Retrieval of Lysosomal Membrane and Acid Phosphatase from Phagolysosomes of *Paramecium caudatum*

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ABSTRACT Little is known about the fate of lysosomal membrane in phagocytic cells. Because the age of the digestive vacuoles in *Paramecium caudatum* can be easily determined, we have been able to study the dynamic membrane events in the older vacuoles. Late in the phagolysosomal stage (DV-III) the vacuole membrane undergoes a burst of tubule formation. The tubules expand into vesicles which have characteristics resembling lysosomes in both thin sections and freeze-fracture replicas. The tubules also contain acid phosphatase activity when they arise from acid phosphatase-reactive vacuoles. We conclude that after active digestion lysosomal membrane is retrieved in whole or in part along with some membrane-associated hydrolases. A logical extension of these results is that the lysosome-like vesicles, after being recharged with hydrolases by fusing with primary lysosomes, are recycled back to DV-II for reuse.

Digestive vacuoles in *Paramecium* can be grouped into four stages, DV-I to DV-IV, based on a combination of parameters including their age, size, vacuolar pH, the presence or absence of acid phosphatase activity, membrane ultrastructure, and freeze-fracture morphology (1–4). In addition, vacuoles undergo a definite processing ("maturation") period during which they are not defecated (1). A recent cytochemical study in our laboratory (2) has shown that only DV-III (phagolysosomes) contain acid phosphatase activity, thus confirming an earlier observation (5) that the oldest digestive vacuoles (DV-IV) in *Paramecium* are devoid of acid phosphatase activity. These DV-IV are found throughout the ventral half of the cell and accumulate in the posterior region near the cytoproct or cell anus (3) and are defecation competent. The exact reason why DV-IV no longer give a positive cytochemical reaction for acid phosphatase, as do the DV-III, was not determined in previous studies. We now report a morphological and cytochemical study of older vacuoles of known age and/or stage which suggests the retrieval of lysosomal membrane from these vacuoles in the form of long tubules, some containing acid phosphatase activity. These tubules become transformed into vesicles having characteristics similar to secondary lysosomes.

MATERIALS AND METHODS

Paramecium caudatum growing in axenic medium (6) was fed polystyrene latex spheres (0.1% solid) for 3 min and chased in axenic medium to indicate the age of labeled vacuoles in sectioned cells (7). Some of these cells were fixed for transmission electron microscopy without further treatment (as in reference 7), while others were incubated in the Gomori medium (8) containing β-glycerophosphate as substrate for acid phosphatase (as in reference 2). Freeze-fractured replicas of polystyrene latex sphere–labeled vacuoles were also examined. These were the same replicas used in an earlier pulse-chase study where cells grown in a bacterized medium rather than an axenic medium were used (4). Electron micrographs were taken with a Hitachi HU-11A or Zeiss 10 electron microscope operated at 75 and 80 kV, respectively.

RESULTS

Thin-section Morphology

The digestive vacuoles of *P. caudatum* pass through at least four distinguishable stages from formation to defecation (1, 9). Vacuole defecation has been shown to begin at ~20 min and is essentially completed by 60 min in axenically grown cells (1) as well as in bacterized cells (unpublished observation). During the last two stages, referred to as DV-III and DV-IV, tubular profiles that had a regular diameter (~45 nm) and extended from the vacuole membrane into the cytosol could often be seen (Fig. 1). That these profiles were indeed tubular was supported by the observation of many circular cross-sectioned tubules (arrowheads, Fig. 2) in our sections and the complete lack of flattened cisternae lying within the section plane between the tubular profiles. These tubules were observed on the DV-III surface from shortly after the lysosomes fused with the vacuole (~10-min old), and on the DV-IV constantly until the vacuoles were defecated.

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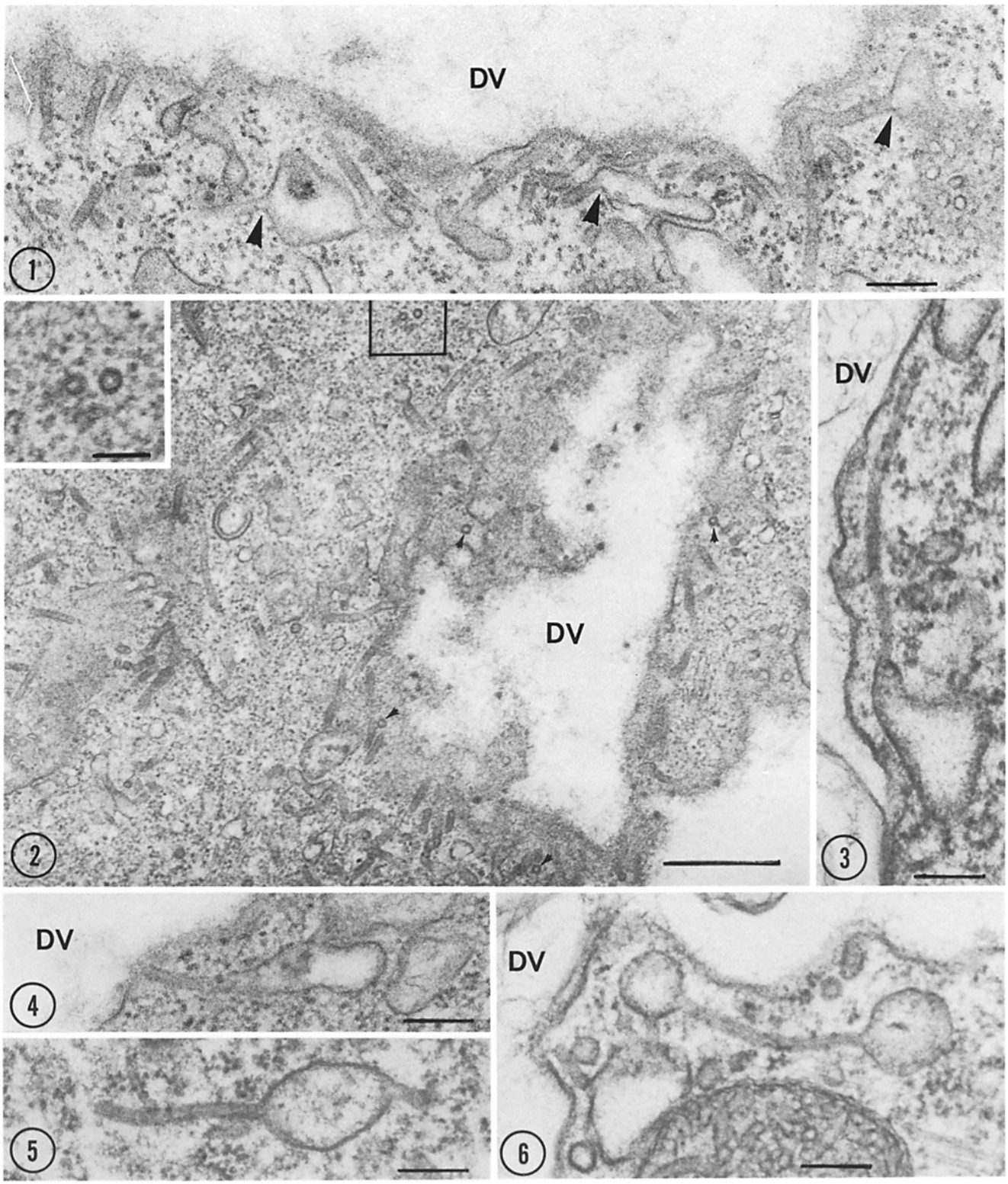


FIGURES 1–6 Fig. 1: Portion of late DV-III vacuole of *Paramecium caudatum* with numerous tubules next to its membrane. Tubules sometimes branch and expand at their distal ends (arrowheads). Bar, 0.2 μm. × 60,000. Fig. 2: Tangential section through late DV-III vacuole with many tubules lying longitudinally within the section plane as well as cross-sectioned tubules (arrowheads) next to the vacuole membrane. Bar, 0.5 μm. × 40,000. (*inset*) An enlargement of the cross-sectioned tubule at top of figure showing its glycocalyx. Bar, 0.1 μm. × 100,000. Fig. 3: A long tubule lying parallel to the vacuole with an expanded distal end. The expanded portion bears a distinct lysosome-like glycocalyx. Bar, 0.2 μm. × 60,000. Fig. 4: Tubule arising from the digestive vacuole membrane. Bar, 0.2 μm. × 60,000. Fig. 5: Lysosome-like vesicle with two tubular extensions. Lysosome lies free in cytoplasm. Bar, 0.2 μm. × 60,000. Fig. 6: An extended tubule with expansions at the distal end and at two separate places along the tubule. Bar, 0.2 μm. × 60,000.

Tubule formation appeared to reach a maximum at a time when vacuoles were being transformed from the DV-III to the DV-IV stage (Fig. 1). These tubules were more often lying along the surface than extending perpendicular from this surface; sections tangent to these vacuoles frequently contained longitudinally sectioned tubules (Fig. 2). Since the diameter of the tubules was nearly the same thickness as the thin sections, continuity between the tubules and the vacuole membrane was usually indistinct, though continuity was observed often enough to support the contention that most tubules were continuous with the vacuole membrane.

A striking feature of many of these tubules, particularly evident at the time of DV-III to DV-IV transition, was their tendency to balloon out either at their distal ends (Figs. 1, 3, and 4) or somewhere along the length of the tubule (see Fig. 6). The membrane of these bulbous enlargements bore a glycocalyx (Fig. 3) similar in thickness and appearance to that on the DV-III vacuole membrane and to the secondary lysosomes commonly found around DV-II vacuoles (3). A glycocalyx could also be seen within cross-sectioned tubules (Fig. 2, inset), but here it was more compact and not fibrous as on the DV-III membrane.

Lysosome-like vesicles lying free in the cytoplasm (Fig. 5) sometimes bore tubular tails of similar diameter to the tubules next to DV-III (e.g., Fig. 6). Occasionally, secondary lysosomes with tails were seen lying next to DV-II vacuoles.

Acid Phosphatase Cytochemistry

About 50% of the vacuoles 8–20-min old were acid phosphatase positive following incubation in the Gomori medium (2). Late in this time period tubules extending from the vacuole surface (see Fig. 8) as well as their bulbous distal ends frequently contained regions of acid phosphatase reaction product (Figs. 7 and 8). Some tubules were completely filled, but more often tubules contained only localized regions of reaction product (Figs. 8 and 9) or none at all. At times, reaction product also filled the expanded portions of the tubules (Fig. 10). In the vacuole lumen the reaction product was always localized along the membrane and around particles such as the latex spheres used to label the vacuoles. Tubules extending from acid phosphatase-unreactive vacuoles (such as DV-IV) (Fig. 10) never contained reaction product.

Freeze-Fracture Morphology

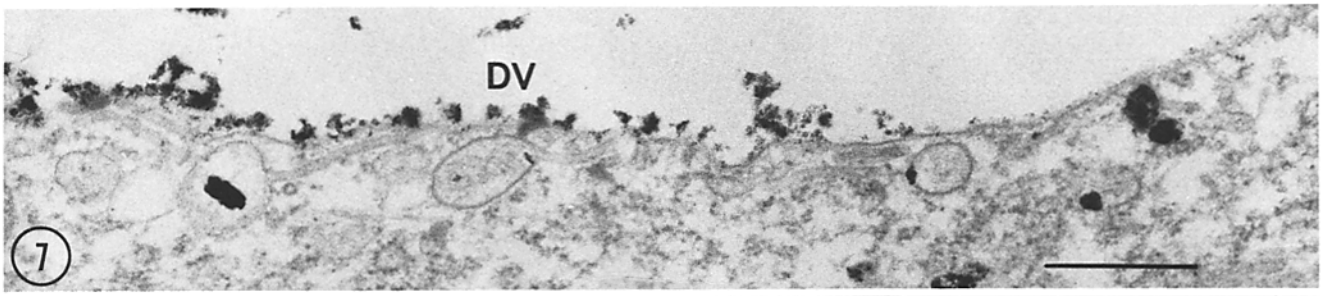
Late DV-III membranes, when fractured to show large expanses of membranes, revealed fractured necks of a uniform diameter arranged in clumps of three to six (Fig.11). Such necks represent sites at which tubules had been fractured from the vacuole. Some prominent intramembrane particles were scattered on the E fracture face of longitudinally fractured tubules and their expansions (Figs. 12 and 13) whereas the P fracture face contained a more heterogeneous assortment of these particles (Fig. 12). Although no attempt was made to quantitate the number of prominent intramembrane particles per square millimeter on these tubules, there was little doubt that the fractured membrane of the tubules and their bulbous expansions resembled the lysosomal membrane and the DV-III membrane. A few lysosomes with tubular tails apparently lying free in the cytoplasm or near DV-II (Fig. 14) were also observed in freeze-fracture replicas.

DISCUSSION

Little is known about the fates of the hydrolases and the lysosomal membrane once the lysosomes have fused with phagosomes. In macrophage and fibroblasts there appears to be a bidirectional flow of at least some of the membrane components between the plasma membrane and phagosomes (10–13). Shuttling of membranes between the Golgi apparatus and the lysosomes and between prelysosomal compartments and lysosomes is suspected but difficult to prove (14). Our results indicate that some acid phosphatase along with an unknown portion of the lysosomal membrane are retrieved from the vacuoles (phagolysosomes) of *Paramecium* by a process of tubule formation and pinching-off of the vacuole membrane. Evidence in support of this conclusion includes (*a*) the large number of tubules extending from vacuoles ≥20-min old, a time when vacuoles in *Paramecium* become defecation competent; (*b*) the rounding of the distal end of these tubules to form lysosome-like bodies whose membrane also possesses a distinctive lysosome-like glycocalyx; (*c*) the presence of acid phosphatase reaction product both in the tubules and in the expanded distal portions of the tubules; (*d*) the fact that membrane removed from late DV-III and DV-IV has a freeze-fracture appearance similar to that of the secondary lysosomes around the DV-II; and (*e*) our previous observation (1) that DV-IV tend to become smaller in diameter than DV-III.

Steinman et al. (15) and Farquhar (14) have discussed the possibility that one type of membrane may be sorted from another membrane type during membrane recycling. This is probably the case in *Paramecium* digestive vacuoles. Thin-section electron microscopy supports the selective removal of the prominent glycocalyx of DV-III which leaves an inconspicuous glycocalyx on the spent vacuole membrane (3, 16) much like that of the discoidal vesicles. Freeze-fracture electron microscopy demonstrates the similarity of the intramembrane appearance of the retrieved membrane to that of the secondary lysosomes. Together these observations are consistent with the sorting out and removal of lysosomal membrane components from the vacuole membrane. Whether the retrieved portions of the membrane were present in the vacuole membrane as distinct microdomains or as homogeneously dispersed components is not known, but the freez-fracture replicas indicate the probable homogeneous distribution. On the other hand, a lack of mixing of membranes from various sources may be easily overlooked if a rapid replacement of one type of membrane by another takes place such as in the DV-I to DV-II transformation (17) or if membrane fusion is rapidly followed by the fission of the same membrane.

Sorting of membrane components for retrieval would explain the observation that the lysosomal membrane proteins in mammalian systems have heterogeneous half-lives (18–20). We see no morphological evidence for autophagic engulfment of lysosomes during the log-phase of growth in *Paramecium* (21) and thus no pinching-off and return of selected membrane components from the lysosomal membrane to the autophagic vacuole membrane as proposed by Dean (22). (Turnover of lysosomes through autophagosomes does not seem to be required by *Paramecium.*) However, removal and recycling of selected lysosomal components combined with the "processing" of those membrane components remaining in the digestive vacuole membrane could explain heteroge-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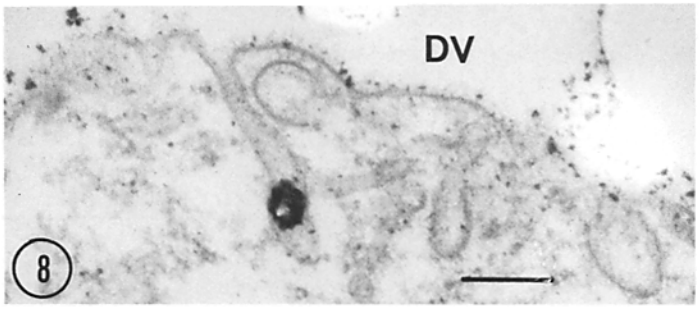
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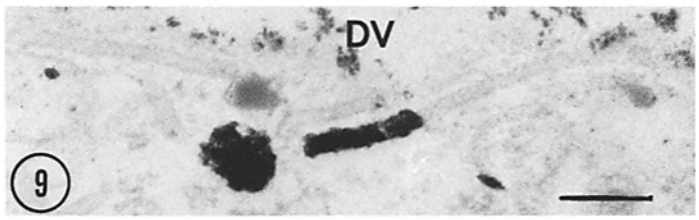
DV
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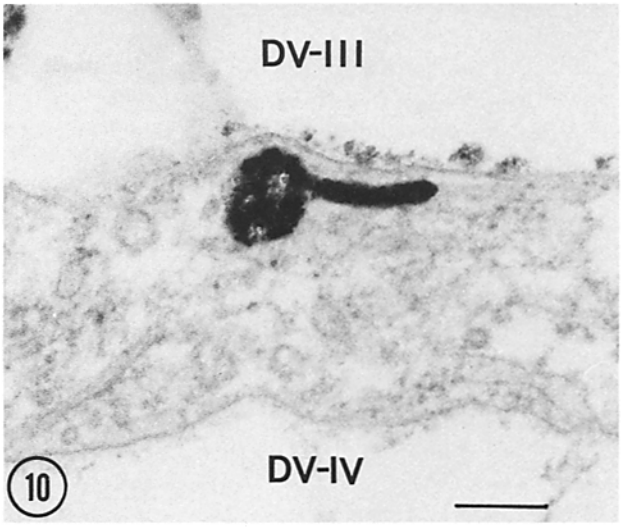
DV-III
DV-IV
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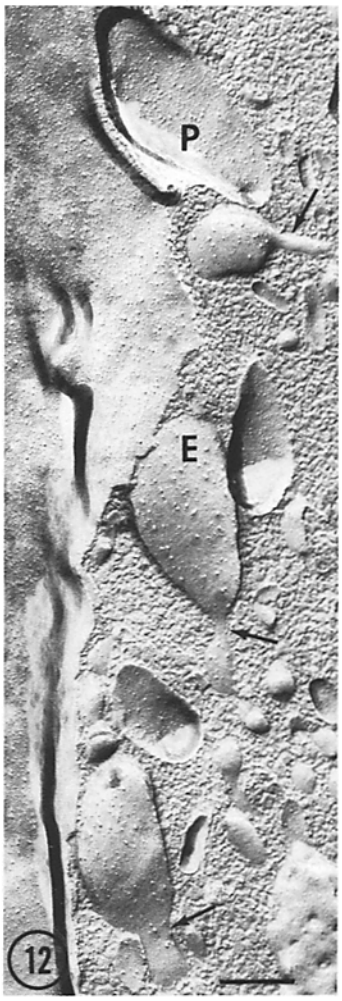
11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neous half-lives of lysosomal membrane proteins in cells where autophagocytosis is not occurring.

As observed recently in another study (3), it appears that, in *Paramecium*, whenever membrane is removed from a relatively planar surface, it is usually by tubule formation followed by a pinching-off of the tubules. Tubules may then take on different shapes (e.g., discoidal or spherical) as the forces holding the membrane in cylindrical form are relaxed. This may occur either before or after the tubules are removed from the larger membrane plane. Freeze-fracture pictures make it clear that tubule formation in DV-III occurs in clumps of up to five to six tubules within a small area. (Tubule formation of vacuole membrane also occurs in DV-I, but here the freeze-fracture pictures show a more random distribution of the fractured necks.) The forces causing tubule formation of phagolysosomal membrane must be stronger at scattered foci over the vacuole membrane. We do not know the nature of these forces or what triggers the reshaping of these membranes. Also, we do not know how rapidly the tubules pinch off the vacuole once they have formed or whether tubules remain for an extended period while one or more vesicles pinches off their distal ends.

Removal of membrane as long tubules of small diameter rather than as spherical vesicles effectively restricts the uptake of the vacuole content while allowing the removal of a relatively large membrane area. The lumen of the tubule is further occluded by a compact glycocalyx leaving a central electron-transparent channel of only 10 nm diam (Fig. 2, inset). Such a small channel would permit passage between the vacuole lumen and the expanded distal ends of the tubules only of solution or minute suspended particles. Undigested particulate material would remain in the vacuoles for eventual defecation.

These observations suggest that both components of the lysosomal membrane (if not the complete membrane) and some acid phosphatase are being retrieved from the old vacuoles for recycling. We do not know where primary lysosomes enter the digestive vacuole system, but it is conceivable that retrieved lysosome-like vesicles may need to be recharged with hydrolases, specific membrane components, and surface recognition factors. These may be obtained by fusion with small primary lysosomes arising from the Golgi apparatus.

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FIGURES 7–14 Fig. 7: Acid phosphatase reaction product is found along the vacuole membrane in a 21–24-min-old vacuole as well as in the expanded portions of tubules arising from the digestive vacuole membrane. Bar, 0.5 μm. × 40,000. Fig. 8: A tubule showing a clear continuity with the vacuole membrane and containing a clump of acid phosphatase reaction product. Bar, 0.2 μm. × 60,000. Fig. 9: Acid phosphatase activity is found in a portion of a tubule as well as filling a circular profile. Bar, 0.2 μm. × 60,000. Fig. 10: The top vacuole is a 21–24 min-old vacuole containing polystyrene latex spheres. A tubule next to this vacuole with an expanded end is heavily reactive for acid phosphatase. Tubules next to acid phosphatase-negative vacuoles (lower vacuole) lack acid phosphatase. Bar, 0.2 μm. × 60,000. Fig. 11: A freeze fracture replica of a digestive vacuole membrane showing the E face and fractured necks arranged in clumps. Tubules with expanded ends (black and white arrows) lie beside the vacuole. Bar, 1 μm. × 25,000. Fig. 12: Lysosome-like vesicles with tubular extensions (arrows) next to the P face of a 32–35 min-old vacuole. *E*, E fracture face; *P*, P fracture face. Bar, 0.25 μm. × 40,000. Fig. 13: A tubule continuous with the vacuole (P face exposed) has an expanded distal end (E face exposed). Bar, 0.2 μm. × 80,000. Fig. 14: The E fracture face of a lysosome-like body with tubular "tail" next to a late DV-I vacuole. Bar, 0.2 μm. × 60,000.