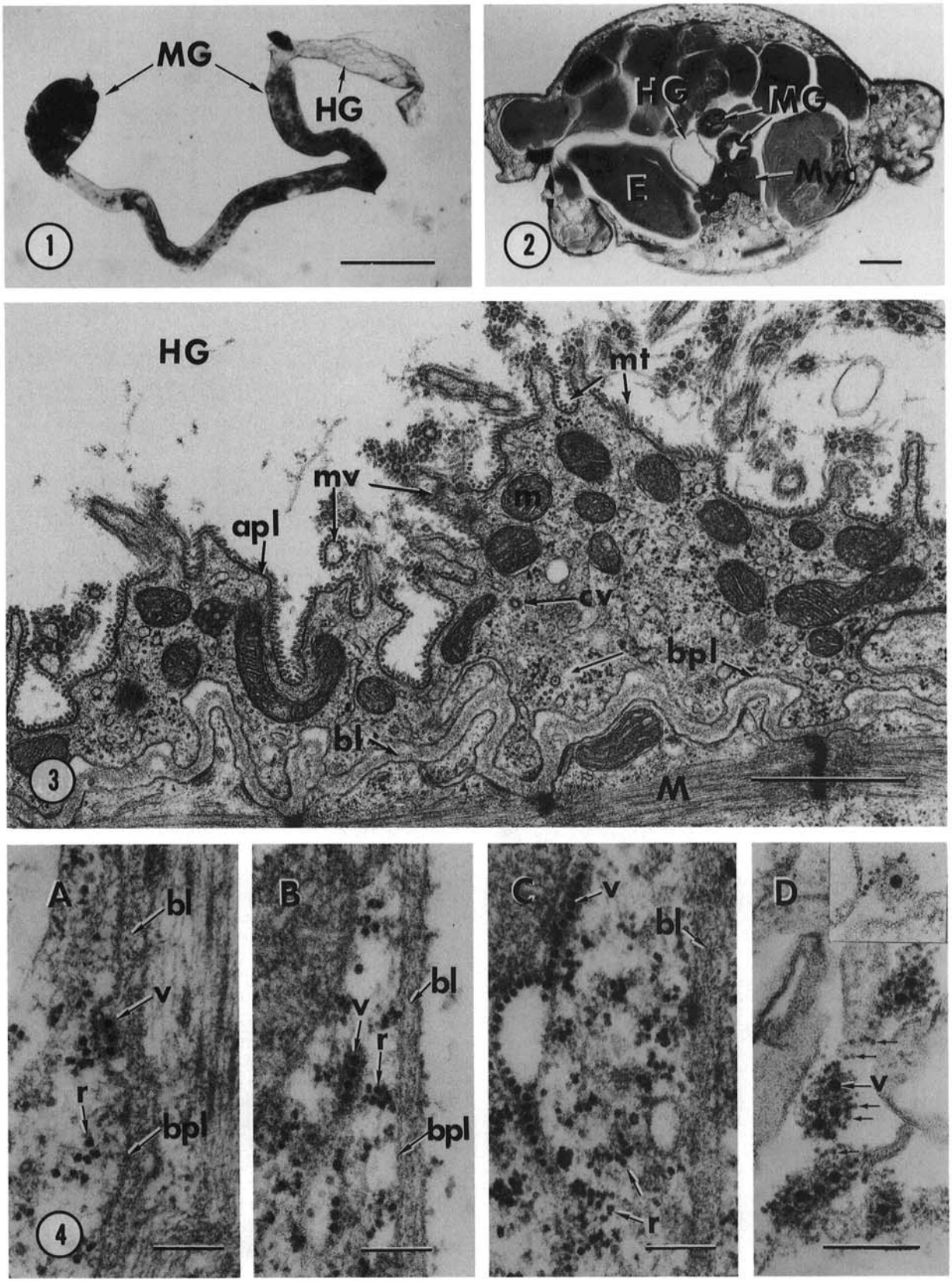


Figs. 1–4. Aphid alimentary canal structures of *Rhopalosiphum padi*. **1,** Unstained whole mount of a dissected alimentary canal of *R. padi* showing the stomach and intestinal regions of the midgut (MG) and the fragile thin-walled hindgut (HG) by bright-field light microscopy. Bar = 0.5 mm. **2,** Thick-section through the abdomen of *R. padi* showing transverse sections through the hindgut (HG) and four sections of midgut (MG), and mycetocytes (Myc) and embryos (E) by interference contrast microscopy. Bar = 0.1 mm. **3,** Electron micrograph of hindgut cell showing extracellular microtubules (mt) lining the apical plasmalemma (apl) and microvilli (mv) in the lumen of the hindgut. Other indicated structures are mitochondria (m), coated vesicles (cv), basal plasmalemma (bpl), and the basal lamina (bl). Note particle in coated vesicle (unlabeled arrow). Bar = 1 μm. **4,** Virions of the RPV isolate of BYDV in hindgut cells of *R. padi* fed on RPV-infected oats and injected with rabbit antiserum to **A,** RPV; **B,** *R. padi* virus (RhPV); **C,** the MAV isolate of BYDV; and **D,** RPV, before injection with ferritin-conjugated goat antirabbit antiserum. Note aggregation of virions (v) in the hemocoel between the plasmalemma and basal lamina only in aphids injected with anti-RPV. Cytoplasmic ribosomes (r) are smaller than virions with irregular outlines and ferritin (**D,** unlabeled arrows and inset) is associated only with labeled virions. Bar = 200 nm.

previously described (10), occurred extracellularly in the lumen associated specifically with the apical plasmalemma (apl). Mitochondria (m) were closely associated with the apical plasmalemma and Golgi bodies and coated vesicles (cv) were frequently observed. The basal plasmalemma (bpl) was not invaginated, unlike the midgut cells, and the entire hindgut was surrounded by an obvious extracellular basal lamina (bl).

Virus particles, approximately 25 nm in diameter, were consistently associated only with cell membranes of the hindgut epithelium in aphids fed on RPV-infected oats. Similar particles were not associated with membranes of the midgut of the same aphids. Occasionally viruslike particles of similar size, shape, and staining intensity were observed free in the midgut and hindgut lumen. Such particles, however, were never numerous. No virus particles were observed associated with the midgut or hindgut cells of *R. padi* fed on healthy oat plants (Table 1).

To identify these particles as barley yellow dwarf virus, virions were immunologically labeled in vivo as described. Thin sections of *R. padi* fed on RPV-infected oats and then injected with anti-RPV, anti-MAV, or anti-RhPV antiserum, before injection with ferritin-conjugated goat anti-rabbit antiserum were examined (Table 1). Virions located between the basal plasmalemma and the basal lamina surrounding the hindgut were positively labeled in *R. padi* injected with anti-RPV antiserum (Fig. 4A,D). Virions were not labeled in any aphids injected with anti-RhPV antiserum (Fig. 4B) or antiserum to MAV (Fig. 4C), even though virions in tubular arrays occurred in the cytoplasm adjacent to the plasmalemma. Particles being released from the hindgut cells were identified as the RPV isolate of BYDV and were not an aphid virus. Furthermore, the clone of *R. padi* used in this work had previously been reported to be free of the RhPV aphid virus (1). Only virions released from the hindgut cells into the hemocoel were labeled when antiserum was injected into the hemocoel. Virions were not observed labeled in the cytoplasm of the hindgut epithelium.

Virions in the lumen of the hindgut (Fig. 5) were most frequently observed in shallow pitlike regions of the apical plasmalemma (apl) underlying the extracellular layer of microtubules (mt). Individual virus particles were also observed in coated pits (cp) and coated vesicles (cv) adjacent to the apical plasmalemma (Figs. 6 and 7). These static images suggest that endocytosis may function in virus ingress into the aphid epithelial cell. No obvious association between the extracellular microtubules and virus was observed.

In the epithelial cell cytoplasm, virions were commonly observed singly in small vesicles and were aligned in tubular vesicles (Fig. 8) similar to those described in salivary gland cells (4). Not all cells making up the circumference of the hindgut contained virus. Within a given aphid, some hindgut cells contained no visible virus, others contained small numbers of virions in vesicles or associated with the plasmalemma, and some cells contained very high concentrations of virions within a variety of membrane-bound structures (Fig. 9). Large concentrations of virions were also observed in several types of lysosomelike bodies (Figs. 10–12). Figure 11 shows a crystalline array of virus particles within a lysosome. Similar arrays have also been observed in the salivary gland of *R. padi* fed on RPV-infected oats (*unpublished*).

At the basal end of the cells, adjacent to the hemocoel, virions were occasionally embedded in the extracellular basal lamina or between the basal lamina and the plasmalemma. The mechanism for virus release from hindgut epithelial cells is not clear. Virions in tubular vesicles or in coated vesicles were occasionally observed in close proximity to the basal plasmalemma, but coated pits releasing particles, such as those described for the salivary gland (4,5), were not observed. A tubular vesicle containing several virions was fused to the basal plasmalemma and might illustrate a mechanism for releasing virions from the cell into the hemocoel (Fig. 13). In this case a coated vesicle containing a virion (arrow) was either forming or had fused with the tubular vesicle.

Virions were rarely observed between the basal lamina and plasmalemma in uninjected aphids or aphids injected with anti-MAV or RhPV. Virions were readily observed in this location, however, in anti-RPV injected aphids. This is shown in Fig. 14 in which an aggregate of labeled virions (V) occurred between the basal lamina and the plasmalemma and unlabeled virions occurred in membrane-bound vesicles (arrows) in the cytoplasm. Figure 15 shows two labeled particles adjacent to a discontinuity of the plasmalemma, which suggests they had been recently released into the hemocoel. Most labeled particles between the basal lamina and plasmalemma were found in aggregates similar to those shown in Figs. 4A, 14, and 15, further suggesting that virus particles may not be released individually into the hemocoel.

TABLE 1. The number of *Rhopalosiphum padi* fed on healthy oats (HO) or oats infected with the RPV isolate of barley yellow dwarf virus in which RPV was observed in the hindgut by transmission electron microscopy following injection of antiserum specific for RPV, MAV, or *R. padi* virus (RhPV), as a control[a]

Exp.	Host plant	Rabbit antiserum	Antirabbit ferritin	Virus observed	Virus labeled
1	RPV	RPV	+	4/5[b]	4/5
2	RPV	RPV	+	4/6	4/6
3	RPV	RPV	+	5/5	5/5
1	RPV	RhPV	+	5/5	0/5
2	RPV	RhPV	+	6/6	0/6
1	RPV	MAV	+	3/3	0/3
1	HO	RPV	+	0/5	0/5
2	HO	RPV	+	0/6	0/6
3	HO	RPV	+	0/8	0/8
3	RPV	–	–	10/10	0/10

[a] Aphids were reared 5–14 days on caged healthy or infected cultivar Coast Black oats at 20 C and a 24-hr photoperiod. Each aphid was injected with antiserum diluted to 0.2 mg/ml in 0.01 phosphate buffer (pH 7). Injected aphids were allowed to feed 24 hr and then were injected with ferritin-conjugated goat anti-rabbit antiserum. Following a second 24-hr feeding, the aphids were fixed for electron microscopy.

[b] Number of aphids containing virus/total number observed.

DISCUSSION

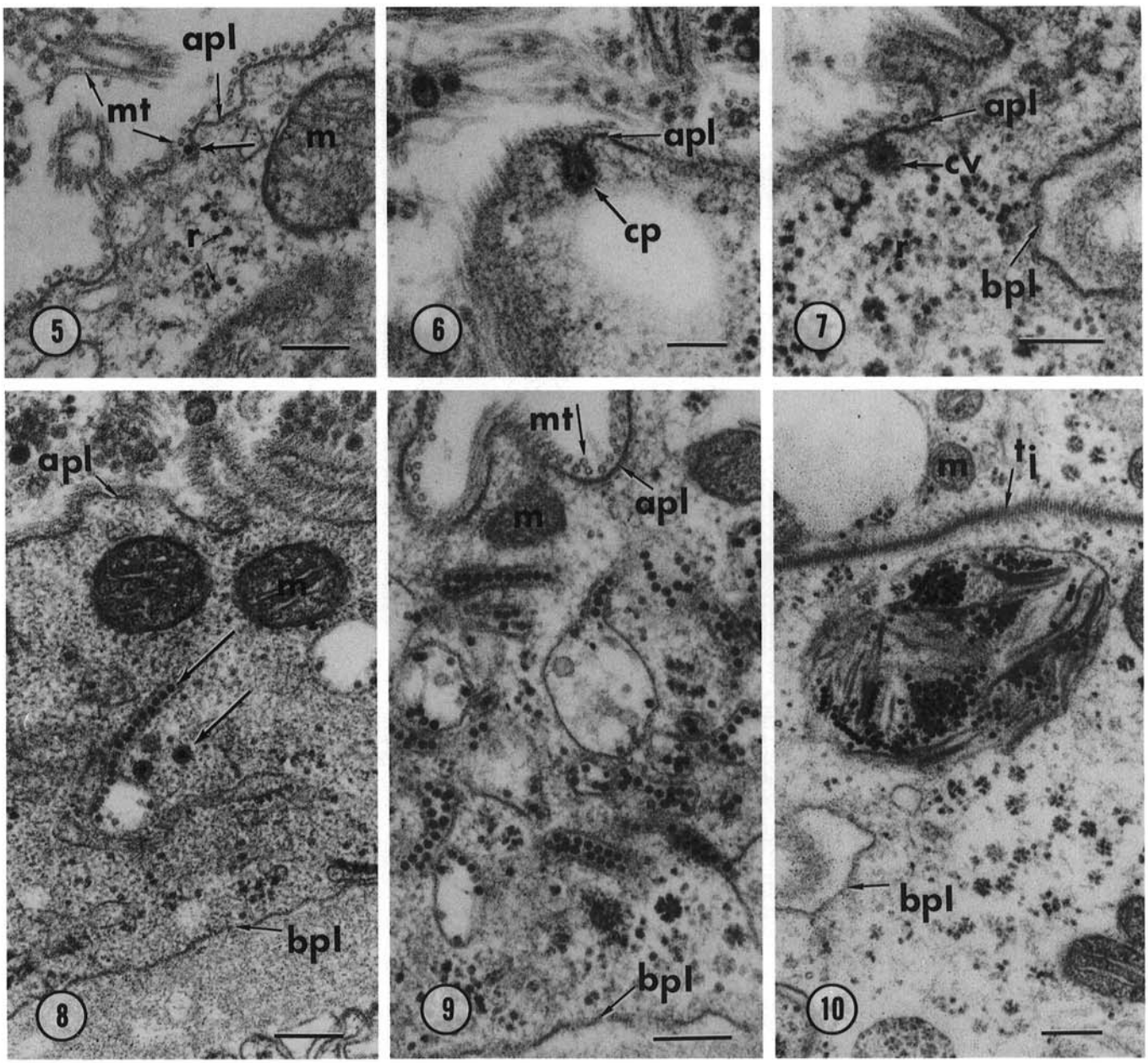
Observations indicated that the hindgut epithelium is a site for luteovirus ingress into the aphid hemocoel. Virions of the RPV isolate of BYDV were consistently associated only with the hindgut epithelial cells of the vector, *R. padi*. The static images reported suggest that virions in the lumen of the hindgut adsorb in a specific manner to the apical plasmalemma and initiate coated pit development and subsequent endocytosis of the particles into coated vesicles. The fate of virus particles in coated vesicles is uncertain. Accumulations of virions in lysosomes and in tubular vesicles, however, suggests that these organelles may be the destination for many virus-containing coated vesicles. Observations to date suggest that virus is released from the cell by fusion of tubular vesicles, containing virus, with the basal plasmalemma. The infrequent occurrence of virus particles embedded in the basal lamina, and between it and the plasmalemma, except when trapped by homologous antiserum, indicated that virions normally moved quickly into the hemocoel once released from the cell.

The role of coated vesicles and tubular vesicles in luteovirus transport through aphid salivary gland cells has been discussed (4,5), and a role for coated pits and vesicles in virus penetration of host cells has been described for several groups of animal-infecting viruses (2,8,17). Semliki Forest virus particles have been shown to be internalized by endocytosis and transported in coated vesicles to intracellular lysosomes where they may accumulate prior to release into the cytoplasm (8). The fate of RPV virions accumulated in lysosomes of hindgut cells (Figs. 10–12) is unknown. St. Louis encephalitis virus (SLEV) accumulates in cisternae of endoplasmic reticulum (20). In this system, SLEV is released from mosquito cells by fusion of the virus-containing endoplasmic reticulum with the plasmalemma. The accumulation of SLEV within distended cisternae of endoplasmic reticulum is reminiscent of the accumulation of RPV in tubular vesicles and distended vacuoles in aphid hindgut cells (Figs. 8 and 9), and these structures may play a

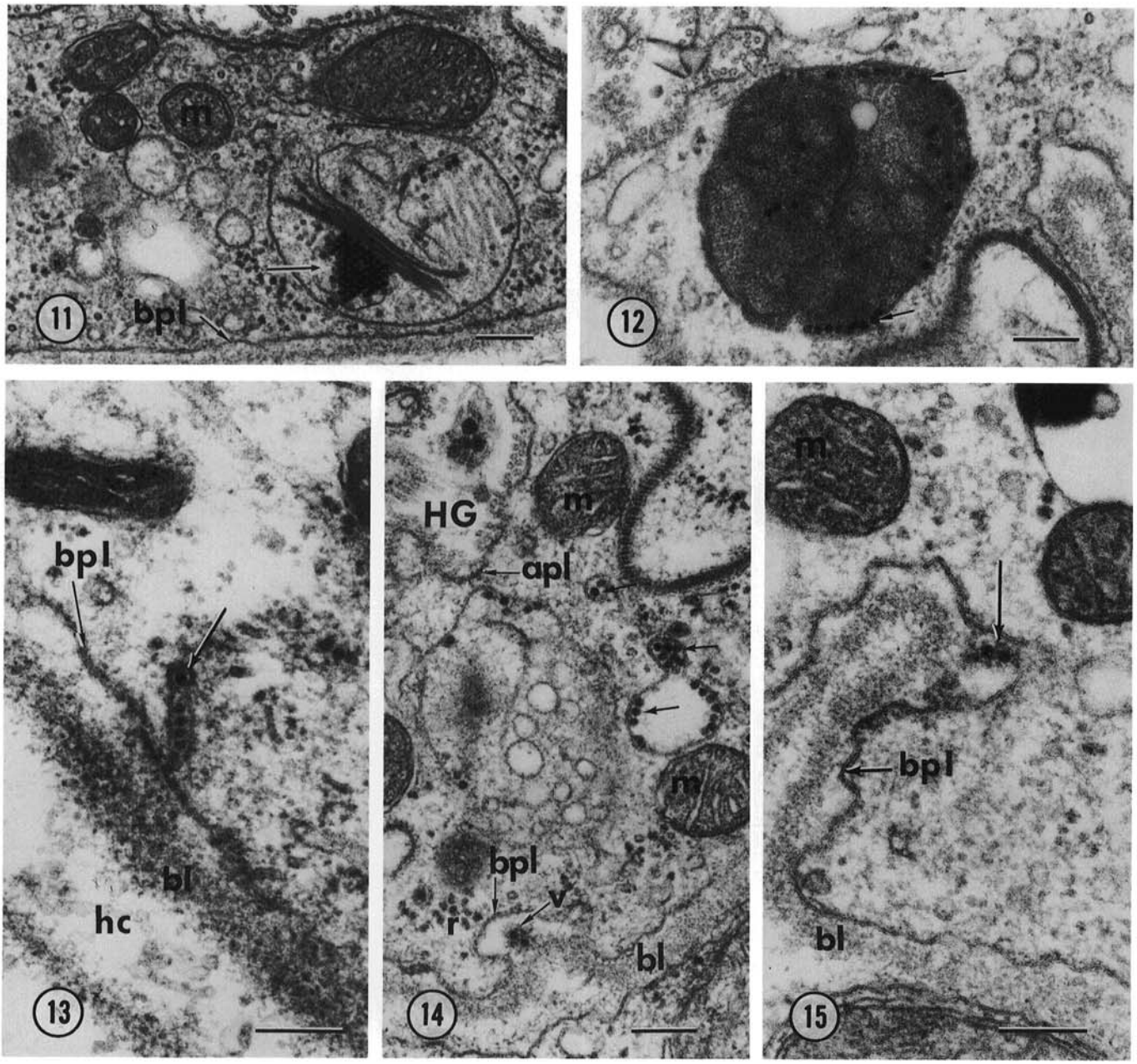
similar role in RPV release into the hemocoel. The relationship of the tubular vesicles and vacuoles with endoplasmic reticulum has not been verified for *R. padi*.

At the present time, transmission data and serological tests (3,11,19) do not support the idea that luteoviruses replicate in their aphid vectors. Extensive ultrastructural studies of *Sitobion avenae* F. (5) and *Myzus persicae* Sulzer (4) salivary glands have failed to detect cytopathological alterations or large accumulations of luteoviruses within salivary gland cells. The accumulation of RPV in membrane-bound cisternae and lysosomes of some cells of the hindgut, however, is suspicious. The large number of virions observed could arise from accumulation and sequestering of particles in these organelles, without virus replication. On the other hand, Hardy et al (7) point out that in several arthropod-borne virus systems only one in five midgut cells may possess detectable virus, that virus concentration in the midgut may decline rapidly following infection, and that no cytopathological symptoms are associated with virus replication. Therefore, the remote possibility that BYDV could replicate at low levels in a few cells of the aphid hindgut could be another explanation for the observed accumulation. More data are required, however, to substantiate or refute this hypothesis.

Visualization of virions in coated pits on the plasmalemma and endocytosis into coated vesicles suggest that virus recognition and adsorption to the cell membrane may be a mechanism regulating virus uptake into the hindgut. Although a gut barrier does not prevent movement of some barley yellow dwarf virus isolates into some nonvector aphids (16), it might explain why aphids transmit relatively few plant viruses in a circulative manner. A specificity mechanism, analogous to that hypothesized for the salivary gland



Figs. 5–10. Electron micrographs of sections through hindgut cells of *Rhopalosiphum padi* fed on oats infected with the RPV isolate of BYDV. **5,** A virion (arrow) beneath the extracellular microtubules (mt) lining the hindgut and in contact with the apical plasmalemma (apl). Smaller, irregularly shaped ribosomes (r) occur free in the cytoplasm. **6,** A virion in a coated pit (cp) invaginated into the cytoplasm. **7,** A coated vesicle (cv), containing a virion, adjacent to the apical plasmalemma (apl). The basal plasmalemma (bpl) indicates the thinness of the hindgut in some areas. **8,** A string of virions in a tubular membrane structure and two individual membrane-bound particles, which could be tubular vesicles in transverse section. **9,** A concentration of virions in various membrane-bound vesicles. **10,** Virions concentrated in a lysosomelike vesicle next to a tight junction (tj) between membranes of adjacent cells. Bars = 200 nm.



Figs. 11–15. Sections of hindgut cells of viruliferous *Rhopalosiphum padi* fed on oats infected with the RPV-isolate of BYDV. **11,** A triangular crystalline array of particles in a lysosome. **12,** Virions (arrows) lining the perimeter of a membrane-bound vesicle. **13,** Virions in a tubular vesicle which appears continuous with the basal plasmalemma (bpl). **14,** Labeled virions (v) external to the basal plasmalemma (bpl) and aggregated with anti-RPV rabbit IgG and anti-rabbit goat IgG conjugated to ferritin. Unlabeled membrane-bound particles (arrows) occurred in the cell. **15,** Two labeled virions (arrow) in the hemocoel adjacent to a discontinuity of the basal plasmalemma (bpl). Bars = 200 nm.

(6), could operate in the hindgut. If such a mechanism were operable, then the majority of plant viruses may not be able to enter the hemocoel of nonvectors due to the inability of viruses to adsorb to the hindgut.

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