

# Tubular Early Endosomal Networks in AtT20 and Other Cells

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**Abstract.** Using horseradish peroxidase (HRP) as a fluid-phase endocytic tracer, we observed through the electron microscope numerous tubular endosomes with a diameter of 30–50 nm and lengths of  $>2 \mu\text{m}$  in thick sections (0.2–0.5  $\mu\text{m}$ ) of AtT20 cells. These tubular endosomes are multibranching and form local networks but not a single reticulum throughout the cytoplasm. They are sometimes in continuity with vesicular endosomal structures but have not been observed in continuity with AtT20 cell late endosomes. Tubular endosomal networks are not uniformly distributed throughout the cytoplasm, but are particularly abundant in growth cones, in patches below the plasma membrane of the cell body, and surrounding the centrioles and microtubule organizing center (MTOC). Tubular endosomes at all these locations receive HRP within the first 5 min of endocytosis but  $\sim 30$  min of endocytosis are required to load the tubular endosomal networks with HRP so that their full extent can be visualized in the electron microscope. After 10 min of endocytosis, complete unloading occurs within 30 min of chase, but between 30 and 60 min are required to

chase out all the tracer from the tubular endosomes loaded to steady state during 60 min endocytosis of 10 mg/ml HRP. In interphase cells, neither the loading nor unloading of tubular endosomes depends on microtubules but in cells blocked in mitosis by depolymerization of the mitotic spindle with nocodazole, HRP does not chase out of tubular endosomes. The thread-like shape of tubular endosomes is not dependent on microtubules. Furthermore, HRP is delivered to AtT20 tubular endosomes at 20°C. All these properties indicate that AtT20 cell tubular endosomes are an early endocytic compartment distinct from late endosomes. Tubular endosomes like those in AtT20 cells have been seen in cells of the following lines: PC12, HeLa, Hep2, Vero, MDCK I and II, CCL64, RK13, and NRK; they are particularly abundant in the first three lines. In contrast, tubular endosomes are sparse in 3T3 and BHK21 cells. The tubular endosomes we have observed appear to be identical to the endosomal reticulum observed in the living Hep2 cells by Hopkins, C. R., A. Gibson, H. Shipman, and K. Miller. 1990. (*Nature (Lond.)*. 346:335–339).

**M**EMBRANE-bound cytoplasmic organelles can in a broad sense be classified into two morphological groups, the vesicular and the reticular. In animal cells, mitochondria, peroxisomes, secretory granules, clathrin-coated vesicles, and in most cases, lysosomes, fall into the first category. Although these organelles are far from perfectly spherical, in most cells they have a discrete shape and a regular outline. The smooth ER, the rough ER, and the *trans*-Golgi network (for review see Griffiths and Simons, 1986), which is also known as the *trans*-Golgi reticulum (for example, Geuze et al., 1987), fall into the second category. These organelles do not have a discrete shape and regular outline, but consist of sheets and tubes joined together in a net that extends in three dimensions. There is also compelling morphological evidence, from electron microscopy of thick sections (Rambourg et al., 1981; Rambourg and Clermont, 1986) and optical microscopy of living cells (Cooper et al., 1990), to indicate that the apparently discrete but stacked cisternae of the Golgi apparatus are in fact joined together at least horizontally by tubular elements. The different cisternae of the Golgi stack are, therefore, each part of

a two-dimensional network. Whether there are also tubular connections between the successive cisternae in the stack to generate a truly three-dimensional network is still an open question.

The difference in appearance of the Golgi apparatus in thick and thin sections under the electron microscope is an object lesson. It makes the obvious but still far from trivial point that as the volume of cytoplasm that is sampled goes down—in other words as the sections get thinner—structures that are connected in space by, for example, narrow meandering tubules, increasingly appear to be discrete and unconnected vesicles. In micrographs of thin sections, circular profiles will be overrepresented and liable to be interpreted as sections through spherical objects. It is equally obvious that unlike roughly spherical, vesicular organelles, the reticular organelles are particularly vulnerable to disruption by shear. When a cell is homogenized, reticulae are usually destroyed; pancreatic microsomes produced by homogenization of the ER in exocrine pancreatic cells constitute the paradigm. In cell homogenates vesicular structures will be massively overrepresented.

How do early and late endosomes fit into the broad classification of cytoplasmic organelles as vesicular or reticular structures? Microscopic observations indicate that in most mammalian cells, late or multivesicular endosomes (which are distinguished from lysosomes by their content of cation-independent CI-M6PR) (Griffiths et al., 1988, 1990; for reviews see Courtoy, 1989; Gruenberg and Howell, 1989; Hubbard, 1989) are vesicular organelles. That is not to say that late endosomes never fuse with each other or exchange material by vesicular transport, but only that they do not have a reticular fine structure. The same is true of lysosomes in most animal cells, which are usually described as small spherical or ovoid dense vacuoles (for review see Kornfeld and Mellman, 1989). There are exceptions, however; as Swanson et al. (1985, 1987) have shown, in the macrophage line J774 and in primary peritoneal macrophages treated with phorbol ester, late endosomal structures, termed lysosomes, have a clearly reticular morphology, the maintenance of which depends upon microtubules and is also influenced by the endocytic load (Knapp and Swanson, 1990).

Accepting that in most cell types late endosomes and lysosomes appear to be vesicular, what about the early endosomes? These structures, which are hard if not impossible to identify in thin sections under the electron microscope in the absence of an electron-dense marker introduced by endocytosis, are highly pleiomorphic. The diversity and the irregularity of the shapes of early endosomes seen in the electron microscope have defied a short, precise description and they are therefore referred to as tubulocisternal and tubulovesicular organelles, since in many cell types early endosomes include vesicular or cisternal elements with tubular extensions (for example, Wall et al., 1980; Geuze et al., 1983, 1987; Müller and Hubbard, 1986; Griffiths et al., 1989; for reviews see Helenius et al., 1983; Hubbard, 1989). Tubular early endosomal elements are particularly prominent below the apical plasma membrane of the absorptive cells of the suckling rat ileum, as Wilson et al. (1987) have shown. These tubular endosomes connect to endocytic vesicles and may also form networks.

Given the high fusion activity of early endosomes with one another *in vitro*, Gruenberg and Howell (1989) suggested that early endosomes by fusion and fission events might transiently form networks *in vivo*, rendering them in effect a single compartment. But before the work of Hopkins et al. (1990), early and late endosomes in tissue culture cells had not been described as a reticulum or network, however else their structure had been described. We draw a distinction here between a dynamic network as envisaged by Gruenberg

1. *Abbreviations used in this paper:* CI-M6PR, cation-independent mannose-6-phosphate receptor; CURL, compartment for uncoupling of receptor from ligand; HRP, horseradish peroxidase; MTOC, microtubule-organizing center; POMC, proopiomelanocortin.

and Howell (1989) and a morphological reticulum as described by Hopkins et al. (1990). The latter authors reported the presence in Hep2 cells of extensive tubular endosomal networks that could be visualized in living cells using transferrin conjugated to Texas Red as an endocytic tracer and allowing its uptake for 60 min. Here we report electron microscopic observations of thick sections of cells loaded with HRP or BSA conjugated to 7 nm colloidal gold. We find networks of tubular endosomes in many cell lines including AtT20, Hep2, HeLa, MDCK, and PC12 but not in others, for example BHK21. Our experiments with AtT20 cells indicate that in these cells, and by extrapolation in the other cell types studied, the networks of tubular endosomes are part of the early endocytic compartment and distinct from late endosomes. Our results therefore confirm the presence of endosomal reticulae in many cell types but do not support the notion that these networks are part of the late endosomal compartment. Early endosomes can be added to the growing list of organelles known to have in at least some animal cells a reticular rather than vesicular structure.

## Materials and Methods

### Cell Culture

AtT20, PC12, HeLa, MDCK I and II, Vero, NRK, and 3T3 cells maintained at the European Molecular Biology Laboratory and Hep-2, RK13, BHK-21 (C13), and CCL64 cells from the American Type Culture Collection (Rockville, MD) were propagated under normal cell culture conditions, except that the MDCK I cells were grown on polycarbonate filters (Transwell 3412; Costar Corp., Cambridge, MA).

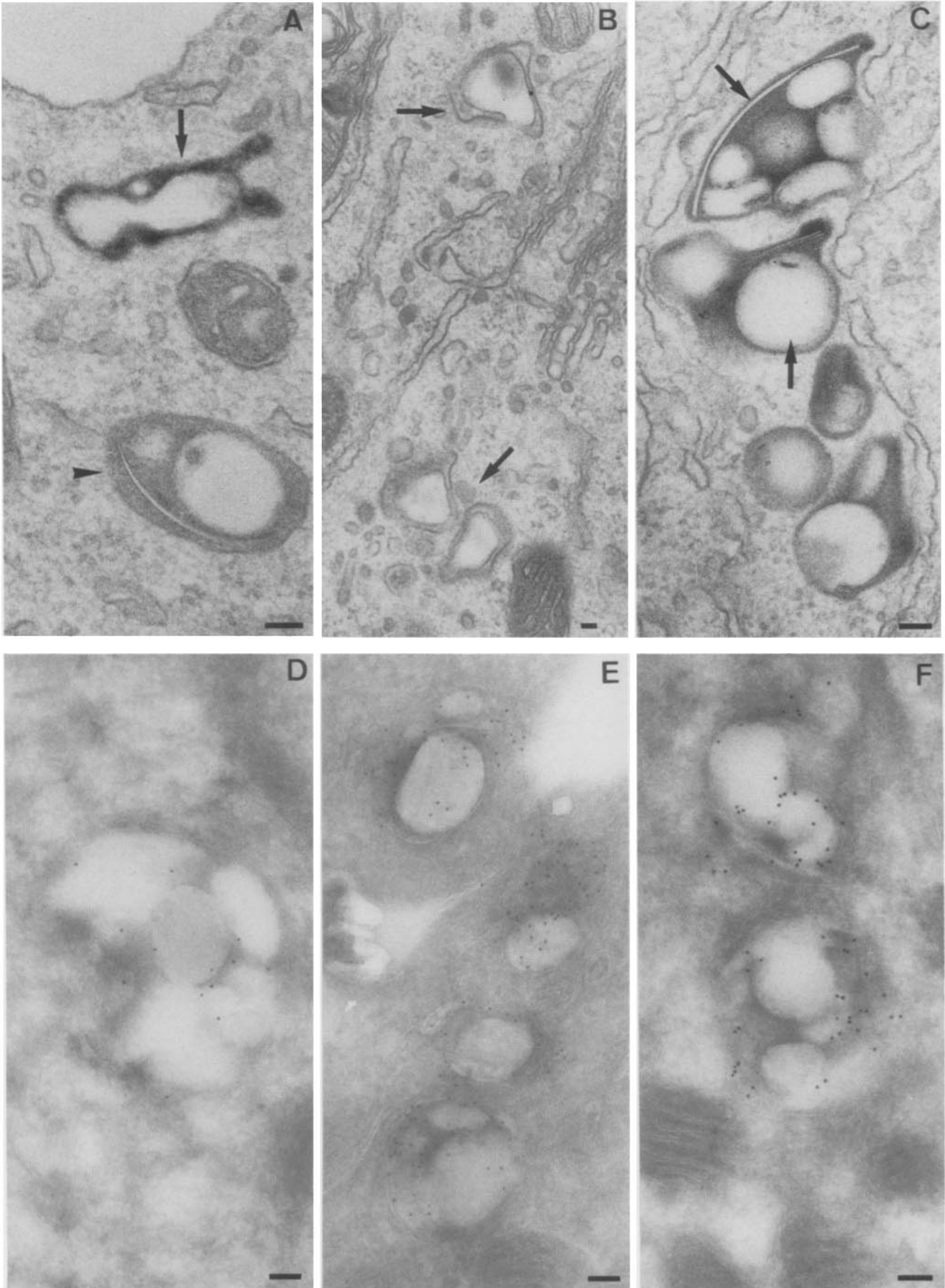
### Antibodies

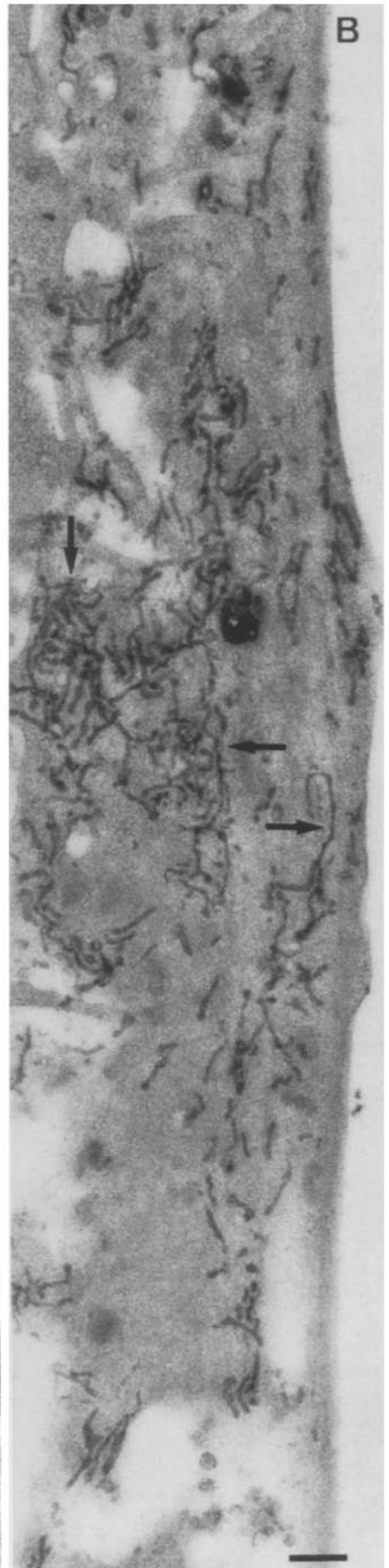
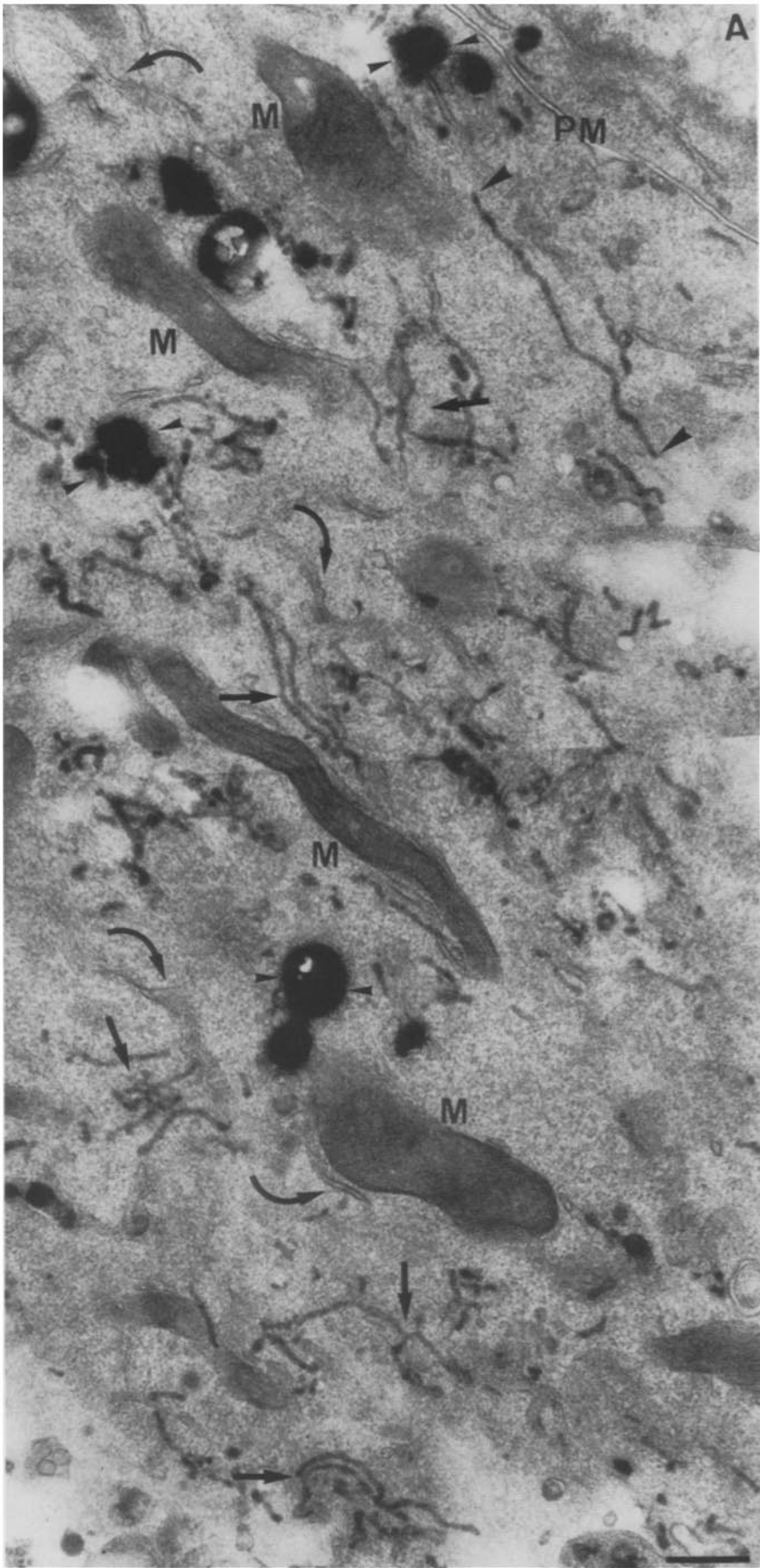
For immunocytochemistry we used the following: affinity-purified rabbit antibody against rat cathepsin D, rabbit antibody against bovine CI-M6PR and rabbit antibody against tubulin. Soluble fragments of bovine CI-M6PR conjugated to 9 nm colloidal gold were used as previously described (Tooze et al., 1990).

### Electron Microscopy

Cells were incubated for varying times from 5 to 120 min with horseradish peroxidase (HRP) type II sp act 200 U/mg (Sigma Chemie GmbH, Deisenhofen, Germany) or sp act 1,362 U/mg (Serva Feinbiochemica, Heidelberg, Germany) at a concentration of 10 mg/ml in medium at 37°C and 5% CO<sub>2</sub>. Cells were then washed with ice-cold PBS and fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate pH 7.4 for 30 min. Immediately after fixation, cells were washed with cacodylate buffer and then reacted with diaminobenzidine (DAB), either 1 min DAB followed by 2 min DAB with H<sub>2</sub>O<sub>2</sub> (short DAB), or 1 min DAB and then 30 min DAB with H<sub>2</sub>O<sub>2</sub> (long DAB). The reaction was stopped by washing with cacodylate buffer, and the cells were postfixed in osmium tetroxide, washed in water and then in 0.5% Mg uranyl acetate overnight at 4°C. The cells were then processed for Epon embedding and EM. Cells were also incubated with BSA stabilized colloidal gold, 7.5 nm. The BSA-gold was dialyzed against PBS and added to the cells for 60 min in varying concentrations ranging from OD<sub>520</sub> 30 to OD<sub>520</sub> 5, then the cells were fixed and processed for electron microscopy. Cells were also incubated in 20 μM nocodazole to depolymerize microtubules ei-

*Figure 1.* Vesicular early and late endosomes in AtT20 cells. *A* shows a vesicular-cisternal early endosome (*arrow*) labeled with HRP after 5 min endocytosis of the tracer and 3 min incubation with substrate. The arrowhead indicates an unlabeled late endosome. *B* shows early endosomal structures (*arrows*) in an Epon section of a conventionally fixed and embedded cell in a culture not exposed to HRP. *C* shows late endosomes in the same preparation as *B*. Note the electron lucent cores and sheets (*arrows*). The sections shown in *A–C* were contrasted with lead citrate. *D* shows late endosomes in a thin cryosection immunogold labeled with rabbit antibody against cathepsin D. *E* shows late endosomes immunogold labeled with rabbit antibody against bovine CI-M6PR. *F* shows late endosomes labeled with soluble fragments of bovine CI-M6PR conjugated to 9 nm colloidal gold. Bars, 100 nm.





ther before HRP uptake or together with HRP. Microtubule depolymerization was checked by indirect immunofluorescence microscopy with an antitubulin antibody. Cells were also incubated first with 10  $\mu\text{g}/\text{ml}$  brefeldin A (Epicentre, Madison, WI) for 60 min and then with brefeldin A and HRP for 60 min. Sections were cut varying in thickness from 60 nm to 500 nm, and were then examined either at 60 or 80 kV on a Zeiss EM10 microscope.

For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 250 mM Hepes, pH 7.4 at 4°C for 10 min and then in 8% PFA at room temperature for 50 min, then scraped with a rubber policeman and spun to a pellet. The cell pellet was infiltrated with 2.1 M sucrose and frozen in liquid nitrogen. Ultrathin sections were cut and labeled according to Tokuyasu (1980) and Griffiths et al. (1984).

## Results

### AtT20 Cell Early Endosomes

AtT20 cells were allowed to endocytose HRP for 5, 7.5, and 10 min and then after fixation incubated with DAB and hydrogen peroxide for only 3 min according to the procedure of Griffiths et al. (1989). In thin sections early endosomes were revealed as compartments of variable shape, size, and morphology; the majority, however, consisted of labeled cisternae enclosing a central lucent region or enclosing membranous material (Fig. 1 A). Compartments with this structure were also seen in cells not incubated in tracer (Fig. 1 B). Other AtT20 cell early endosomes had spherical or more irregular shapes and a multivesicular internal structure.

### AtT20 Late Endosomes

In thin sections of conventionally fixed and embedded AtT20 cells, late endosomes with structural features essentially identical to those of the well-characterized late endosomes of BHK cells (Griffiths et al., 1989; Gorvel et al., 1991) could be readily recognized (Fig. 1 C). The late endosomes are characterized by one or more electron lucent cores and in addition straight or curved sheets of electron lucent material that extend through the depth of the compartment and are apparently rigid enough to distort the bounding membrane of the organelle (Fig. 1 C).

The late endosomes in AtT20 cells began to receive HRP between 10 and 15 min after the cells were exposed to the tracer; before that none contained HRP reaction product. After 15 min of HRP uptake, ~5% of late endosomal profiles contained reaction product, after 30 min uptake 40–50% were labeled and after 120 min, >90% of the late endosomal profiles contained HRP. However, by examining serial sections we established that even after 120 min of continuous HRP uptake, or 60 min HRP uptake followed by 60 or 120 min chase, a small number (<5%) of the late endosomes identified by their morphology were still unlabeled. Delivery of HRP to late endosomes in AtT20 cells is, therefore, highly asynchronous, as in BHK cells (Griffiths et al., 1989; Gruenberg et al., 1989). Experiments with BSA con-

jugated to 7 nm colloidal gold, another unspecific endocytic marker, led to the same conclusion (not shown).

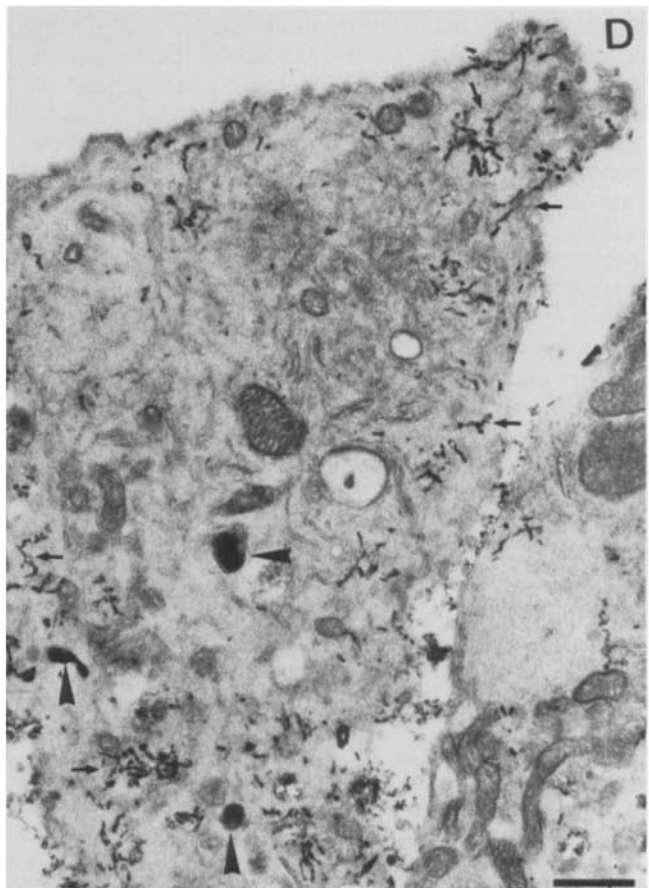
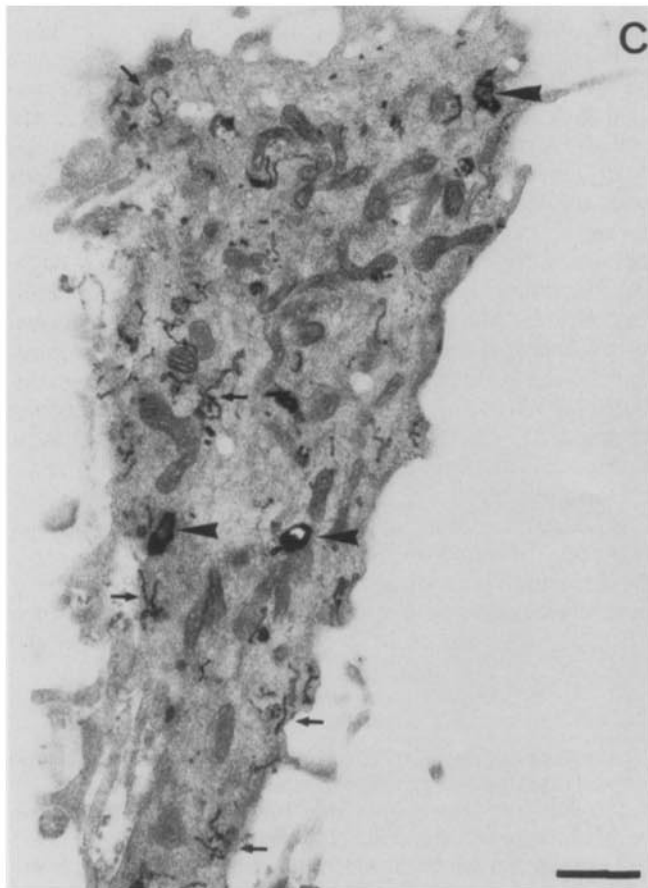
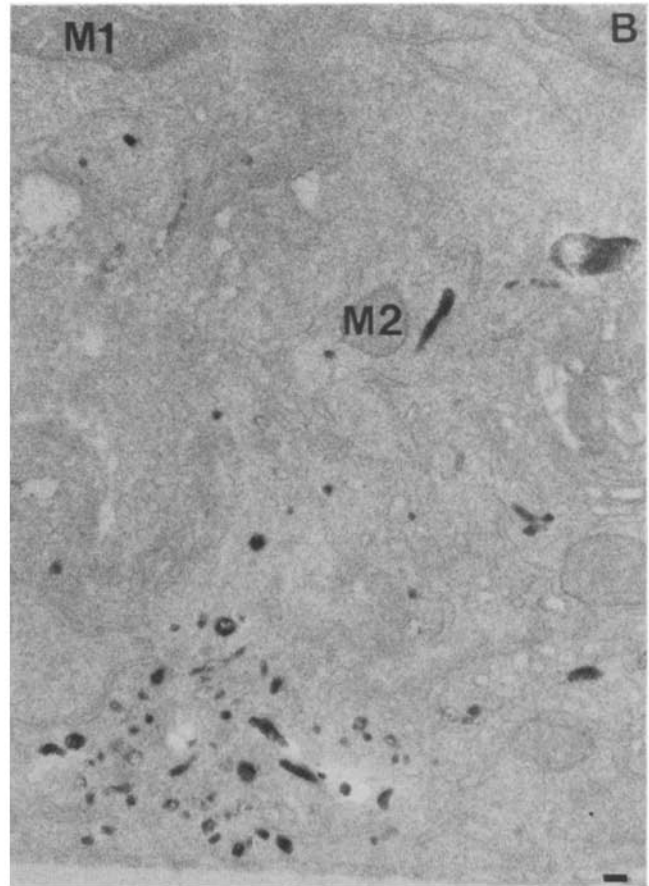
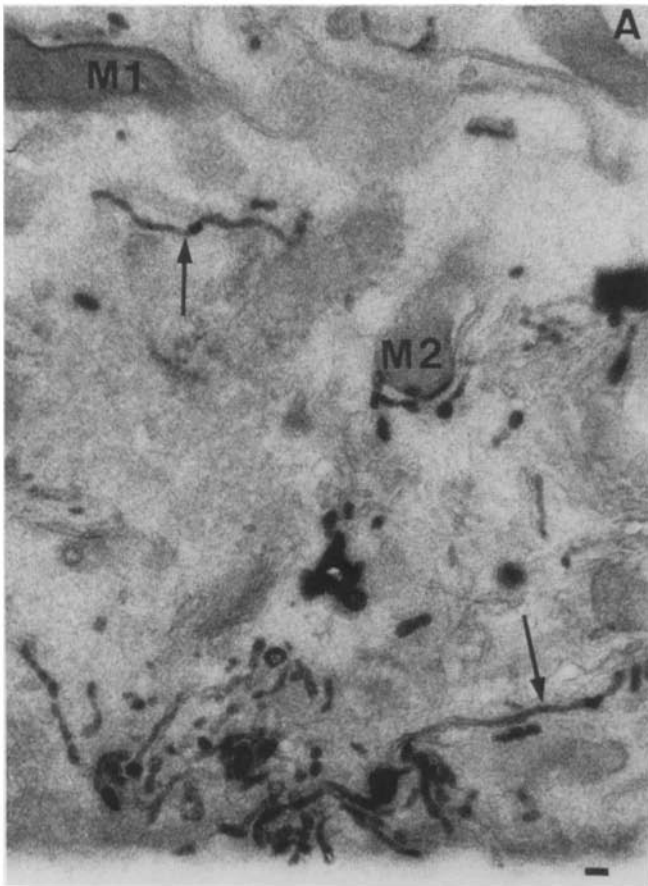
After fixation in either paraformaldehyde or glutaraldehyde and embedding in sucrose, immunogold labeling of AtT20 cells with specific antibodies showed that the late endosomes contain cathepsins D (Fig. 1 D) and B (not shown), cation independent mannose-6-phosphate receptor (CI-M6PR) (Fig. 1 E), and the lysosomal glycoproteins LAMP1 and 2 (Chen et al., 1985, 1988; data not shown). Soluble fragments of CI-M6PR conjugated to colloidal gold (Tooze et al., 1990) also labeled the late endosomes (Fig. 1 F), indicating the presence of M6P residues on the lysosomal enzymes in these compartments.

### Tubular Endosomes and Networks in AtT20 Cells

We incubated AtT20 cells for 60 min in medium containing 10 mg/ml of HRP with a high specific activity. Then, after washing and relatively mild glutaraldehyde fixation, the cells were immediately incubated with substrate and DAB for 30 min rather than 3 min. After this procedure additional extensive tubular endosomal structures were revealed (Fig. 2) when we examined single and serial sections ranging in thickness from ~0.1 to ~0.5  $\mu\text{m}$ , i.e., considerably thicker than the thin sections usually examined by electron microscopy. We should stress here that to observe thread-like structures such as tubular endosomes in sections of epon embedded cells under the electron microscope, it is essential to use sections at least 0.1–0.2  $\mu\text{m}$  thick. This is illustrated by Fig. 3, A and B, which shows micrographs of immediately serial sections of the same cell, one section ~0.2  $\mu\text{m}$  thick, the other ~0.05  $\mu\text{m}$  thick. In the latter tubular endosomes are not visible.

The AtT20 tubular endosomes had the following characteristics. They were tubules, rather than sections of sheets or cisternae, with a remarkably constant diameter of 30–50 nm and, in individual sections, lengths of up to >2  $\mu\text{m}$  (Fig. 2). Many tubular endosomes within the thickness of one section were straight rods, but others were sharply curved, sinuous, forked or multibranching; the ends of branches were often bulbous. Figs. 2–4 show some of the range of shapes of tubular endosomes. In several of our micrographs, for example Fig. 4 G, the bulbous ends of branches from tubular endosomes appeared to be coated, suggesting budding or fusion of vesicles but as yet we cannot say whether or not such coats contain clathrin. Fig. 4, K and L show rosette structures containing HRP which we observed in AtT20 cells (not shown) and several other cell types including HeLa cells and BHK21 cells, as illustrated. To eliminate the formal possibility that tubular endosomes are an elaborate system of invaginations of the plasma membrane we incubated AtT20 cells with HRP for 60 min at 4°C, washed and fixed them at 4°C, and carried out the peroxidase reaction. Neither tubular endosomes nor

**Figure 2.** Tubular endosomes in AtT20 cells. A and B show tubular endosomes in thick sections of AtT20 cells from two separate experiments after endocytosis of HRP for 60- and 30-min reaction with substrate. In A, the tubular endosome marked with arrowheads is 2  $\mu\text{m}$  long. Note the uniform diameter (30–40 nm) of the tubular endosomes and their branching and looping to form networks (arrows). Vesicular endosomes are indicated by small arrowheads. Mitochondria are labeled M, the plasma membranes of two adjacent cells, PM; curved arrows indicate some of the cisternae of the RER. The cells were soaked in uranyl acetate before embedding but the sections were not contrasted with lead citrate. The same is true of all the subsequent micrographs unless otherwise stated. Bars, 500 nm.



any vesicular early or late endosomes were labeled (data not shown).

### Cellular Distribution of Tubular Endosomes

The distribution of tubular endosomes within the cytoplasm was far from uniform. They were very abundant in the growth cones of AtT20 cells (Fig. 3, *C* and *D*) which resemble the growth cones of neurites (Tooze et al., 1989) and correspond to the ruffling edges of other cells. They were also seen accumulated in the region of the Golgi apparatus. Elsewhere in the cells, tubular endosomes were frequently seen clustered in patches below the plasma membrane, but some regions of the cytoplasm contained few if any tubular endosomes. Examination of serial sections indicated that within these patches the tubular endosomes form networks, however it also appeared to be the case that tubular endosomes do not form one continuous reticulum throughout the entire cytoplasm, at least following the fixation conditions we used.

### Tubular Endosomes Are Early Endocytic Elements

We initially observed tubular endosomes in AtT20 cells that had been incubated in medium containing 10 mg/ml of HRP for 60 min at 37°C, by which time the concentration of the tracer within the early endosomal compartments should have reached a steady state. To determine whether the tubular endosomes were part of the early rather than the late endocytic compartment, we allowed cells to endocytose HRP for 5, 10, 15, 30, and 60 min and, after fixation, incubated them with substrate for 30 min. After 5 and 10 min uptake of HRP, the tracer was seen in peripheral tubular endosomes, especially those in growth cones (Fig. 3, *C* and *D*), but also in tubular endosomes encircling the centrioles and the MTOC (Fig. 7, *A* and *B*). Tubular endosomes can therefore be regarded as part of the early endocytic compartment of AtT20 cells. As the length of endocytosis was increased from 5 to 30 min the intensity of labeling and the number of labeled tubular endosomes increased, as did between 5 and 15 min the number of labeled vesicular early endosomes. Comparison of cells that had endocytosed HRP for 30 and 60 min suggested that the tubular endosomal system became saturated by 30 min because the extent of these structures reached a plateau. In contrast, after 30 min continuous endocytosis of HRP, counts showed that only ~40–50% of typical late endosomes contained reaction product (see above).

When AtT20 cells were incubated with 10 mg/ml HRP at 20°C instead of 37°C for 60 min, the tubular endosomes contained reaction product but none of the late endosomes did. However, after 3 h continuous incubation with HRP at 20°C, both the tubular endosomes and ~40–50% of the late endosomes contained reaction product (data not shown). In short, transfer of HRP from early to late endosomes was more sensitive to the reduction in temperature than was the uptake of HRP into the tubular endosomes; this is consistent

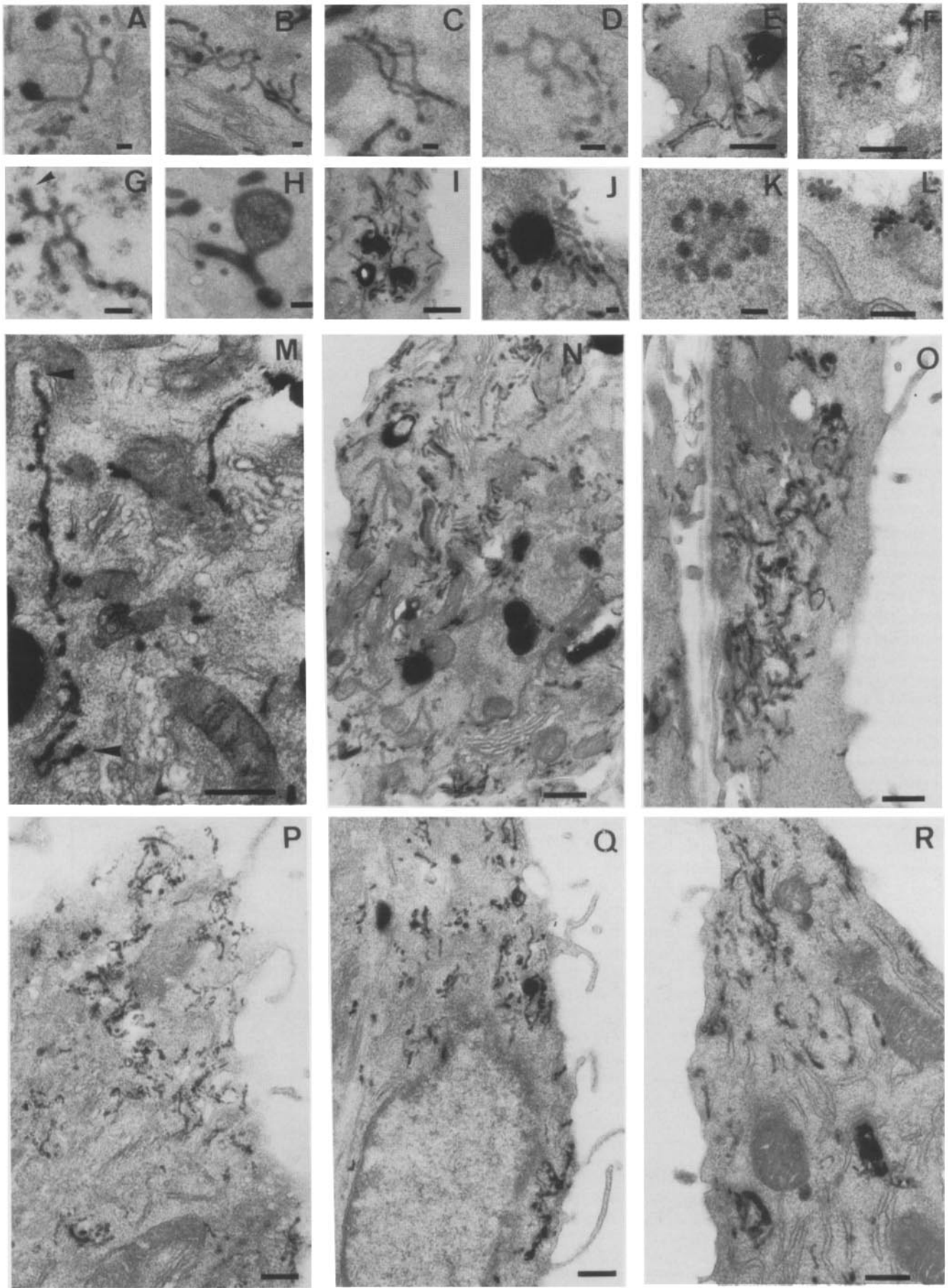
with the other evidence indicating that tubular endosomes are early endosomes.

In single or serial sections of HRP labeled cells we could find no morphological evidence for direct connections between tubular endosomes and late endosomes, identified by their characteristic morphology. On the other hand, in thick sections tubular endosomes at the cell periphery were often seen clustered around vesicular endosomes, interpreted as early endosomes (Fig. 4, *I* and *J*). Unfortunately with sections 0.2–0.4  $\mu\text{m}$  thick, which are optimal for visualization of tubular endosomes, it is impossible to demonstrate unambiguously possible continuity of the bounding membrane of tubular and vesicular endosomes since the membrane is not resolved. Using thinner sections, which are not optimal for visualizing tubular endosomes, a small number of images revealed direct continuity between tubular endosomes and vesicular early endosomes (for example, Fig. 4 *H*). It must be stressed, however, that the examination of serial thick and thin sections showed that many patches of tubular endosomes were not in the vicinity of cisternal-vesicular early endosomes.

### Tubular Endosomes in Other Cells

To assess whether the extensive arrays of tubular endosomes seen in AtT20 cells were the exception or the rule, we decided to survey a relatively large number of other cell lines. After incubation in medium containing 10 mg/ml HRP for 60 min at 37°C to achieve steady-state labeling of the endocytic pathway, tubular endosomes with the same characteristics as those in AtT20 cells were seen in cells of the following lines: HeLa (Fig. 4 *M*), PC12 (Fig. 4 *N*), Hep2 (Fig. 4 *O*), MDCK II grown on plastic (Fig. 4 *P*), CCL64, MDCK I grown as a polarized monolayer on semipermeable filters (not shown), RK13 (Fig. 4 *Q*), NRK cells (not shown) and Vero (Fig. 4 *R* and see also Figs. 6 *B*, 8, and 9, *B–F*). Tubular endosomes were particularly abundant in PC12 and Hep2 cells, as in AtT20 cells; moreover, additional experiments showed that some Hep2 tubular endosomes received HRP within 5 min, but steady-state labeling of the entire tubular endosomal system in these cells required at least 30 min incubation with the tracer, as with AtT20 cells. In semi-thin sections of BHK-21 cells that had endocytosed 10 mg/ml of HRP for 15 min, Marsh et al. (1986) observed, particularly in the perinuclear regions (see below), tubular endosomes apparently similar to those described here; we found tubular endosomes to be relatively sparse in BHK-21 cells and 3T3 cells compared with AtT20, PC12, and Hep2 cells, for example. However, BHK-21 cells contained numerous rosette-shaped endosomes (Fig. 4 *L*), which were seen less frequently in cells of the other lines (for example, Fig. 4 *K*). We drew two conclusions from this survey: first, tubular endosomes occur in cells of many lines and second, the extent of tubular endosomes varies greatly between different cell

**Figure 3.** Tubular endosomes in AtT20 cells. *A* and *B* show immediately consecutive sections of ~0.2 and 0.05  $\mu\text{m}$ , respectively, of a cell after 60 min endocytosis of HRP and 30 min reaction with substrate. Comparison of the two micrographs shows that tubular endosomes cannot readily be visualized in thin sections. The two tubular endosomes marked by arrows are 1.2  $\mu\text{m}$  long. *M1* and *M2* indicate the same mitochondria in the two micrographs. This pair of micrographs also illustrates the greater stretching of thin sections compared to thick ones. *C* shows tubular endosomes (*small arrows*) and vesicular endosomes (*arrowheads*) in the growth cone of an AtT20 cell after 5 min endocytosis of HRP and *D* in a growth cone after 10 min endocytosis of HRP. Bars: (*A* and *B*) 100 nm; (*C* and *D*) 1  $\mu\text{m}$ .





types. We have not directly established that the tubular endosomes in all the cell types mentioned above are early endosomes; to do so would require repeating with each cell line all the experiments with AtT20 cells that are reported here. However, since both the morphology and the intracellular distribution of tubular endosomes in these other cell types are essentially identical to those of the tubular endosomes in AtT20 cells (see, for example, the clustering of tubular endosomes around centrioles, described below) it seems reasonable to conclude that the tubular endosomes in all the cell types we have studied are indeed early endosomes.

### *HRP Chases out of Tubular Endosomes*

We incubated AtT20 and Hep2 cells with the tracer for 60 min to load fully the tubular endosomes and the other early endosomes. After thoroughly washing the cells, they were chased for 15, 30, and 60 min, and in the case of AtT20 cells also for 18, 48, and 72 h. After 15 and 30 min of chase, many of the tubular endosomes still contained reaction product. However, after 60 min of chase, the AtT20 and Hep2 tubular endosomes had lost their HRP and they could no longer be identified (Fig. 5 *A*). The only structures with HRP reaction product were late endosomes, identified by their characteristic morphology, and some smaller multivesicular structures. Examination of serial sections of AtT20 cells, each  $\sim 0.1 \mu\text{m}$  thick, showed that the typical late endosomes extended on average through eight to nine sections, whereas the more spherical multivesicular structures extended through three to four sections (not shown). In short, the two compartments differed in their fine structure and size; the multivesicular structures resembled the putative transport vesicles in BHK cells (Gruenberg et al., 1989) and MDCK cells (Borns et al., 1990). We concluded that between 30 and 60 min of chase were required to reduce the amount of HRP in tubular endosomes from a steady state level to below the level for enzymatic detection. When, however, AtT20 cells were allowed to internalize HRP for only 10 min the tracer was completely chased out all of the tubular endosomes, and most of the vesicular early endosomes within 30 min. After 10 min endocytosis of HRP none of the late endosomal profiles contained reaction product but after the 30-min chase counts showed that between 60 and 65% were labeled. The fact that HRP chases out of tubular endosomes, either to late endosomes or by recycling to the medium, or both, provides further evidence that tubular endosomes are proximal to the typical late endosomes.

### *Tubular Endosomes Labeled with BSA Gold*

BSA conjugated to colloidal gold has been used as an endocytic marker (Geuze et al., 1988). We therefore incubated AtT20 and HeLa cells with a high concentration of BSA-7 nm gold in PBS (OD<sub>520</sub> 30) for 60 min to determine whether

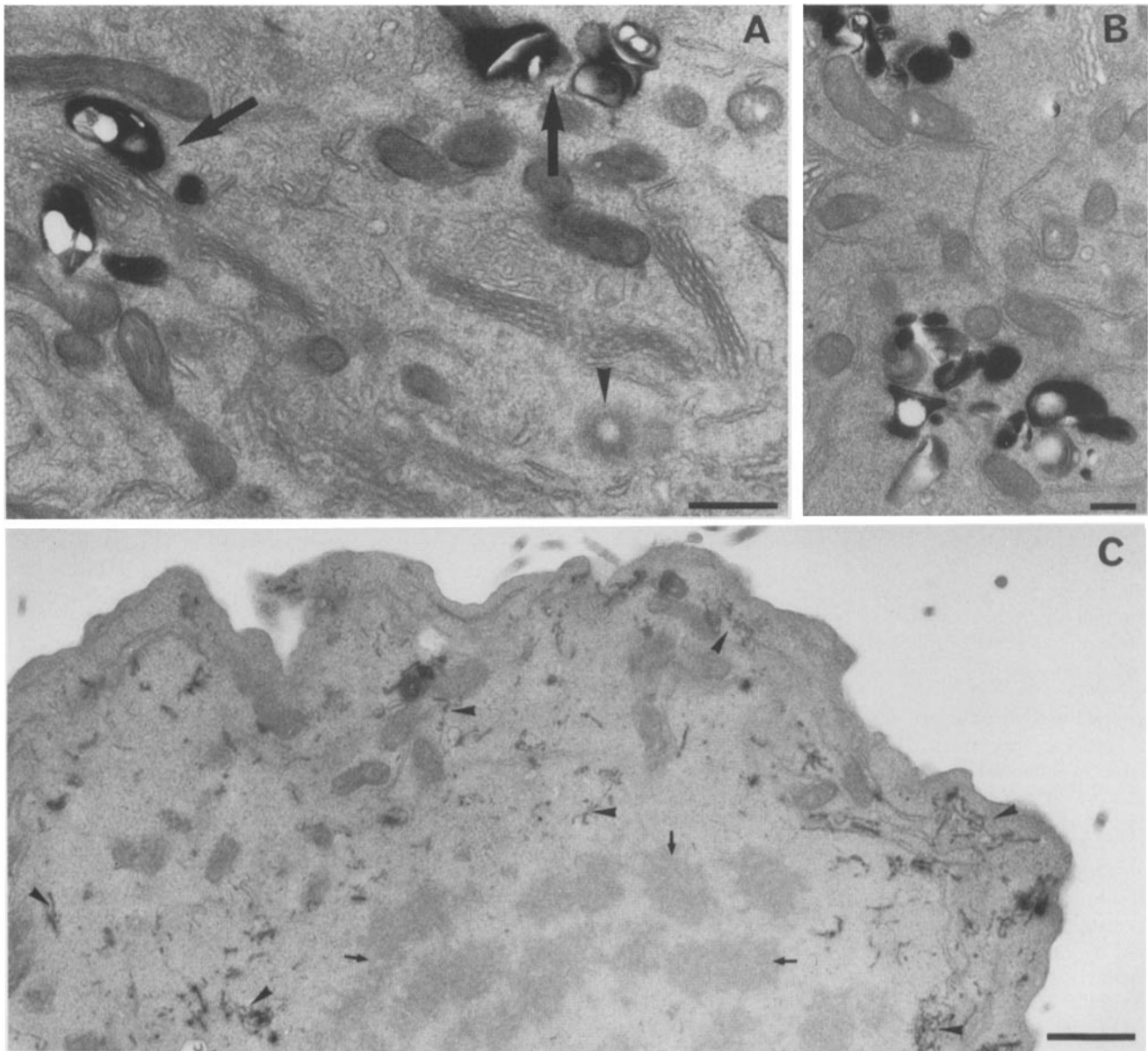
or not we could identify tubular endosomes with this alternative endocytic tracer. Many late endosomes contained heavy deposits of gold particles after 60 min endocytosis of BSA-gold (Fig. 9 *F*). Some of the tubular endosomes in the same cells contained single gold particles or very small numbers of them. For example, Fig. 9 *G* shows a cluster of tubular endosomes in a mitotic HeLa cell labeled with BSA gold. While confirming that tubular endosomes are not artifacts of HRP uptake, these experiments showed that when the lumen of tubular endosomes is not filled with electron-dense material, the extent of this compartment goes unrecognized.

### *Tubular Endosomes Not Affected by Nocodazole*

To test whether or not the integrity of interphase microtubules is essential to maintain the tubular form of these endosomes we incubated AtT20, HeLa and Hep2 cells in 20  $\mu\text{M}$  nocodazole for 30 min or longer at 37°C. By indirect immunofluorescence microscopy using antitubulin antibody we established that this procedure results in the essentially total depolymerization of the microtubules; only a very few short microtubules scattered throughout the cytoplasm resist depolymerization (not shown). Cells were then incubated for 60 min in HRP, to achieve steady-state loading, followed by 30 or 60 min in HRP and 20  $\mu\text{M}$  nocodazole, or they were incubated in nocodazole for 30 min followed by 60 min with HRP and nocodazole. In neither case did the depolymerization of the microtubules cause the AtT20, HeLa, or Hep2 tubular endosomes to change shape; they did not for example round up (Fig. 6, *A* and *B*). Moreover, prior depolymerization of the microtubules did not block delivery of endocytosed HRP to the tubular endosomes (Fig. 6 *A*). In addition, the shape and distribution of tubular endosomes in mitotic cells (see below) indicates that microtubules are not required to maintain their tubular form.

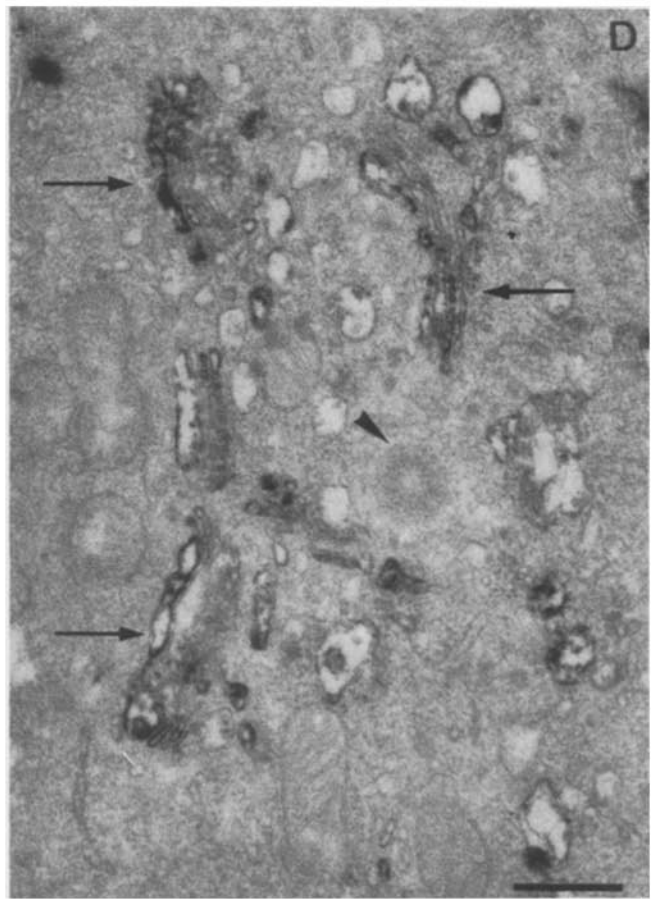
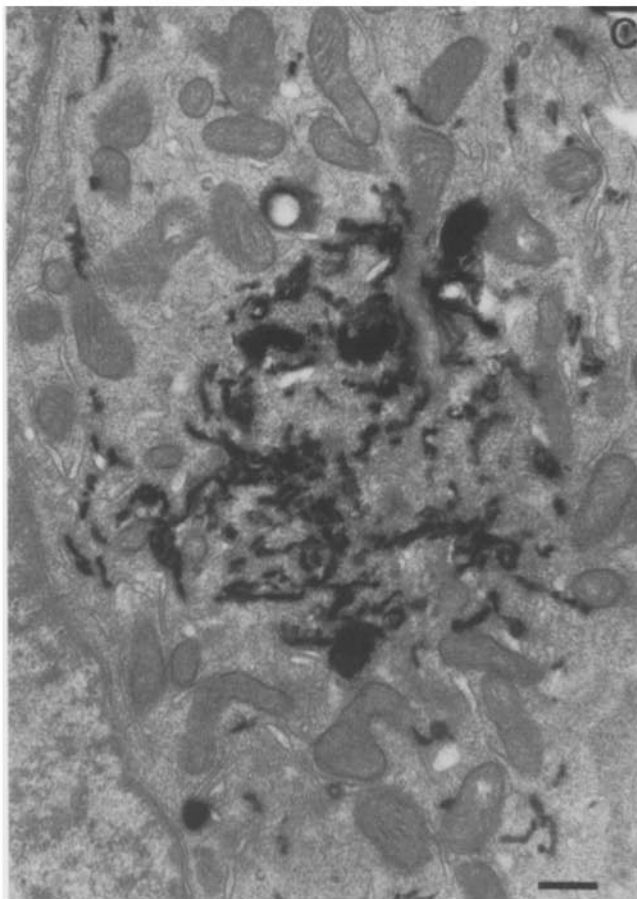
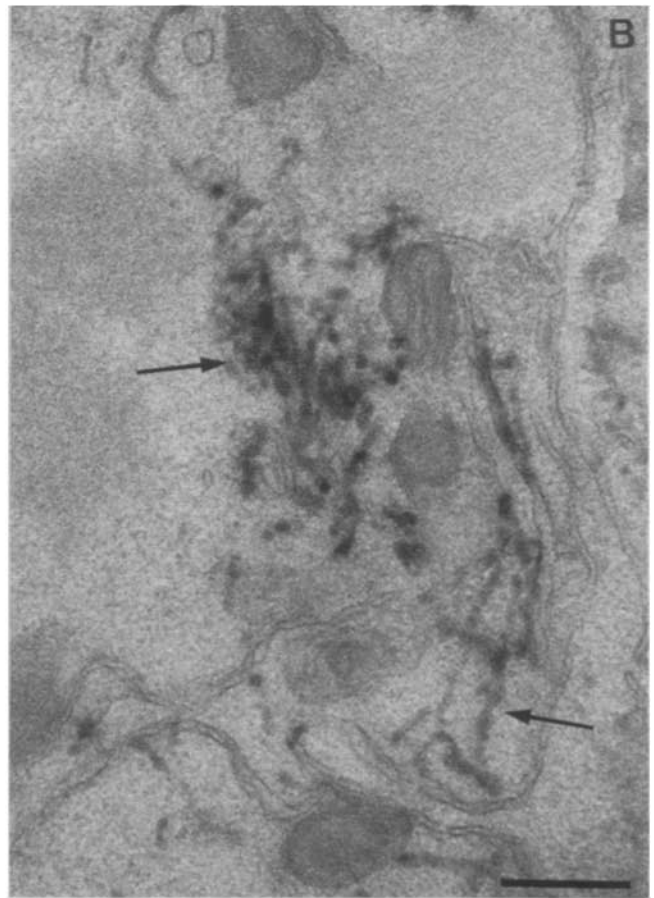
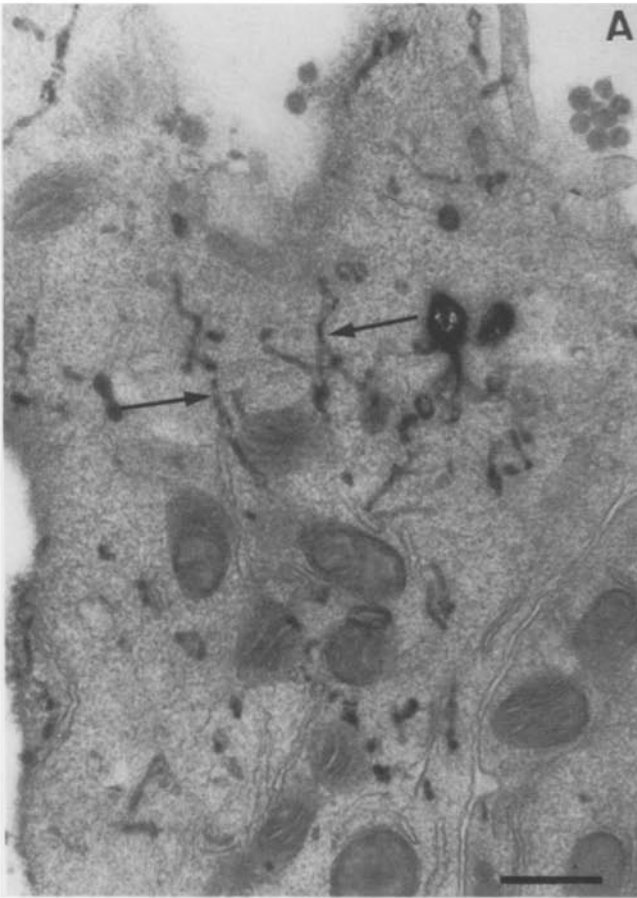
To determine whether or not HRP could be chased from tubular endosomes in the absence of microtubules, we performed the following experiments with AtT20, HeLa, and Hep2 cells. The cells were incubated at 37°C with 20  $\mu\text{M}$  nocodazole for 30 min, then with HRP and nocodazole for 60 min, followed by a chase of 60 min in the presence of nocodazole and the absence of HRP. Alternatively, the cells were incubated at 37°C for 60 min with HRP followed by 30 min in HRP and 20  $\mu\text{M}$  nocodazole and then a 60-min chase in medium containing nocodazole but lacking HRP. In both sorts of experiment, all the HRP chased out of the tubular endosomes in interphase AtT20, HeLa, and Hep2 cells during a 60-min chase in the presence of 20  $\mu\text{M}$  nocodazole (see, for example, Fig. 5 *B*). In the experiments using the second protocol, after the 60-min chase labeled tubular endosomes remained in mitotic cells, which had taken up HRP before the addition of nocodazole but which could not progress through mitosis in the absence of the mitotic spindle

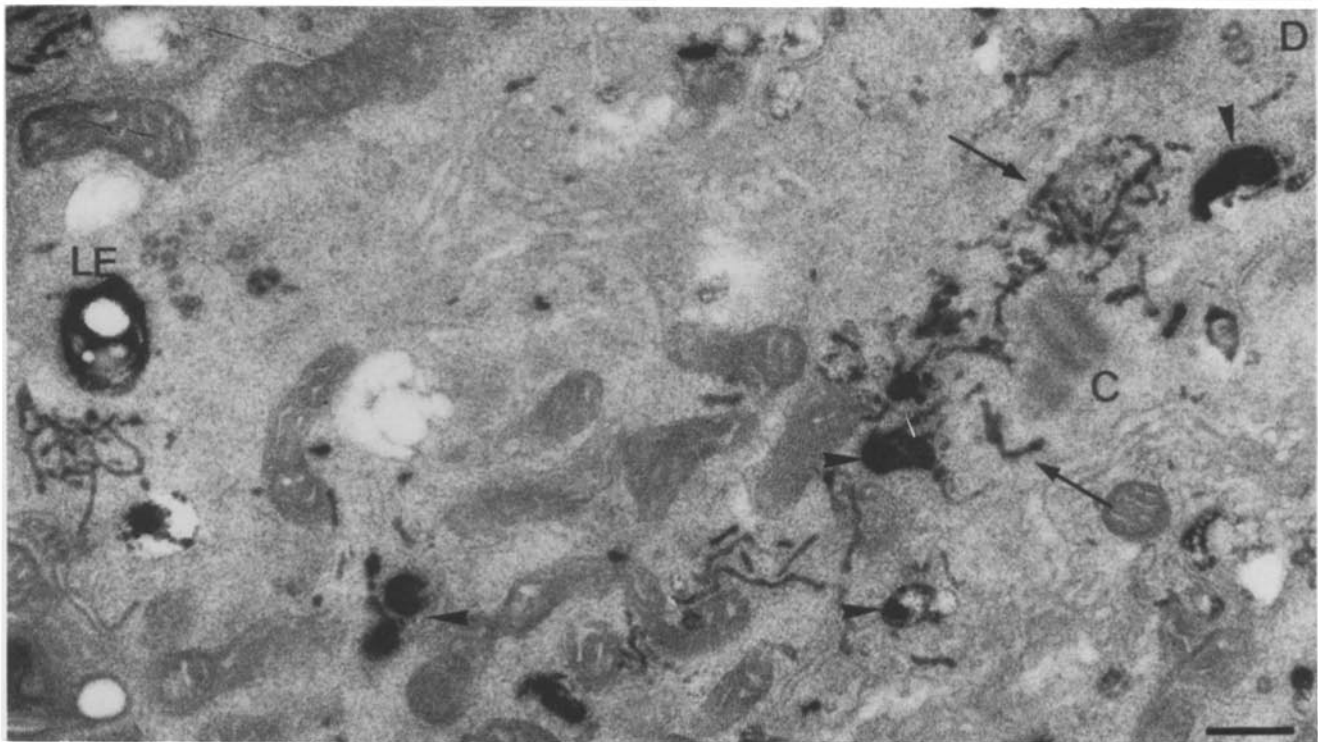
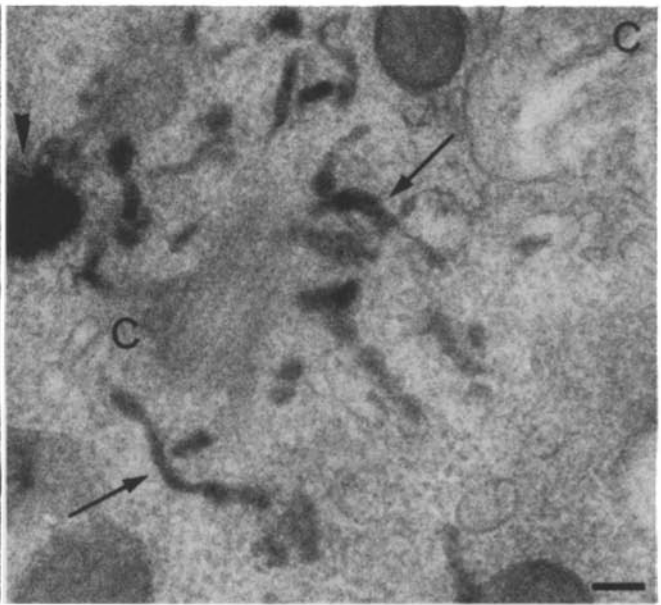
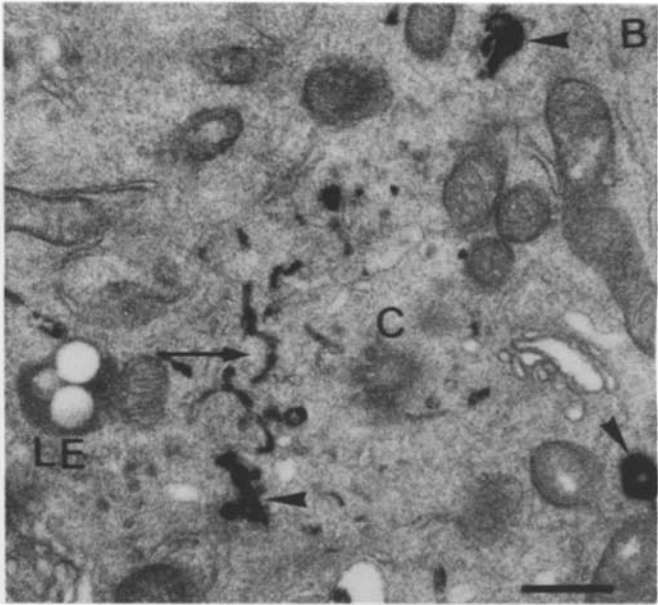
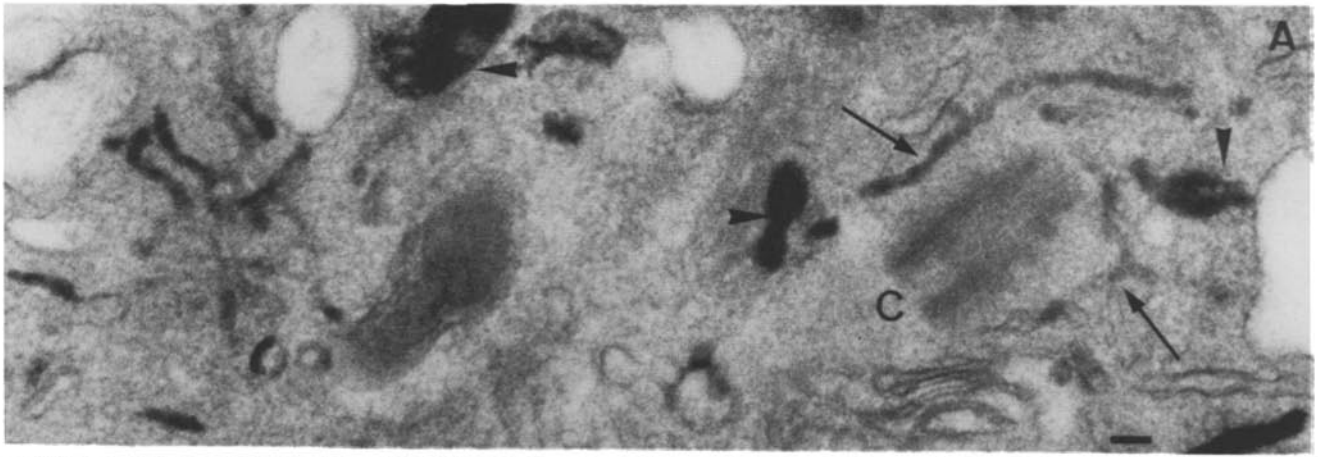
**Figure 4.** The range of shapes of tubular endosomes in AtT20 and other cell types. *A–J* show details of various aspects of tubular endosomes in AtT20 cells. Note the ring form shown in *D*, while *G* shows a tubular endosome in a lysed AtT20 cell with what appears to be a coat on the surface of a bulbous branch (*arrowhead*). *H* shows a vesicular endosome in continuity with a tubular endosome in a thin section. *I* and *J* show tubular endosomes clustered round vesicular endosomes. *K* shows a rosette-shaped endosome in a HeLa cell and *L* shows a cluster of similar structures at the periphery of a BHK21 cell. *M* shows a 3.8- $\mu\text{m}$ -long tubular endosome (*arrowheads*) in a HeLa cell. *N–R* show tubular endosomes, and vesicular endosomes, in interphase cells of the following lines: PC12, Hep2, MDCK II, RK13, and Vero, respectively. Bars: (*A–D*, *G*, *H*, and *J*) 100 nm; (*E*, *F*, *I*, and *L*) 500 nm; (*M–R*) 500 nm.



**Figure 5.** HRP can be chased out of tubular endosomes. *A* shows part of an Aft20 cell which had endocytosed HRP for 60 min to achieve steady state labeling and then was chased for 60 min in the absence of HRP. Note the labeled late endosomes (*arrows*) but the complete absence of tubular endosomes even around the centrioles (*arrowhead*), where they accumulate (see Figs. 7 and 8). *B* shows labeled late endosomes but not tubular endosomes in an interphase Aft20 cell that was incubated in medium with nocodazole for 30 min followed by nocodazole and HRP for 60 min and then a 60-min chase in nocodazole without HRP. *C* shows a mitotic Hep2 cell that was incubated in HRP for 60 min, in HRP and 20  $\mu$ M nocodazole for 30 min and chased in medium containing nocodazole but not HRP for 60 min. During the 60 min chase in nocodazole the tubular endosomes (*arrowheads*) in this cell blocked in mitosis remained labeled. Small arrows indicate chromosomes. Bars: (*A* and *B*) 500 nm; (*C*) 1  $\mu$ m.

**Figure 6.** Neither nocodazole nor brefeldin A alter tubular endosomes. *A* shows tubular endosomes (*arrows*) in an Aft20 cell that was incubated for 30 min in medium containing 20  $\mu$ M nocodazole and then 60 min in nocodazole and HRP. Nocodazole does not block loading of tubular endosomes or change their shape. *B* shows tubular endosomes (*arrows*) in a mitotic Hep2 cell in a culture that was incubated with medium containing HRP for 60 min and then in medium containing HRP and 20  $\mu$ M nocodazole for 30 min. *C* shows tubular endosomes clustered around the MTOC in an Aft20 cell incubated for 60 min in medium with 10  $\mu$ g/ml of brefeldin A and then for 60 min in 10  $\mu$ g/ml of brefeldin A and 10 mg/ml of HRP. Compare this figure with Fig. 7. *D* shows a centriole (*arrowhead*) surrounded by Golgi stacks (*arrows*) in an Aft20 cell labeled by the preembedding immunoperoxidase method with an antibody specific for POMC and ACTH. Note the absence of labeled structures resembling the pericentriolar tubular endosomes seen in *C* and Fig. 7. This section was contrasted with lead citrate for 1 min. Bars, 500 nm.





(see, for example, Fig. 5 C). Finally we allowed AtT20 cells to endocytose HRP for only 10 min, then chased them immediately for 30 min at 37°C, or incubated them on ice with 20  $\mu$ M nocodazole for 30 min and then chased them for 30 min in the presence of nocodazole at 37°C. In both cases at the end of the 30-min chase the tubular endosomes in interphase cells were devoid of HRP. We concluded from this set of experiments that intact microtubules are not essential for the complete unloading of steady state levels of a fluid phase marker from the tubular endosomes of interphase AtT20 or Hep2 cells. By contrast, in cells blocked in mitosis the HRP remains in the tubular endosomes, as Fig. 5 C shows.

### ***Tubular Endosomes and Centrioles***

In interphase cells of several lines that we examined, tubular endosomes were particularly abundant in the region of the Golgi apparatus, where many late endosomes are also located. In sections of AtT20 cells it is sometimes possible to identify the *trans*-Golgi network of the Golgi apparatus by virtue of the presence of condensing secretory proteins destined for the cores of secretory granules (Tooze and Tooze, 1986). After careful examination of many such Golgi stacks we failed to find any example of HRP reaction product in the *trans*-Golgi network, even in cells that had internalized the tracer for up to 120 min (not shown).

Many of our sections of AtT20 and other cells included centrioles in the vicinity of the Golgi stacks and these showed that tubular endosomes frequently surround the centrioles but are separated from them by the amorphous pericentriolar material of the microtubule organizing center (MTOC) (Figs. 7 and 8). Within the first 5 min of exposure of AtT20 cells to HRP, the tracer reached the pericentriolar tubular endosomes (Fig. 7 A) and the extent of labeling of tubular endosomes at this site increased with increasing times of endocytosis (Fig. 7, B–D). Since they are labeled by HRP within 5 min we consider the pericentriolar tubular endosomes in AtT20 cells to be early endosomes despite their location in the cell. As Fig. 7 A also shows, vesicular endosomes containing HRP were also present in the pericentriolar region after 5 min uptake of the tracer.

In AtT20 cells treated with 20  $\mu$ M nocodazole to depolymerize the microtubules, we found no evidence for the accumulation of tubular endosomes in the vicinity of the dispersed Golgi stacks or in perinuclear positions. In these cells we failed to observe any centrioles, which presumably also lose their central location in the MTOC close to the nucleus in the absence of microtubules. These findings indicate that the accumulation of tubular endosomes around the centrioles is dependent on intact microtubules.

We incubated cells for 60 min in medium with 10  $\mu$ g/ml of brefeldin A and then for 60 min in medium with brefeldin A and HRP. The Golgi apparatus was no longer present but the tubular endosomes, and vesicular early and late endo-

somes, were as extensively labeled with HRP in both interphase and mitotic cells as in control cells. Brefeldin A did not block entry into mitosis or inhibit endocytosis. In the brefeldin A-treated cells the accumulation of tubular endosomes in the region of the centrioles was even more marked than in controls (Fig. 6 C), presumably because more space became available around the MTOC following the elimination of the Golgi stacks.

There were no morphological differences between the tubular endosomes at the cell periphery, for example in AtT20 cell growth cones or the neurites of PC12 cells treated with nerve growth factor, and those around centrioles. Furthermore, in AtT20 cells labeled by the immunoperoxidase method with an antibody that specifically recognizes proopiomelanocortin (POMC) and ACTH, and labels all compartments of the regulated exocytic pathway (Tooze and Tooze, 1986), we could find no evidence of pericentriolar tubular compartments containing POMC or ACTH (Fig. 6 D). The tubular early endosomes at this site are clearly distinct from the *trans*-Golgi network.

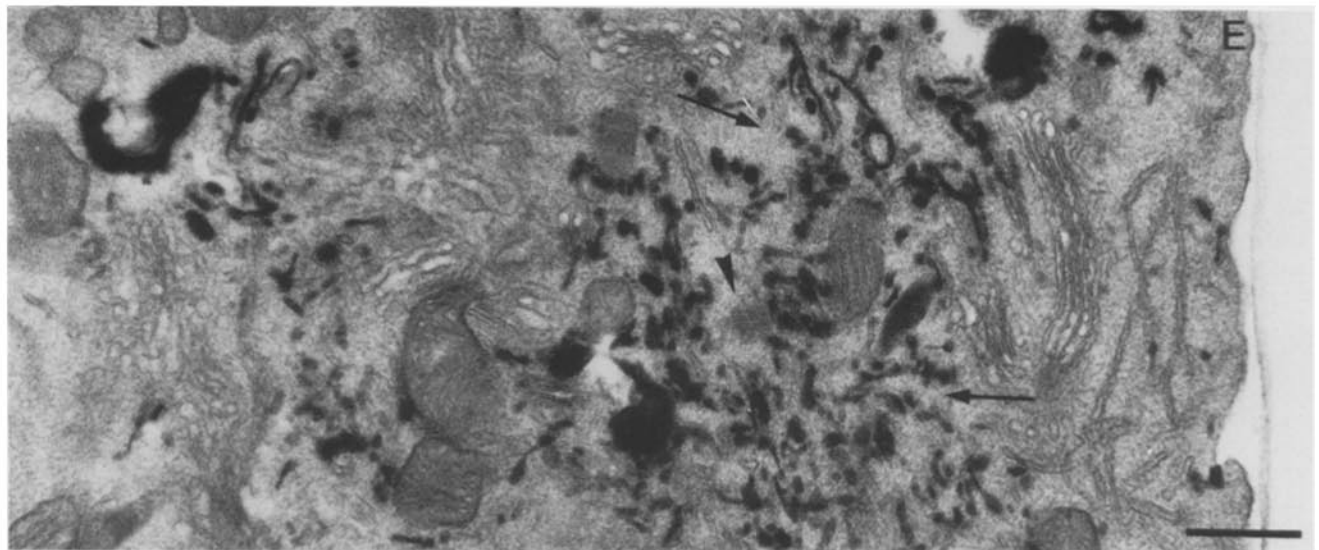
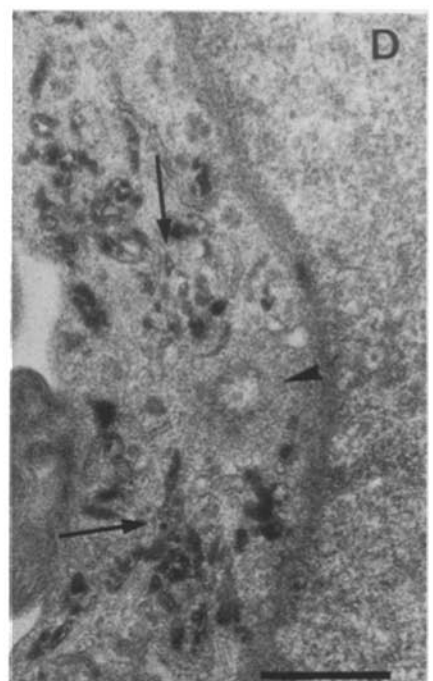
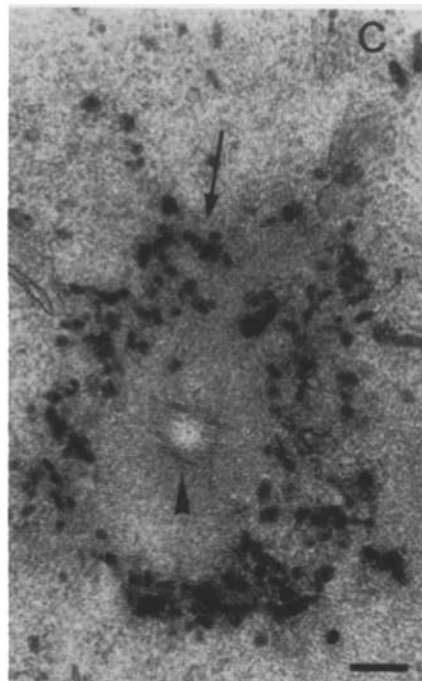
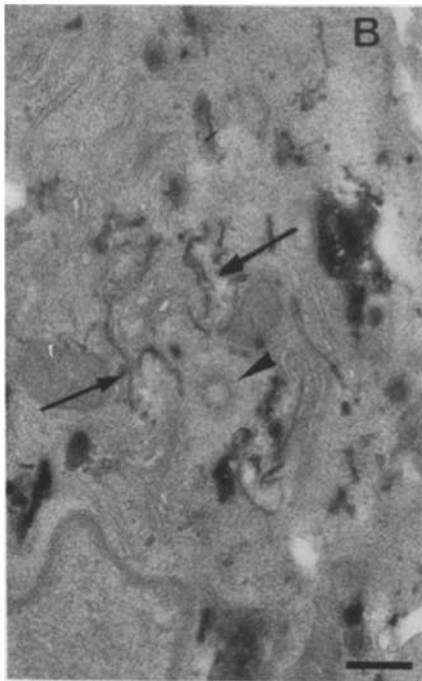
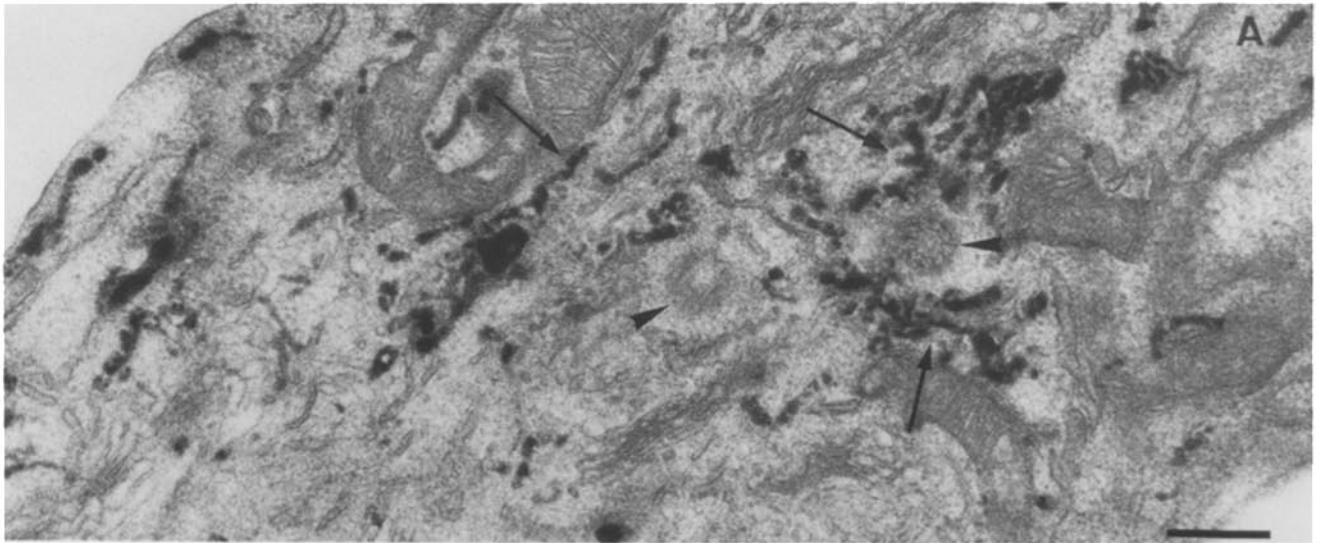
### ***Tubular Endosomes in Mitotic Cells***

After 60 min labeling with HRP, vesicular early and late endosomes were seen at the periphery of mitotic cells, but not deep within the cytoplasm enclosed by the mitotic spindle. Tubular endosomes with their characteristic morphology were particularly prominent in mitotic cells, no doubt in part because in these rounded up cells each section samples a relatively large cytoplasmic volume. Unlike the early and late vesicular endosomes, the tubular endosomes were not excluded from the cytoplasm of the mitotic spindle (Fig. 9, A and B). At late prophase, metaphase and anaphase, tubular endosomes occurred within the spindle volume often close to chromosomes. The distribution of tubular endosomes within mitotic cells indicated that they do not associate with the microtubules of the spindle. Examination of serial sections established that the tubular endosomes within the mitotic spindle were not all connected together, neither were they connected to the distant early and late vesicular endosomes located at the cell periphery outside the mitotic spindle.

Frequently in mitotic HeLa and Hep2 cells, and less often in some of the other cell types in mitosis, we observed very large clusters of tubular endosomes, sometimes together with vesicular endosomes, at the cell periphery (Fig. 9, C–G). Serial sectioning showed that these clusters of tubular endosomes extended in the third dimension for up to  $>1 \mu$ m. Similar clusters of tubular endosomes were observed in mitotic HeLa cells not exposed to any endocytic tracer (not shown) as well as in mitotic HeLa cells in cultures allowed to endocytose BSA gold for 60 min (Fig. 9 G). Such very large tubular endosomal clusters were not observed in interphase HeLa cells. Apparently, therefore, during mitosis in

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**Figure 7.** Tubular endosomes encircling centrioles in AtT20 cells. The cells shown in A–D were allowed to endocytose HRP for 5, 10, 15, and 60 min, respectively, and incubated with substrate for 30 min. Note in all four micrographs the tubular endosomes (arrows) around centrioles (C). In A, the arrowed tubular endosome above the centriole is 1  $\mu$ m long. As these micrographs show, the number of labeled tubular endosomes observed around centrioles increased with increasing periods of endocytosis. Vesicular endosomes, which in A and B at least must be early endosomes, are indicated by arrowheads. Note the unlabeled late endosome in B and the labeled late endosome in D (LE). Bars: (A and C) 100 nm; (B and D) 500 nm.



at least some cell types tubular endosomes form large clusters, and this occurs in the presence and absence of HRP.

## Discussion

Using HRP as a marker of fluid-phase endocytosis, we have revealed networks of tubular endosomes in a variety of cell lines of neuroendocrinal (e.g., AtT20 and PC12), fibroblastic (e.g., Vero), and epithelial (e.g., MDCK, HeLa, Hep2) origin. We attribute our ability to visualize tubular endosomes to several technical factors. First and foremost, we examined thicker sections than are usually used for the electron microscopy of Epon-embedded cells; the crucial importance of section thickness for the visualization of long tubular structures cannot be over-emphasized (see Fig. 3, *A* and *B*). Second, we used HRP with a high specific activity and allowed endocytosis to proceed for up to 60 min. Third, we used relatively mild fixation in 0.5% glutaraldehyde at room temperature (see Materials and Methods). When we fixed HRP loaded cells in cacodylate-buffered 2% glutaraldehyde containing 4.5% sucrose for 2 h at 37°C, a method used by Swanson et al. (1987) to preserve tubular lysosomes in macrophages, we failed to see any tubular endosomes although late endosomes still contained reaction product (data not shown). Obviously detection of endocytosed HRP depends on the tracer retaining its enzymatic activity after fixation and clearly it is possible to overfix the enzyme. Fourth, we incubated the cells immediately after fixation and washing with H<sub>2</sub>O<sub>2</sub> and DAB for 30 and sometimes 60 rather than 3 min. As a comparison of micrographs of sections of equal thickness of cells labeled for 60 min either with HRP or with a high concentration of BSA-gold shows (Fig. 9, *E* and *F*), visualization of tubular endosomes depends upon their lumen being filled with electron dense material. Without prior knowledge of the shape and distribution of tubular endosomes obtained after HRP labeling, we would not have been able to identify this compartment in cells labeled with BSA gold.

### Tubular Endosomes and the ER

We equate the endosomal reticulum observed in living Hep2 cells by Hopkins et al. (1990) with the tubular endosomes we have described here for the following reasons, all morphological but nevertheless compelling. First, tubular endosomes and elements of the endocytic reticulum have the same dimensions. Second, both appear to form patches of three-dimensional network that are found in various parts of the cytoplasm. Third, both do not form one single reticulum that is continuous throughout the cytoplasm. Fourth, both are abundant in the pericentriolar region. Fifth, both can be connected to vesicular endosomal compartments. In addition, tubular endosomes are fully loaded with the fluid-phase marker HRP and the endosomal reticulum is fully loaded with transferrin-Texas Red, a marker of receptor-mediated endocytosis, after 60 min.

The tubular endosomes we have observed cannot, however, be equated to the tubular lysosomes in macrophages (Swanson et al., 1985, 1987; Knapp and Swanson, 1990) because acid phosphatase histochemistry does not label tubular endosomes in AtT20 cells (data not shown) and microtubules are not essential for the maintenance of the shape of tubular endosomes. Likewise, the morphology and the dimensions of the apical tubular endosomes in absorptive suckling rat ileum (Wilson et al., 1987) are clearly different from those of the tubular endosomes in cultured cells described here.

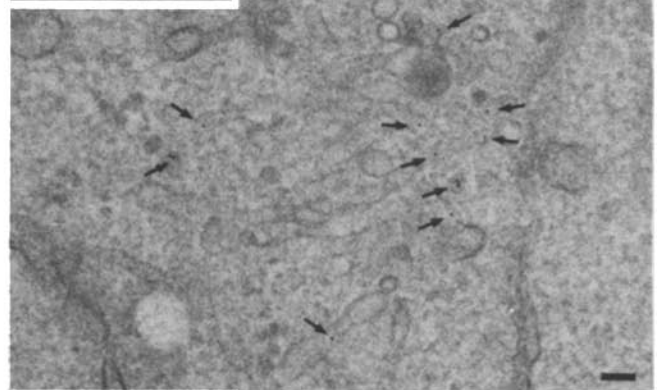
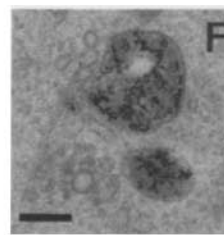
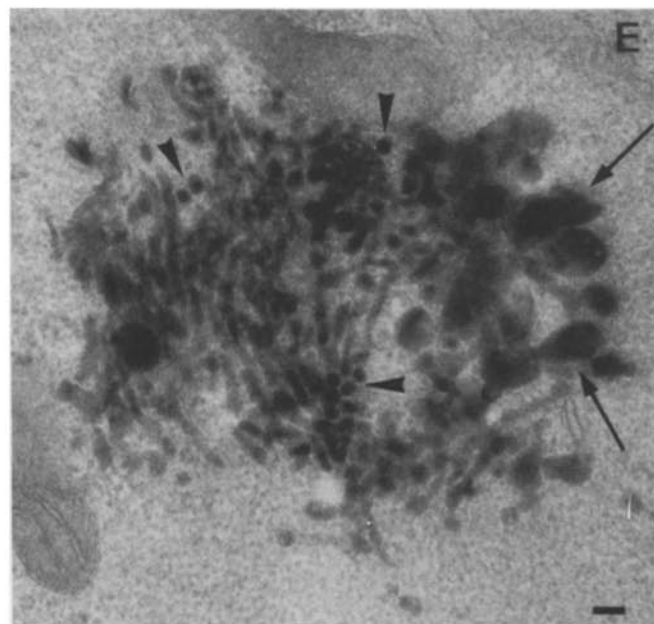
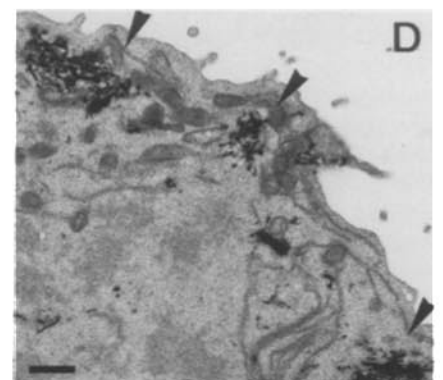
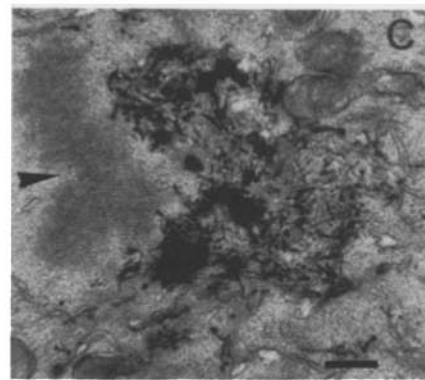
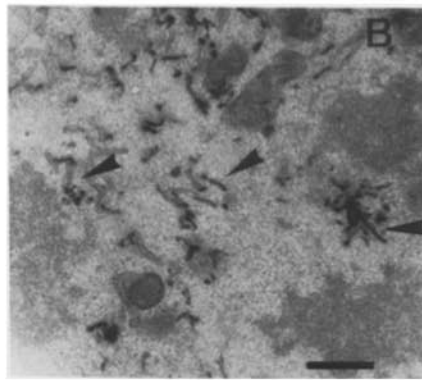
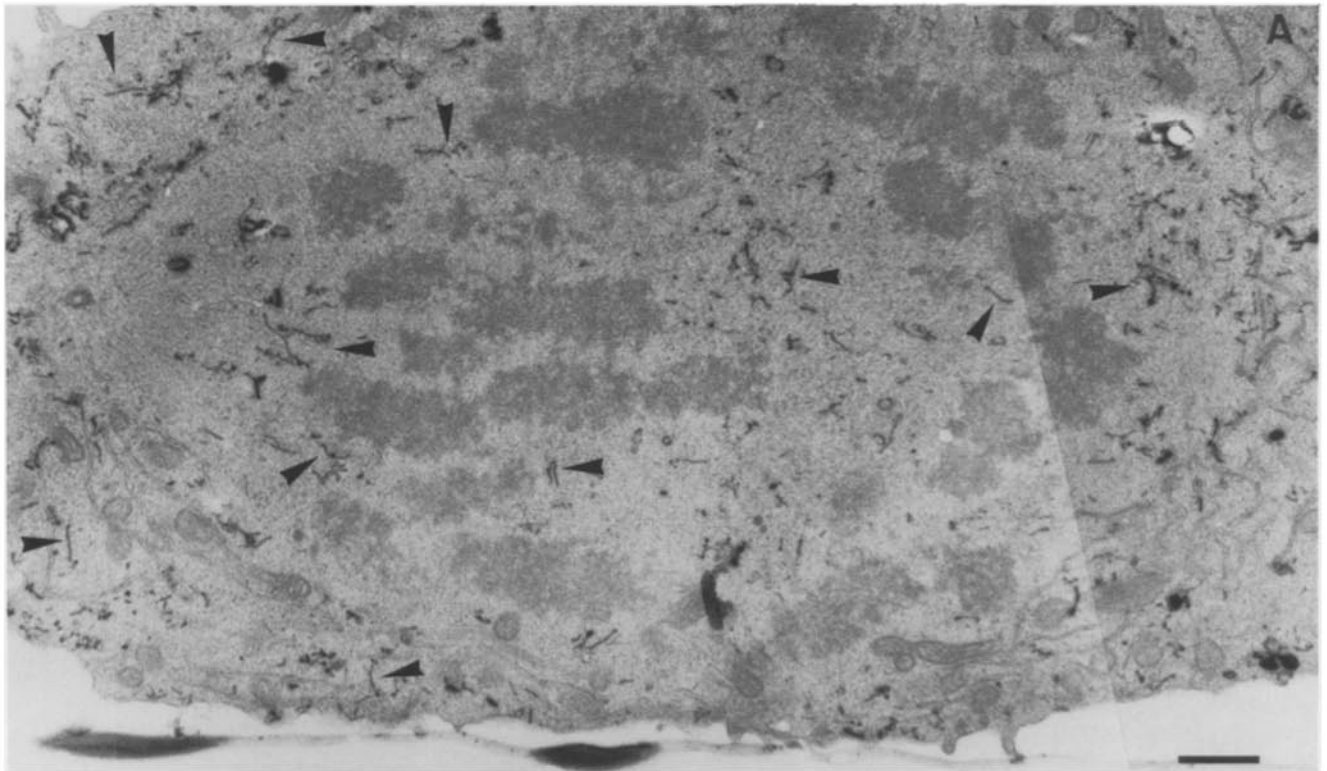
Two pieces of evidence establish that pericentriolar tubular endosomes are not part of the Golgi apparatus, including the TGN. First, after the disassembly of the Golgi stacks with brefeldin A, tubular endosomes surround the MTOC, as in controls, and HRP does not reach the rough ER. Secondly, immunoperoxidase immunocytochemistry with antibody against POMC and ACTH labels the whole Golgi stack and in particular the TGN (Tooze and Tooze, 1986) but does not label any tubules with the dimensions and characteristic pericentriolar distribution of tubular endosomes.

### What Maintains the Shape of Tubular Endosomes?

Is the elongate shape of tubular endosomes a consequence of their being stretched out along a cytoskeleton scaffold or is it an intrinsic property of the membrane of these structures? Two pieces of evidence indicate that microtubules are not essential for the maintenance of the shape of tubular endosomes, which is not to say that tubular endosomes do not move along microtubules. First in mitotic cells, in which the interphase microtubules have completely disassembled and the only microtubules present are those of the mitotic spindle (Vale, 1991), the tubular endosomes are not aligned along the latter but they remain tubular. Second, in interphase cells treated with nocodazole to depolymerize the microtubules the tubular endosomes remain tubular and do not round up. On the other hand, the microtubule dependent accumulation of tubular endosomes around centrioles in interphase cells provides circumstantial evidence that tubular endosomes move in a retrograde direction along microtubules.

AtT20 cells contain only one class of intermediate filaments, namely neurofilaments, and the known distribution of neurofilaments in mitotic AtT20 cells (Tooze et al., 1989) does not coincide with the distribution of tubular endosomes; this excludes the possibility that the tubular endosomes are stretched out along neurofilaments. The possibility that actin filaments are involved in maintaining the elongated shape of tubular endosomes can, we believe, be discounted, because when we incubated AtT20 and HeLa cells with cytochalasin D, and checked by immunofluorescence microscopy that virtually all the actin filaments were depolymerized, we found that both the shape and the intracellular distribution of the tubular endosomes was unaffected (data not shown). We suggest, therefore, that the thread-like shape of tubular endosomes is an intrinsic property.

*Figure 8.* Tubular endosomes investing centrioles in cells of five different lines. The micrographs, all of cells allowed to endocytose HRP for 60 min and then incubated with substrate for 30 min, show accumulations of tubular endosomes (*arrows*) around centrioles (*arrowheads*). The cell types are as follows: *A*, MDCK II grown on plastic; *B*, Hep2; *C*, HeLa; *D*, Vero; and *E*, PC12 cells. The HeLa section was  $\sim 0.1 \mu\text{m}$ , those of the other cells  $\sim 0.2 \mu\text{m}$ . Bars: (*A*, *B*, *D*, and *E*) 500 nm; (*C*) 200 nm.





### **Surface Area-to-Volume Ratio of Tubular Endosomes**

Perhaps the most striking property of tubular endosomes is their very high surface area-to-volume ratio. Simple calculations show that a tube with a diameter of 40 nm and a length of 2  $\mu\text{m}$  has a surface area 2.5 times that of a sphere of equal volume. Why does this endocytic compartment have such a large surface area? One possibility is that the large surface area facilitates the sorting of endosomal membrane proteins before recycling or passage to late endosomes. Tubular endosomal networks could also be an adaptation for storing plasma membrane proteins such as receptors and transporters or endosomal membrane in the cytoplasm of rapidly dividing cultured cells at a minimal cost in terms of cytoplasmic volume. Although we have not made morphometric measurements our conclusion after surveying 12 different cell lines is that some cell types have many more tubular endosomes than others. AtT20, PC12, Hep2, and HeLa cells, for example, have very many more than 3T3 and BHK21 cells. Whether this difference correlates with the absolute endocytic capacity of the cells, their rate of division or some other factor remains to be seen. A relevant additional question, which we have not yet addressed, is do similar networks of tubular endosomes exist in any cells in tissues or are they only found in tissue culture cells of stable lines?

### **Tubular Endosomes Do Not Contact Late Endosomes**

In AtT20 cells, where we can with confidence identify the late endosomes by virtue of their characteristic morphology and by immunocytochemistry, we have not observed in single or serial, thick or thin sections continuity between tubular endosomes and late endosomes. In cells pulsed with HRP for 60 min and then chased for between 60 min and 48 h the late endosomes contained heavy deposits of reaction product but tubular endosomes were completely unlabeled. If tubular endosomes were even transiently in connection with late endosomes in these pulse chased cells, we should have seen at least a few tubular endosomes in the vicinity of late endosomes with reaction product; that was not the case. Examination of serial sections of late endosomes labeled with HRP showed that the latter often had a more irregular three-dimensional shape than we would have concluded from images of single sections. However, tubules with the dimensions of tubular endosomes were never observed radiating from the late endosomal bounding membrane. Since tubular endosomes were never in continuity with late endosomes, we can reach two conclusions. First, tubular endosomes cannot be considered as part of the typical late endosomes, second, by elimination, the vesicular endosomes with which tubular endosomes are sometimes in continuity must be early endo-

somes, or precursors to putative transport vesicles (Gruenberg et al., 1989).

### **Tubular Endosomes Are Early Endosomes**

At least 30 min of continuous endocytosis of HRP at a concentration of 10 mg/ml are needed to load all the tubular endosomes such that their full extent can be visualized by electron microscopy; these structures might, therefore, be considered on kinetic grounds to be late endosomes (see Gruenberg and Howell, 1989). However, tubular endosomes are clearly not typical late endosomes and the following of their properties led us to conclude that they are an early endosomal compartment. First, the distribution of tubular endosomes in the cytoplasm is consistent with this. Although some tubular endosomes accumulate at the MTOC most are seen in clusters below the plasma membrane, often surrounding peripheral vesicular endosomes and in ruffling edges. In AtT20 cells they are very abundant in growth cones which, at the ends of neurites distal from the cell bodies (Tooze et al., 1989), contain the ruffling edges of these neuroendocrinal cells. Second, some AtT20 tubular endosomes, both at the cell periphery and around the MTOC, receive HRP within the first 5 min of endocytosis, that is 10 min before any typical late endosomes receive the tracer. Given the structure of the tubular endosomal networks, along the lumen of which HRP and its reaction product can diffuse and therefore become diluted, the time at which HRP is first detected in limited regions of the networks is, in our opinion, more significant than the time required to load them fully. Third, depolymerization of the microtubules of interphase cells does not impair delivery of HRP to AtT20, HeLa and Hep2 tubular endosomes. Others have shown that delivery of HRP and other markers to early endosomes is independent of microtubules (Gruenberg et al., 1989; Bomsel et al., 1990). Fourth, in both the presence and the absence of microtubules, HRP can be completely chased out of AtT20, HeLa and Hep2 tubular endosomes loaded to steady state. Fifth, in contrast to the situation in interphase cells, HRP does not chase out of tubular endosomes in mitotic cells blocked in mitosis by depolymerization of the spindle with nocodazole. Both endocytosis and recycling have been shown to be inhibited during mitosis (Fawcett, 1965; Berlin et al., 1978; Berlin and Oliver, 1980; Warren et al., 1984; for review see Warren, 1985). Sixth, in AtT20 cells uptake of HRP into tubular endosomes is much less sensitive to a reduction in temperature to 20°C than is delivery of the tracer to late endosomes. Finally, if the tubular endosomes described here and the endosomal reticulum in living Hep2 cells reported by Hopkins et al. (1990) are the same struc-

*Figure 9.* Tubular endosomes in mitotic cells. *A* shows an AtT20 cell at anaphase. Note the tubular endosomes (*arrowheads*) at the cell periphery but also within the mitotic spindle. *B* shows tubular endosomes (*arrowheads*) adjacent to chromosomes in a mitotic PC12 cell. *C* shows a large cluster of tubular endosomes, as well as some vesicular endosomes, close to a chromosome (*arrowhead*) in a mitotic Hep2 cell. *D* shows at low magnification part of a mitotic HeLa cell with clustered tubular endosomes (*arrowheads*) at the cell periphery. *E* shows at higher magnification a large cluster of tubular endosomes, with some vesicular endosomes (*arrows*), in a mitotic HeLa cell. Note the circular profiles of transversely sectioned tubular endosomes (*arrowheads*). *F* shows the accumulation of BSA-gold (7 nm) in a late endosomal structure in a HeLa cell incubated for 60 min in BSA-gold (OD<sub>520</sub> 30.0) in PBS. *G* shows a cluster of tubular endosomes in a mitotic HeLa cell in the same experiment as *F*. The gold particles are indicated by small arrows. Bars: (*A* and *D*) 1  $\mu\text{m}$ ; (*B* and *C*) 500 nm; (*E* and *G*) 100 nm; (*F*) 200 nm.

ture, then it follows that tubular endosomes have abundant transferrin receptor in their membrane.

For all these reasons we consider AtT20 tubular endosomes, and by extension those in other cell types studied, to be an early endocytic compartment proximal to typical late endosomes. While our observations indicate that tubular endosomes are widespread in cultured cells, and therefore confirm and generalize the observations of Hopkins et al. (1990), we differ from that group in our interpretation of this compartment. We do not have any evidence in support of the notion that tubular endosomal networks, the endosomal reticulum, provide continuity between early and late endosomes. That HRP chases out of tubular endosomes in interphase cells lacking microtubules but not from mitotic cells suggests that tubular endosomes could be involved in recycling, which is not microtubule dependent but is inhibited during mitosis. Indeed, recycling might possibly be their sole function and in this context it is significant that the tubular portions of tubulovesicular early endosomes (or compartment for uncoupling of receptor from ligand [CURL]) are intermediates in the recycling pathway in hepatocytes (Geuze et al., 1987). Whether or not the tubular endosomes described here are the same compartment as the tubular portions of CURL remains to be seen. In this context we should emphasize that the tubular endosomes in some cell types, e.g., AtT20, PC12, HeLa, and Hep2 cells, appear to be much more extensive than the CURL described by Geuze and his colleagues. Moreover, in interphase AtT20 cells there are patches of tubular endosomes that are not associated with vesicular endosomes and the same is true of tubular endosomes within the mitotic spindles of several cell types. On the other hand, the kinetics of unloading of tubular endosomes and loading of typical late endosomes in AtT20 cells are consistent with the proposal that some HRP moves from the former to the latter. Moreover, it is hard to believe that the striking microtubule dependent accumulation of tubular endosomes around the MTOC in at least seven cell lines is without functional significance. (We interpret an observation made with A431 cells after their endocytosis of epidermal growth factor conjugated to HRP [see Fig. 1 in Miller et al., 1986] as also showing tubular endosomes accumulated around centrioles.) Likewise, we interpret the small endocytic "vesicles" that contain synaptophysin in their membrane and appear as "clouds" surrounding the centrioles in CHO cells transfected with synaptophysin cDNA and in PC12 cells (Johnston et al., 1989) as images of thin sections of tubular endosomes, which we know accumulate around centrioles in PC12 cells (see Fig. 8 E).

The obvious suggestion is that tubular endosomes accumulate at the MTOC because this brings them in proximity to the Golgi apparatus and to late endosomes, which are abundant in many cell types in the region of the Golgi apparatus and the MTOC. The spatial proximity of these organelles could facilitate transport between them. Further morphological studies, including morphometric analyses and immunocytochemistry to determine, for example, whether or not actin, clathrin, and certain GTP-binding proteins (Chavrier et al., 1990) are associated with the tubular endosomal membrane, as well as biochemical studies are needed to resolve the questions posed by the existence of tubular endosomal networks in several types of cultured cells. It will be equally

if not more important to determine whether or not tubular endosomes occur in any cell types in vivo.

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*Note Added in Proof.* Since this manuscript was accepted for publication we have obtained evidence indicating that the clusters of tubular endosomes seen in mitotic HeLa cells (see Fig. 9, D and E) are the same compartment as the Golgi clusters identified in mitotic HeLa cells by J. M. Lucocq, J. G. Pryde, E. G. Berger, and G. Warren. 1987. (A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.* 104:865-874.)

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