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Calcium-Containing Lysosomes in the Outer Mantle Epithelial Cells of *Amblema*, a Fresh-Water Mollusc

RUTH GWENDOLYN JONES AND WALTER L. DAVIS
Department of Microscopic Anatomy, Baylor College of Dentistry, Dallas, TX 75246

ABSTRACT The cells of the outer mantle epithelium contain numerous large pleomorphic electron dense bodies. In their fine structure they resemble lysosomes. Positive acid phosphatase histochemistry confirms that these supranuclear and subnuclear structures are lysosomes. A major portion of the intralysosomal material is resistant to high-temperature microincineration, indicative of an inorganic component. Subsequent microprobe analyses identified considerable calcium within these organelles. Such entities are similar in structure and ionic content to the lysosomes of avian intestinal absorbing cells, another calcium-transporting epithelium. These mantle lysosomes may function in transcellular calcium transport during shell formation, growth, and repair, especially since the mantle is the shell-forming organ in molluscs.

vertebrate intestinal absorptive cells, lysomes may be involved in the calcium-transabsorptive) process and/or intracellular um homeostasis (Davis et al., 1979; Davis Jones, 1981, 1982; Jones and Davis, 1981). tionally, these organelles may be respon- to the steroid hormone cholecalciferol, i.e., min D_3 (Davis and Jones, 1981) and its active metabolites such as 1,25-dihycholecalciferol (Davis and Jones, 1982). role of vitamin D in intestinal calcium port has been established (DeLuca, 1978). rthermore, this vitamin also affects the scan mantle epithelium (Matthews and 1978).

ecause of the above information, we inves- ed morphologically and analytically the elial cells of the outer layer of the mollus- mantle. This tissue transports the consid- calcium necessary for shell formation ification) and repair (Kirschner et al., Kirschner, 1962; Istin and Maetz, 1964; and Kirschner, 1968; Wilbur, 1972; Petit 1980). Additionally, both acid phospha- activity and numerous lysosomes have described in the epithelial cells of the mantle layer (Kado, 1960; Timmermans, Ganagarajah and Saleuddin, 1972; Chan Saleuddin, 1974). Thus the presence of cal- in the lysosomes of epithelial cells which se a known calcium-transporting epithelium could lend additional credence to our hypothesis (Davis et al., 1979) that these organelles play an important role in intracellular calcium homeostasis and/or calcium-transport mechanisms.

MATERIALS AND METHODS

Mussels were collected, identified, and maintained in the laboratory as previously described (Petit et al., 1978)

For these experiments, after inducing adductor muscle relaxation (Petit et al., 1978) entire mussels were fixed overnight at 4°C in 6.5% cacodylate-buffered (0.1 M, pH 7.4) glutaraldehyde and subsequently osmicated (after several buffer washes) in cacodylate-buffered (pH 7.4) 1% osmium tetroxide. Whole animals were ethanol dehydrated prior to embedment in low-viscosity medium (Spurr, 1969) and cured for 18–24 hours. Twenty-four 2.0-mm sections (slices) were cut with a lapidary diamond saw (Cab-Mate, Graves Company, Del Ray Beach, FL) from an average 5-year-old mussel. From such sections, precise regions of the mantle were identified and selected for further study.

These specific regions were then sectioned for light microscopy at 1.0–2.0 μm with glass

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knives on a Porter-Blum MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newton, CT). Such sections were polychromatically stained (Martin et al., 1967) and evaluated for future electron microscopy.

Ultrathin sections were cut on the above microtome equipped with a diamond knife. Sections were mounted on 200 mesh uncoated copper grids and subsequently double stained with uranyl acetate and lead citrate (Reynolds, 1963). Samples were examined and photographed on a Philips 300 transmission electron microscope (Philips Electronic Instruments, Inc., Mt. Vernon, NY) operated at 40–100 kV.

High-temperature microincineration

For these studies, ultrathin sections from tissues prepared as above were mounted on Formvar-silicon monoxide-coated stainless steel grids (Thomas and Greenwalt, 1968). These sections were not stained. Grids were then placed in a 500°C muffle furnace for 10–15 minutes.

Energy dispersive X-ray spectroscopic analysis

Analytical electron microscopy was performed as described previously (Davis et al., 1979; Petit et al., 1980). Briefly, dark gold sections from tissues prepared as above were mounted on 200 mesh copper grids, left unstained, and carbon coated. Sections were viewed with a JEOL 100 C transmission electron microscope equipped with a high resolution scanning attachement (JEOL, U.S.A., Medford, MA) and a Kevex 30-mm², 158-eV resolution, lithium-drifted silicon, energy dispersive X-ray detector (Kevex Corporation, Burlingame, CA). Microscope conditions were as follows: scanning transmission mode operated at an accelerating voltage of 80 kV; 50 μamp emission current; 30° specimen tilt; and a beam diameter of approximately 40–60 nm. The detector was placed within 20 mm of the sample through the objective pole piece. A total of 25 lysosomes were analyzed for 10–50 seconds each. The X-ray spectra were collected using a Tracor Northern TN-2000 multichannel analyzer operated at 20 eV/channel. These X-ray spectra were then transferred to, and stored in, a Tracor Northern NS-880 Analyzer.

Acid phosphatase histochemistry

To demonstrate mantle lysosomes, whole relaxed mussels were fixed for 4 hours in cold (4°C) 2.5% cacodylate-buffered glutaraldehyde and subsequently washed overnight in cold buffer. Portions of the mantle were removed and rapidly frozen on an IEC model CT1 cryostat (International Equipment Company, Needham, MA). Frozen sections (40 μ) were collected in cold buffer wash prior to incubation for the demonstration of acid phosphatase activity (Novikoff, 1963). Following a 1-hour incubation, samples were washed, dehydrated, osmicated, and embedded in Spurr's medium.

RESULTS

With routine transmission electron microscopy, numerous electron-dense bodies were identified in the cytoplasm of the columnar cells of the outer mantle epithelium (Fig. 1). Such entities were located both apically and basally. They were most numerous, however, in the supranuclear location where they frequently occurred in clusters or aggregates. The dense bodies were pleomorphic (usually ovoid) and nonuniform in size. These organelles were usually dispersed within large accumulations of glycogen rosettes or alpha particles (Figs. 1,2). More apically, the dense bodies were frequently associated with large vacuolar structures (Fig. 1). A close association with both the rough endoplasmic reticulum and the Golgi apparatus was frequently observed (Fig. 2). Mitochondria were not abundant in these cells, and were conspicuously absent in those cytoplasmic regions where the dense bodies prevailed (Fig. 2).

Internally, the contents of these structures were heterogenous and comprised of coarse electron-dense granules or particles (Fig. 2). In the less granular entities, a finely particulate substratum was apparent (Fig. 2). Lipoidal-like and glycogen-like entities were also seen within these organelles (Fig. 2). Limiting membranes were apparent (Fig. 2).

In specimens incubated for the demonstration of acid phosphatase activity, the electron-dense structures described above were richly endowed with this enzyme (Fig. 3). This observation confirms that these electron-dense organelles are lysosomes.

The granular components of these organelles were resistant to high-temperature microincineration (Fig. 4). Lysosomes were clearly identifiable in these sections by their granular ash patterns (Fig. 4). Such data are indicative of the inorganic nature of these intralysosomal particles. Microincineration-resistant deposits were also seen along the lateral intercellular membranes, free in the cytoplasm, and in the nuclei of the epithelial cells.

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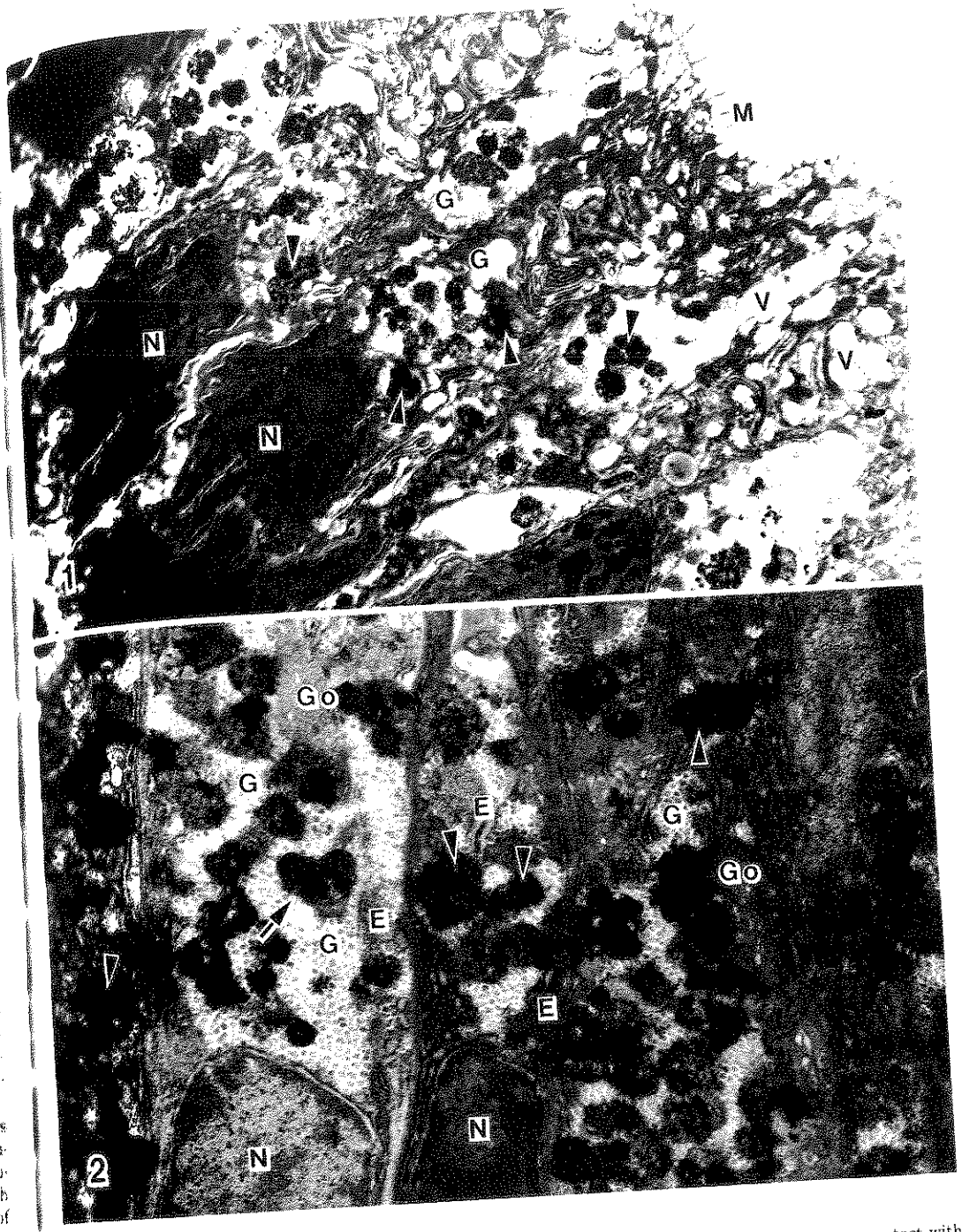
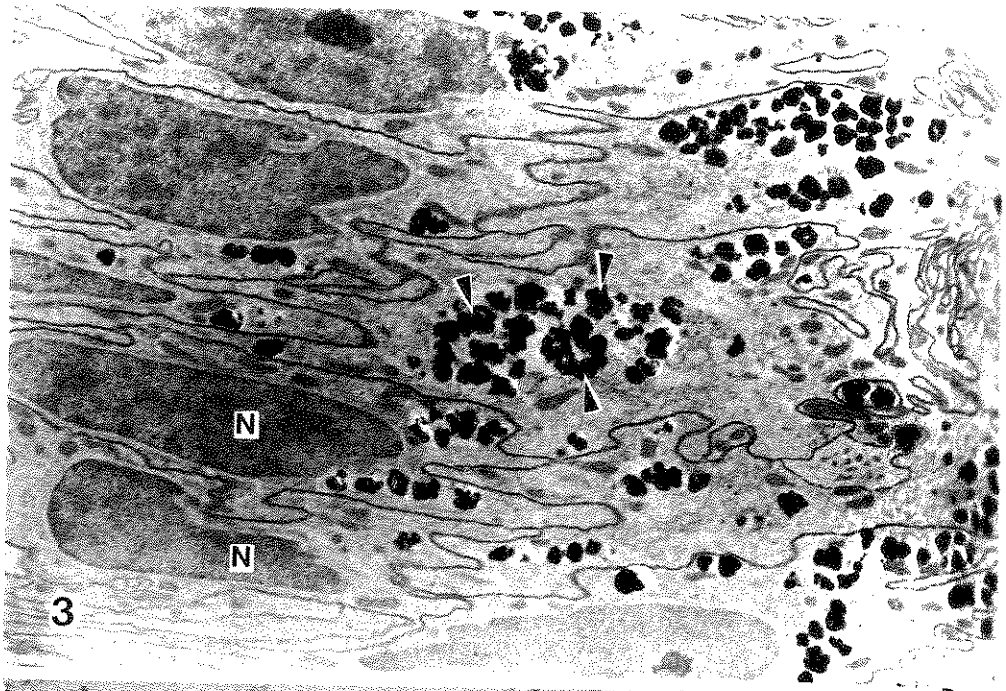


Transmission electron micrograph of the routine-prepared outer mantle epithelium from *Amblema*. Numerous electron-dense pleomorphic lysosome-like bodies (arrowheads) are apparent in the apical cytoplasm of these columnar epithelial cells. N, nucleus; G, glycogen; V, vacuoles; M, microvillar border. × 7,500.

Higher magnification of the apical epithelial cell cytoplasm. The lysosome-like bodies (arrowheads), which are membrane bound (arrow), are in intimate contact with glycogen accumulations (G). Additionally, these electron-dense bodies are in close proximity to the Golgi apparatus (Go) and the rough endoplasmic reticulum (E). Note the conspicuous absence of mitochondria in the apical cytoplasm of these cells. Also note the presence of electron-dense (osmiophilic) lipid-like bodies as well as glycogen-like particles within the lysosomes. N, nucleus. × 16,000.



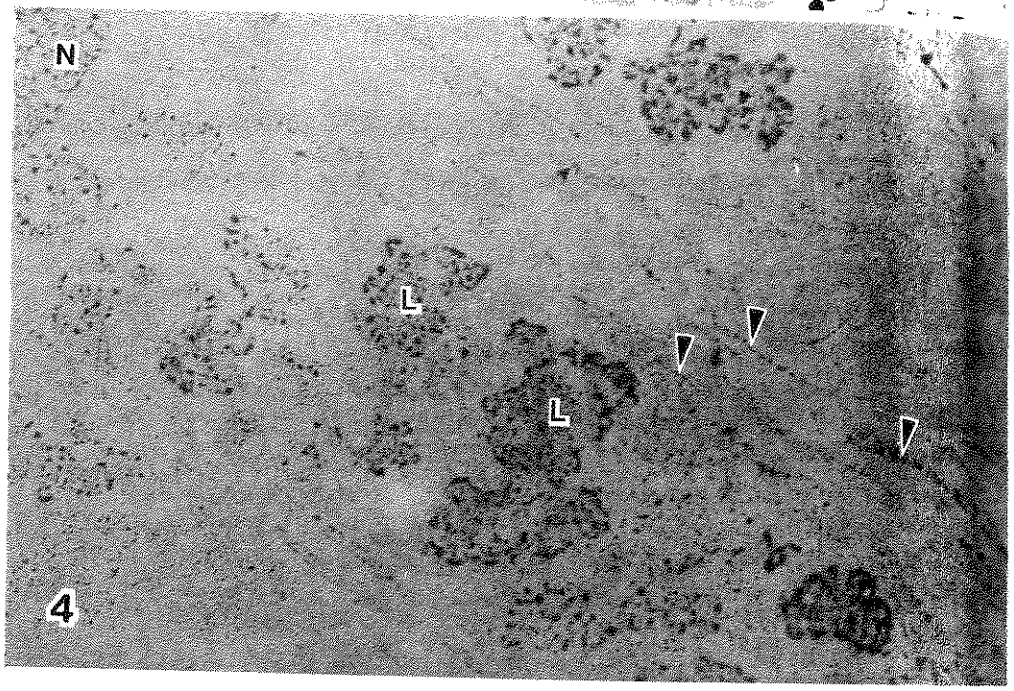
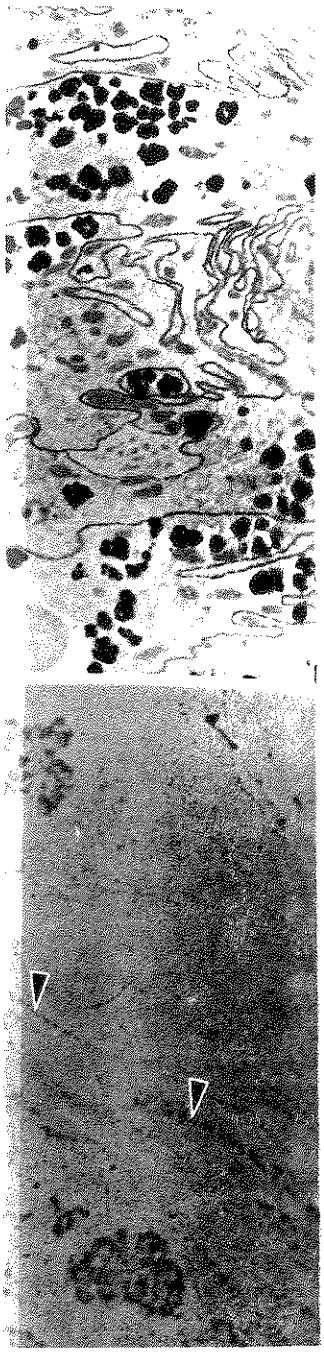


Fig. 3. When incubated for the demonstration of acid phosphatase activity, the pleomorphic bodies were markedly positive for the presence of this enzyme (arrowheads). This observation is indicative of the lysosomal nature of these organelles. N, nucleus. × 6,100.

Fig. 4. Components of the lysosomal matrix (L) were resistant to high-temperature microincineration as demonstrated in this TEM. This is indicative of the inorganic nature of these deposits. Additionally, inorganic deposits were also seen along the cellular membranes (arrowheads) and in the nuclei of these cells (N). × 21,400.



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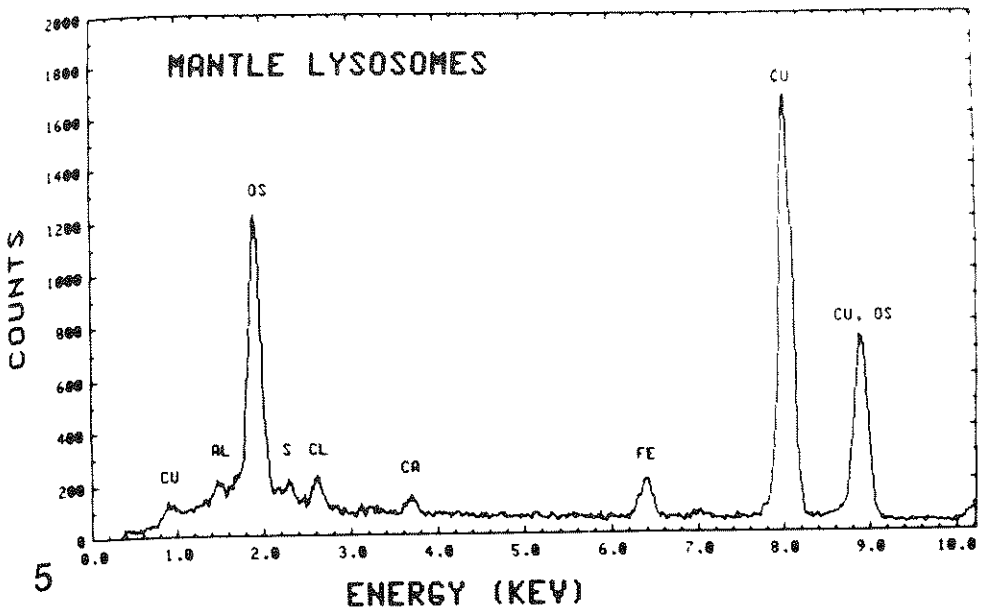

X-ray spectrum obtained from the 50-second analysis of a single apical lysosome. The matrix of this organelle con- considerable calcium (CA), iron (FE), and sulfur (S). Aluminum (AL) and copper (CU) are probably column contami- chlorine (Cl) is from the embedding medium, osmium (OS) from the secondary fixative.

X-ray microanalysis of the lysosomes revealed these organelles to contain high concentrations of calcium (Fig. 5). Other ions present were aluminum, sulfur, and considerable iron (Fig. 5). Phosphorus, if present, was masked by osmium.

DISCUSSION

The lysosomes of the molluscan mantle outer epithelium are quite similar in their morphology, histochemistry, and elemental analysis to those described for the avian intestinal absorptive cells (Davis et al., 1979; Davis and Jones, 1981; Davis and Jones, 1982; Jones and Davis, 1981). Interestingly, these diverse tissues transport considerable calcium and thus play a major functional role in systemic calcium homeostasis and hard tissue formation (Davis et al., 1979; Petit et al., 1980). Such similarities in tissue ultrastructure and function can allow one to speculate that lysosomes, acting in concert with endocytosis (pinocytosis) and exocytosis, may participate (either primarily or secondarily) in directional transcellular calcium transport thus contributing to, and possibly regulating, both intracellular and/or extracellular calcium homeostasis (Davis et al., 1979). Thus, extracellular calcium, probably bound to a calcium-binding protein (perhaps at the level of the cell membrane), enters the epithelial cells via the pinocytotic process. Such vesicles subsequently fuse with lysosomes wherein acid hydrolases cleave the calcium from its binding agent through the denaturation (catabolism) of the latter. During the defecation (exocytotic) process, the free calcium is eventually released to the extracellular space at the opposite pole of the mantle epithelial cells.

A second important similarity between mantle and gut epithelia is the apparent sensitivity of these tissues to vitamin D_3 (cholecalciferol) and its related polar metabolites (1,25-dihydroxycholecalciferol). In rachitic (vitamin D deficient) chick intestine for example, the number of calcium lysosomes is markedly increased following vitamin D therapy with either cholecalciferol or 1,25-dihydroxycholecalciferol (Davis and Jones, 1981; Davis and Jones, 1982). In molluscs, this steroid hormone, when infused into the extrapallial fluid compartment, produced striking changes not only in the morphology of the outer mantle epithelium (as studied by scanning electron mi-

croscopy) but also in the calcium concentration of this fluid compartment, the latter increasing from 10 mg% in controls to 40 mg% in the vitamin-D-infused fluid compartment (Matthews and Petit, 1978). We are currently investigating the ultrastructure of the dense bodies of the mantle epithelial cells after vitamin D treatment to determine if there are experimentally induced changes.

Lysosomal activity in mantle epithelia has been studied by other investigators (Kado, 1960; Timmermans, 1969; Ganagarajah and Saleuddin, 1972). A few reports describe a role for these organelles and acid phosphatase in the process of organic matrix calcification during shell formation, especially in the rendering of calcifiability to the shell organic matrix (Chan and Saleuddin, 1974). A similar notion has also been offered to explain vertebrate cartilage calcification (McLean and Urist, 1968; Matsuzawa and Anderson, 1971). To date, however, no one has acknowledged the potential role of lysosomes in mantle epithelial transcellular calcium transport despite the fact that these organelles play a role in the transepithelial transport of ions and molecules in other tissues (Sohal et al., 1976, 1977; Deutschlander et al., 1975; Cornell et al., 1971; Lev and Orlic, 1972; Rodewald, 1971; DeDuve and Wattiaux, 1966).

Similarly, in mammalian bone, sub-brush border lysosomes in osteoclasts also probably function in calcium uptake, storage, and transport (Vaes, 1969; Bonucci, 1974). Metabolites of vitamin D are active in this process as well (Reynolds, 1975).

Thus, a significant role for dense bodies (lysosomes) may exist in cellular and systemic calcium transport and control (homeostasis) in both vertebrates and invertebrates. The lysosome, or dense body, may be a significant part of a common pathway for calcium transport, beginning with the pinocytosis (endocytosis) of extracellular fluids rich in protein bound or free calcium (and other inorganic ions), progressing to the fusion of loaded vesicles with primary lysosomes (vesicle-lysosome fusion or secondary lysosome formation), followed by the exocytosis (defecation) of the contents of these secondary lysosomes, after translocation. These speculations must await further experimental clarification.

Additionally, the presence of substantial iron in these dense bodies is probably reflective of a role for these organelles in iron metabolism, including the uptake, storage, and denaturation of iron and its associated binding proteins (Deutschlander et al., 1975). Similarly, intralysosomal carbohydrate and lipid are most likely indicative of lysosomal functions in organelle turnover, cell metabolism, and nutrient storage/turnover processes (DeDuve and Wattiaux, 1966).

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