Cell-mediated Extracellular Acidification and Bone Resorption: Evidence for a Low pH in Resorbing Lacunae and Localization of a 100-kD Lysosomal Membrane Protein at the Osteoclast Ruffled Border

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ABSTRACT The extracellular compartment where bone resorption occurs, between the osteoclast and bone matrix, is shown in this report to be actively acidified. The weak base acridine orange accumulates within this compartment but dissipates after incubation with ammonium chloride. Upon removal of ammonium chloride, the cells are able to rapidly reacidify this compartment. The highly convoluted plasma membrane of the osteoclast facing this acidic compartment (ruffled border) is shown to contain a 100-kD integral membrane protein otherwise present in limiting membranes of lysosomes and other related acidified organelles (Reggio, H., D. Bainton, E. Harms, E. Coudrier, and D. Louvard, 1984, *J. Cell Biol.*, 99:1511–1526; Tougard, C., D. Louvard, R. Picart, and A. Tixier-Vidal, 1985, *J. Cell Biol.* 100:786–793). Antibodies recognizing this 100-kD lysosomal membrane protein cross-react with a proton-pump ATPase from pig gastric mucosae (Reggio, H., D. Bainton, E. Harms, E. Coudrier, and D. Louvard, 1984, *J. Cell Biol.*, 99:1511–1526), therefore raising the possibility that it plays a role in the acidification of both intracellular organelles and extracellular compartments. Lysosomal enzymes are also directionally secreted by the osteoclast into the acidified extracellular compartment which can therefore be considered as the functional equivalent of a secondary lysosome with a low pH, acid hydrolases, the substrate, and a limiting membrane containing the 100-kD antigen.

Bone resorption, which involves the removal of both the mineral and the organic constituents of bone extracellular matrix, occurs in areas located directly underneath osteoclasts (20, 45). A characteristic feature of active osteoclasts is the presence of the ruffled border, a complex system of plasmalemmal infoldings extending deep within the cell's cytoplasm. The ruffled border is entirely surrounded by an annular zone rich in contractile proteins (21), where the plasma membrane is closely opposed to the bone matrix, and which mediates the attachment of the osteoclast to its substratum (see Figs. 1 and 4 for general views of osteoclasts). This annular zone is called "sealing zone" as it is thought to seal off a compartment delimited by the ruffled border and bone undergoing resorption (36).

It is generally accepted that organic constituents of bone are degraded by lysosomal enzymes secreted by osteoclasts within the resorbing compartment (11, 19, 24, 44). It has repeatedly been proposed, but never demonstrated, that this compartment is acidified in order to allow the dissolution of the mineral constituents of bone as well as to provide an optimal pH for acid hydrolase activity (19, 28, 30, 31, 44).

Three lines of evidence support this hypothesis. First, early studies by Neuman et al. (30, 31) and by Vaes (44) demonstrated that the parathyroid hormone-induced stimulation of bone resorption in organ culture is associated with increased production of lactate and citrate by the explants and results in the acidification of the culture medium. Second, the observation of increased concentration of Yttrium in bone resorp-

THE JOURNAL OF CELL BIOLOGY · VOLUME 101 DECEMBER 1985 2210-2222

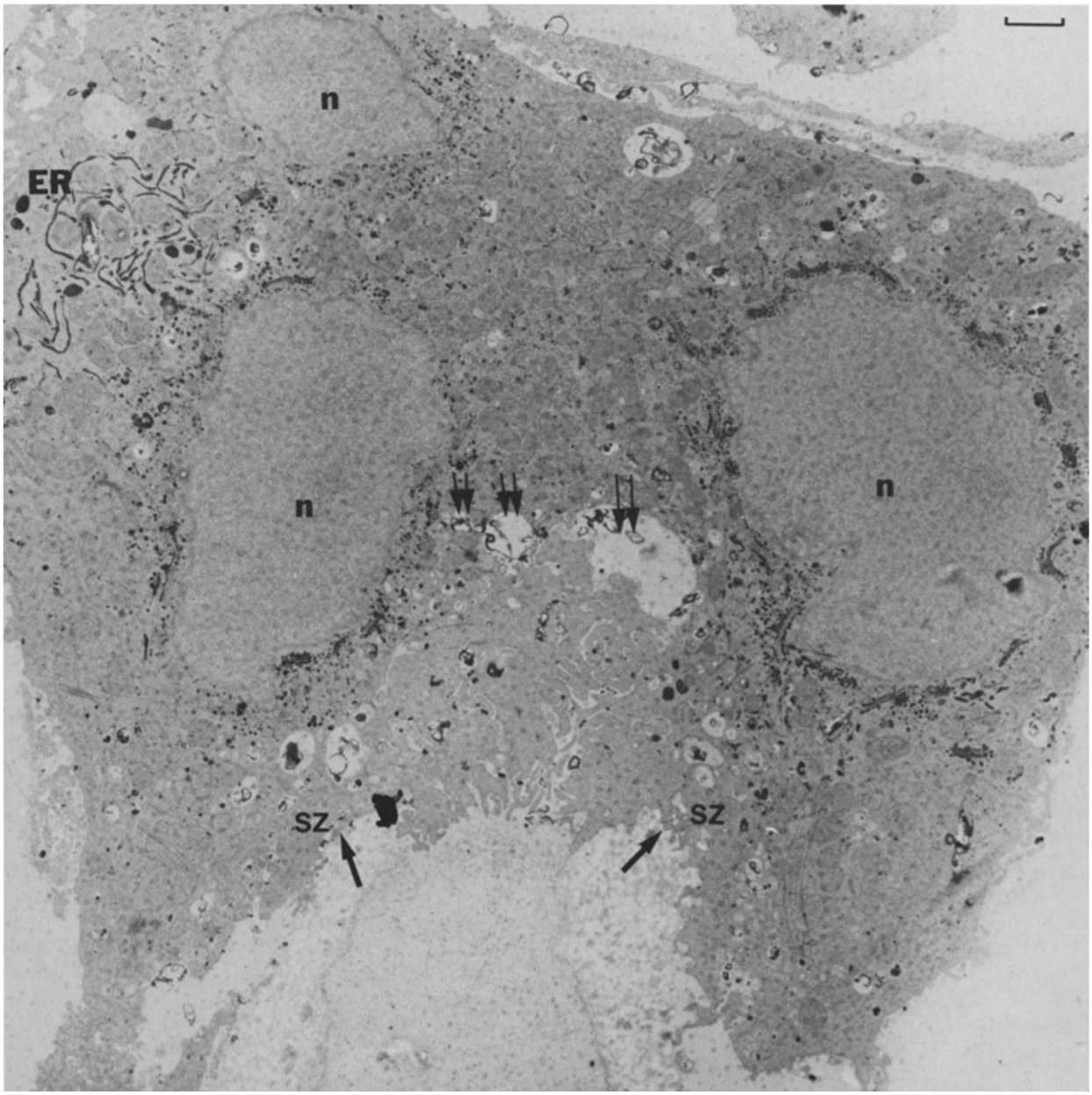


FIGURE 1 General view of an osteoclast after localization of arylsulfatase. This micrograph shows an osteoclast attached to the bone surface at the sealing zones (*SZ*) (arrows), which delimit the ruffled border area (between arrows) and its complex membrane infoldings; large extracellular recesses (double arrows) are found deep into the cell's cytoplasm and are close to the nuclei (*n*) and their surrounding Golgi complexes; abundant mitochondria are found in the osteoclast. Arylsulfatase is localized in all components of the biosynthetic pathway, including the endoplasmic reticulum (*ER*), the numerous perinuclear Golgi complexes, and their associated vesicles, which are often close to the infoldings of the ruffled border membrane (double arrows). Bar, 1 μm. × 9,000.

tion areas was regarded as indicative of a low pH at these sites (31). Third, carbonic anhydrase, an enzyme associated with several acid-secreting epithelia, was demonstrated to be involved in bone resorption (28, 46) and to be specifically concentrated in osteoclasts (2, 14, 15, 43). Despite these suggestions, direct evidence of a low pH at the bone-resorbing lacuna underlying the osteoclast is still lacking and the acidification mechanisms are not yet elucidated.

In other cells and organs, acidification of both extracellular (urinary tract, stomach) and intracellular compartments (endosomes, lysosomes) similarly involves ATP-dependent proton pumps inserted in their limiting membranes (1, 13, 17, 37, 40). Important pharmacological differences may exist, however, between these proton pumps at the various locations (for review see reference 7). These observations and the fact that the conditions prevailing within the extracellular bone-resorbing compartment (high concentration of acid hydrolases, presence of the substrate and, possibly, low pH) are reminiscent of the conditions present within secondary lysosomes, led us to consider the possibility that the osteoclast plasma membrane in the ruffled border area could express related proteins sharing common antigenic determinants with lysosomal membranes and participate in similar ways in the acidification of the resorbing compartment.

Our results demonstrate that the extracellular bone-resorbing compartment is actively acidified and limited by a membrane containing a 100-kD protein otherwise present in lysosomes and other related organelles.

MATERIALS AND METHODS

Acridine Orange Experiments: Newly hatched white Leghorn chicks were placed for two weeks on a calcium-free diet and distilled water, a procedure which leads to a marked increase in the number of bone-resorbing osteoclasts (48). Their tibias and femurs were removed at death and rapidly dissected out, placed in minimal essential medium (MEM)[1] at 4°C, and all muscle, tendons, and periosteum were removed. After a change in medium, the diaphysis were split longitudinally in fresh medium, incubated for 1 h at 37°C, 5% CO_2, and the bone marrow was carefully washed away. Diaphyseal fragments (~5 × 2 mm) were incubated in MEM buffered at pH 7.4 with 20 mM HEPES and containing 5 μg/ml of acridine orange (Sigma Chemical Co., St. Louis, MO) for 15 min at 37°C, washed, and chased for another 15 min in fresh medium without acridine orange. The endosteal surface of these fragments was observed by epifluorescence with a 490-nm excitation filter and a 525-nm arrest filter. In other experiments, the fragments were subsequently incubated in MEM containing 20 mM NH_4Cl and 5 μg/ml acridine orange for 15 min washed, and examined. These preparations were then returned to medium with 5 μg/ml acridine orange alone, incubated for another 15 min, washed, chased in medium without acridine orange, and re-observed.

Cells covering the bone surface were isolated by repeated washing on ice with culture medium and Pasteur pipettes. Filtration of the isolated cells through a 140- a 50-, and a 10-μm nylon filter (nominal pore size, Small Parts, Inc., Miami, FL) resulted in a 20-fold enrichment in osteoclasts in the last filter retentate (14, 33). Incubation with acridine orange was performed either before or after cell isolation. Controls for cell viability were performed by phase-contrast observation, Trypan blue exclusion, neutral red staining, and the ability of the cells to maintain a low pH in some intracellular compartments as demonstrated by acridine orange staining.

Immunocytochemistry: The basic procedures used for the localization of the 100-kD protein by indirect immunoperoxidase were as previously described (8, 9), with the modifications suggested by Brown and Farquhar (6). The nature of the tissue imposed further modifications, described below.

5-d-old Wistar rats were lightly anesthetized by ether and perfused via the femoral arteries with MEM for 1 min, and then for 5 min with either 4% formaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, or the periodate-lysine-formaldehyde (PLP) fixative described by McLean and Nakane (27). The femoral distal and the tibial proximal growth plates were quickly dissected out and fixation was pursued by immersion at 4°C for 15 min in formaldehyde-glutaraldehyde followed by a 15-min quenching in 0.1 M phosphate-buffered saline (PBS) or 4 h in PLP, respectively. The preparations were next incubated for 1 h in PBS containing 10% dimethyl sulfoxide, and frozen in liquid nitrogen. Frozen sections (40 μm) were prepared on a Bright Cryostat (Huntingdon, England) equipped with Jung K tungsten carbide knives and a special holder. When using PLP as a fixative, the sections were further fixed in PLP for an additional 2 h and washed (PBS) before incubation.

The antiserum used to localize the 100-kD lysosomal membrane protein has been previously characterized by Reggio et al. (35). Controls for permeability of the sections were run with an antiserum prepared in rabbits against rat liver Golgi membranes (23), and with an antiserum prepared in rabbits against rat liver mannose-6-phosphate receptors (6).

All sections were incubated and processed as described by Brown and Farquhar (6), using all primary antisera at a dilution of 1:100. Undecalcified sections were dehydrated and embedded in Polybed (Polysciences, Inc., Warrington, PA). Areas containing osteoclasts were selected on semi-thin sections and thin sections stained with lead citrate. For controls, primary antibodies were replaced by non-immune rabbit IgG or were omitted. Kidney tissue, fixed by perfusion via the abdominal aorta, was processed in exactly the same manner.

Enzyme Cytochemistry: 5-d-old Wistar rats were perfused via the femoral arteries with MEM for 1 min followed by 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for ~5 min. Growth plates were dissected out and further fixed by immersion in the same fixative containing 7% sucrose and 10% dimethyl sulfoxide for 1 h, frozen in liquid nitrogen, and 40-μm thick frozen sections were prepared. The sections were decalcified in 4% EDTA, 5% Polyvinyl-pirrolidone, and 7% sucrose, pH 7.4, at 4°C for 15 to 20 h, washed in 0.1 M cacodylate for 24 to 48 h, and incubated in the appropriate medium.

Arylsulfatase was demonstrated according to Goldfischer (18), as modified by Bentfeld-Barker and Bainton (5), using *p*-nitrocathecol sulfate as a substrate. For the demonstration of acid phosphatase, the Barka and Anderson medium was used (3), with β-glycerophosphate as substrate. Control preparations were incubated without substrate. Sections were postfixed in 1% OsO_4 at 4°C for 1 h, dehydrated, and embedded in Polybed. All grids were stained with uranyl acetate and lead citrate.

RESULTS

Evidence for Acidification of the Bone Resorbing Lacunae

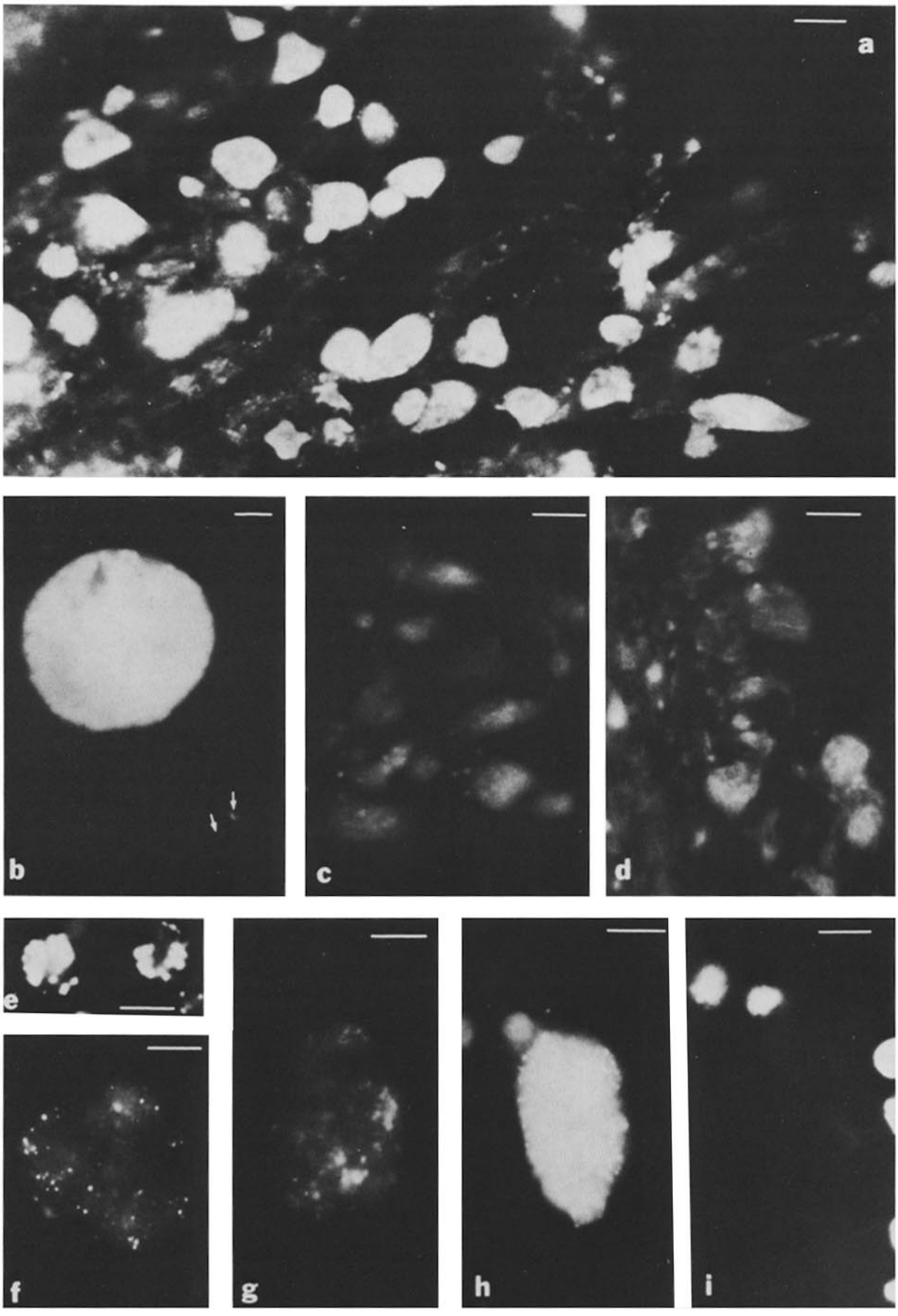
To directly investigate the pH of the bone-resorbing lacunae, we performed acridine orange labeling experiments. Tibias removed from calcium-deprived chicken were cultured in HEPES-buffered MEM at pH 7.4 containing 5 μg/ml acridine orange, washed, and examined in epifluorescence; this allowed us to observe living cells in situ at the endosteal surface, where intense resorption was occurring.

Upon incubation with acridine orange, intense orange fluorescence was seen as either large (>50 μm in diameter) uniformly brilliant orange discs (Fig. 2, *a* and *b*), or as small (~1–2 μm) clustered spots (Fig. 2*b*). At this stage, the large discs were tentatively attributed to bone-resorbing lacunae or to osteoclasts as no other structures of such size could be found. The punctate pattern corresponded to intracellular acidic compartments within mononuclear cells, identified by a faint green nuclear and cytosolic fluorescence due to the binding of acridine orange to cellular RNA (Fig. 2*b*).

Upon 15 min further incubation with 20 mM ammonium chloride, the orange fluorescence vanished from both large discs and small spots (Fig. 2*c*). At places corresponding to previously brilliant discs, fluorescent green nuclei could be

[1] *Abbreviations used in this paper:* MEM, minimal essential medium; PLP, periodate-lysine-formaldehyde.

FIGURE 2 (*a–d*) In situ: bones from calcium-deprived chicken were washed of their marrow, incubated for 15 min with acridine orange (5 μg/ml), and chased in normal culture medium; the endosteal surface was observed in epifluorescence. Intense orange fluorescence is associated with osteoclasts in situ, recognizable by their size and their multiple nuclei seen here as faint fluorescence (*a*). *b* compares at higher magnification the pattern in osteoclasts (*top*) with the small orange dots in mononuclear cells present on the bone surface (bottom arrows). After a 15-min incubation in 20 mM NH_4Cl, the orange fluorescence disappears from all cells (*c*), but reappears if preparations are returned for another 15 min to normal culture medium containing acridine orange and washed (*d*). (*e–i*) *Isolation:* cells lining the bone surface were isolated after incubation in the presence of acridine orange and examined. e shows that mononuclear cells conserve their intracellular staining with the fluorescent dye. With respect to isolated osteoclasts, most of them (50–70%) show a limited number of discrete acridine orange spots (*f* and *g*), in striking contrast with their pattern in situ. A minority (5–10%) of the isolated osteoclasts show a prominent accumulation of such intracellular fluorescent spots (*h*), giving to these cells a staining intensity comparable to what was observed in situ, but a different pattern (compare *b* and *h*). Finally, 25–40% of the isolated cells show no staining at all (*i*): these cells are considered nonviable. Bars, (*a*, *c*, and *d*) 0.1 mm; (*b* and *e*) 10 μm; (*f–i*) 20 μm. (*a*, *c*, and *d*) × 100; (*b*) × 700; (*e*) × 1,000; (*f–i*) × 550.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seen clustered, with a pattern corresponding to that of multinucleated osteoclasts. Preparations were then returned to normal medium containing acridine orange; fluorescence reappeared in both large discs and small vacuoles in <15 min (Fig. 2*d*), indicating thereby the integrity of the cells and the presence of active acidification mechanisms.

To discriminate whether each uniformly fluorescent disc reflected a single large extracellular compartment, corresponding to the resorbing lacuna, or merely the superimposition of numerous intracellular acidic lysosomes, we removed osteoclasts and other cells from their bone substratum by gentle pasteur pipetting. Cell-depleted bone fragments exhibited a considerable decrease in the number of large fluorescent discs and clustered spots, whether cell removal took place before or after acridine orange staining. Given the multinucleation and high vacuolization of the osteoclasts, we found that classical viability tests such as Trypan blue exclusion and neutral red staining were difficult to interpret. We believe that acridine orange staining is a more reliable viability criterion since the maintenance of a low pH within a membrane-bound compartment requires the integrity of ATP-dependent cell functions. More than 90% of the retrieved mononuclear cells retained their punctate acridine orange pattern after isolation (Fig. 2*e*). In contrast, most osteoclasts demonstrated a major change from their in situ appearance. The majority of the isolated osteoclasts (50–70%) demonstrated a punctate pattern of acridine orange–positive intracellular vacuoles, very similar to what was observed in mononuclear cells in situ or after isolation (Fig. 2, *f* and *g*). This pattern was very different from the bright homogeneous fluorescence found in situ and indicated the viability of these isolated cells, also shown by phase-contrast microscopy. About 5–10% of the retrieved osteoclasts were intensely labeled (Fig. 2*h*), due to the superposition of multiple acridine orange–positive spots in their cytoplasm. Finally, 25–40% of the retrieved osteoclasts failed to show any fluorescent vacuoles and were consequently considered as non-viable (Fig. 2*i*).

Since all the osteoclasts observed in situ on their bone substratum were intensely and uniformly labeled whereas very few, if any, retained this labeling pattern after isolation, we take these observations to indicate that the fluorescent discs observed in situ generally correspond to bone-resorbing lacunae, i.e., large extracellular compartments located underneath the cells and actively acidified by their limiting osteoclast. Clearly, osteoclasts also contain intracellular acidic compartments (Figs. 2, *f* and *g*). For reasons not yet determined, the number of these intracellular acidic vacuoles varies from cell to cell. When they reach a high density (Fig. 2*h*), the observation of qualitative changes after isolation is less easy. Nevertheless, the majority of isolated osteoclasts showed only few acidic vacuoles, allowing the distinction between intra- and extracellular acid compartments.

Evidence for the Presence of a 100-kD Lysosomal Membrane Antigen at the Osteoclast's Ruffled Border Membrane

The functional data reported above suggested that, similarly to lysosomes, bone-resorbing lacunae were acidified. Therefore, we next asked whether the plasma membrane of the osteoclast ruffled border and the limiting membrane of lysosomes contained related protein(s) sharing common antigenic determinants.

This possibility was examined by immunocytochemistry of bone cells, using as a primary reagent antibodies specific for a 100-kD rat lysosomal membrane protein, which have been demonstrated to co-localize with acridine orange–stained intracellular vacuoles (35, 42).

In the kidney proximal tubule, used as control, immunoperoxidase staining was exclusively localized to the multivesicular bodies and secondary lysosomes (Fig. 3). The plasma membrane of the brush border, the basolateral membrane, and the other intracellular membranes were not stained. In the particular case of multivesicular bodies, the reaction product was localized along the luminal side of the limiting membrane and around the membranes of the vesicles contained in these large vacuoles; the cytoplasmic side of the limiting membrane was devoid of reaction product (Fig. 3*b*). Within the other cells of kidney sections, the reaction product was also specifically localized at the luminal side of multivesicular bodies or large vacuoles that could be interpreted as secondary lysosomes. We only occasionally observed minor labeling of plasma membranes or other intracellular membranes in these specimens.

In undecalcified sections of bone tissue, and with both fixation procedures, a highly specific localization of the reaction product was also observed. At the light microscopic level, bone marrow cells, osteoblasts, and osteocytes were stained for this antigen only in intracellular vacuoles (Fig. 4), corresponding by electron microscopy to multivesicular bodies and lysosomes.

In contrast, a strong positive reaction was consistently observed at the level of the ruffled border in osteoclasts both at the light and electron microscopic levels (Figs. 4 and 5; see also Fig. 7). The plasma membrane of the ruffled border was strongly and exclusively labeled on its external surface and not its cytoplasmic face (see Fig. 7*a*). No reaction product

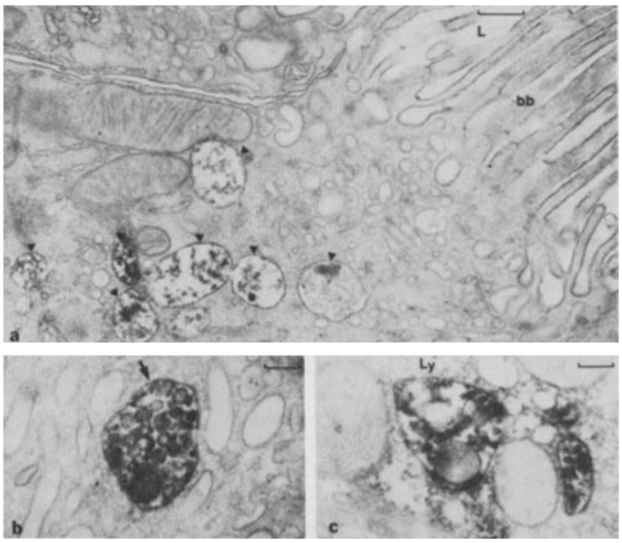


FIGURE 3 Immunolocalization of the 100-kD lysosomal membrane protein in the proximal rat kidney tubule, glutaraldehyde–formaldehyde fixation. The reaction product is localized to multivesicular bodies in the apical portion of the cells (*a*, arrowheads); brush border membrane (*bb*) and other membranes in these cells are devoid of reaction product. At higher magnifications, the staining in multivesicular bodies (*b*) and secondary lysosomes located deeper in the cells (*c*) is found on the luminal side of the limiting membranes. *L*, lumen of tubule. Bars, (*a*) 200 nm; (*b* and *c*) 100 nm. (*a*) × 15,000; (*b*) × 28,000; (*c*) × 25,000.

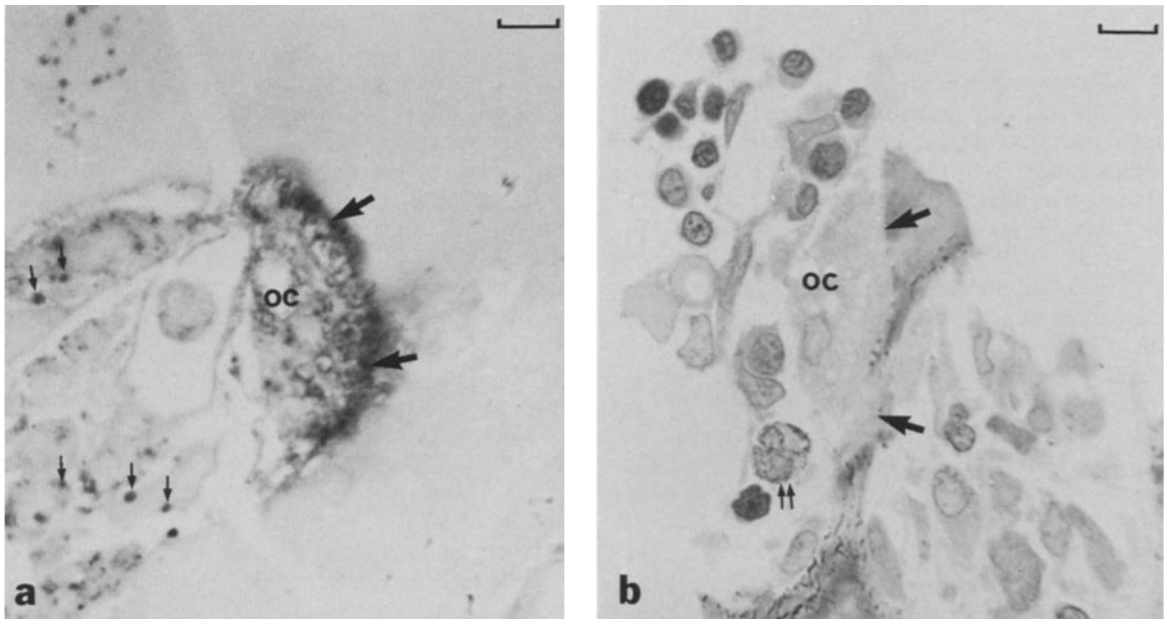


FIGURE 4 Light microscopic view of the distribution of the 100-kD membrane protein in osteoclasts and other endosteal and bone marrow cells, PLP fixation. (*a*) Section incubated with the specific antibodies; the osteoclast (*oc*) shows a prominent immunoperoxidase staining at the ruffled border (large arrows) and large vacuoles associated with it; in addition, a scarce punctate staining pattern can be seen in all cells, including the osteoclast (small arrows). (*b*) Control section incubated with non-immune IgG; no reaction product is found in osteoclast (*oc*) on their ruffled border (large arrows); only small granules containing endogenous peroxidase are stained in one marrow cell (double arrows). Bar, 10 μm. × 700.

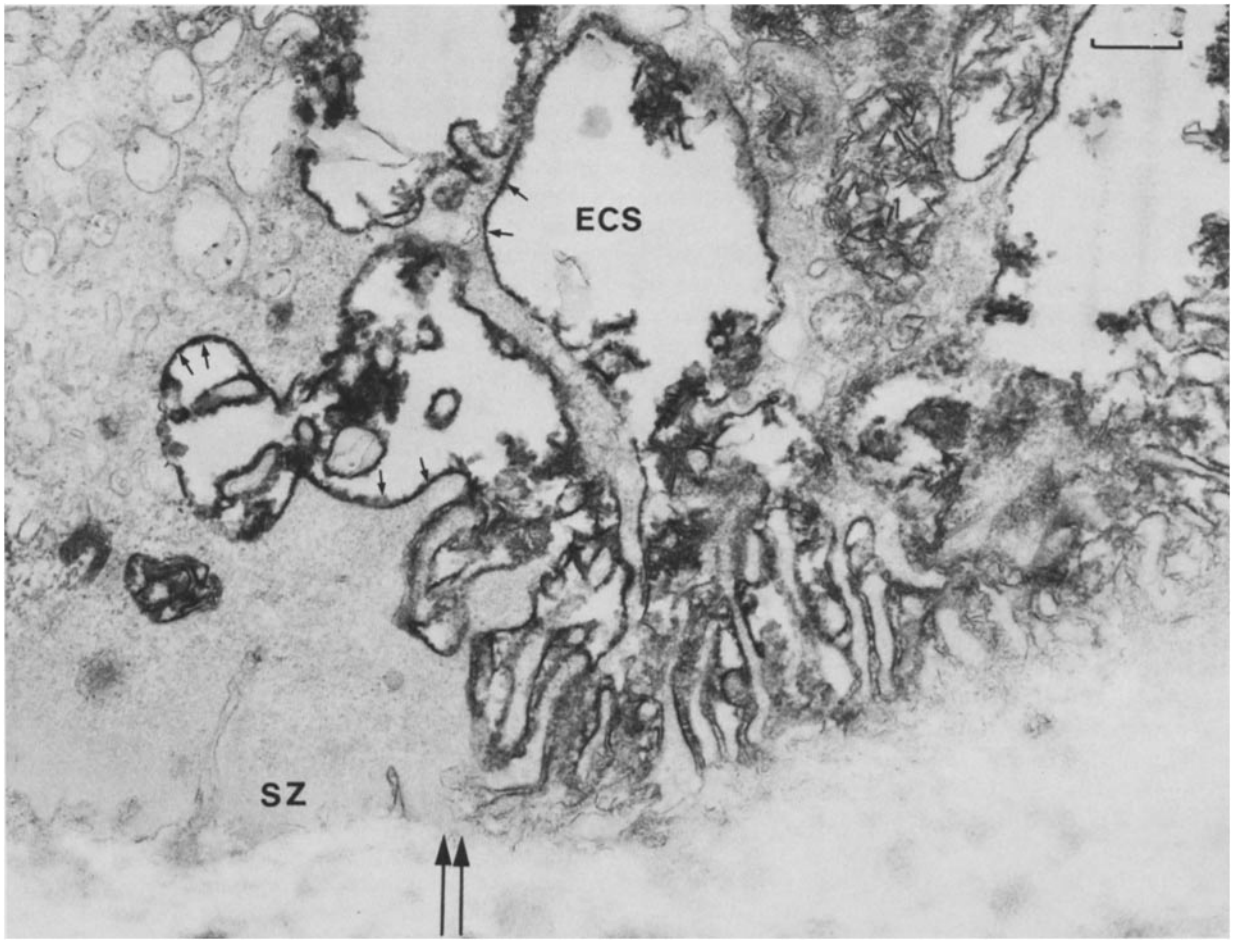


FIGURE 5 Immunolocalization of the 100-kD membrane protein in an osteoclast, PLP fixation. The reaction product is present along the ruffled border membrane but is lacking at the sealing zone membrane (*SZ*); double arrow indicates clear cut limit between these two membrane domains. Note the ampullar dilations of extracellular space (*ECS*) at the cytoplasmic end of the plasma membrane infoldings of the ruffled border, which might appear in sections as intracellular vacuoles (compare with Figs. 3 and 10). Small arrows show the reaction product exclusively at the luminal side of the membrane. Bar, 600 nm. × 20,000.

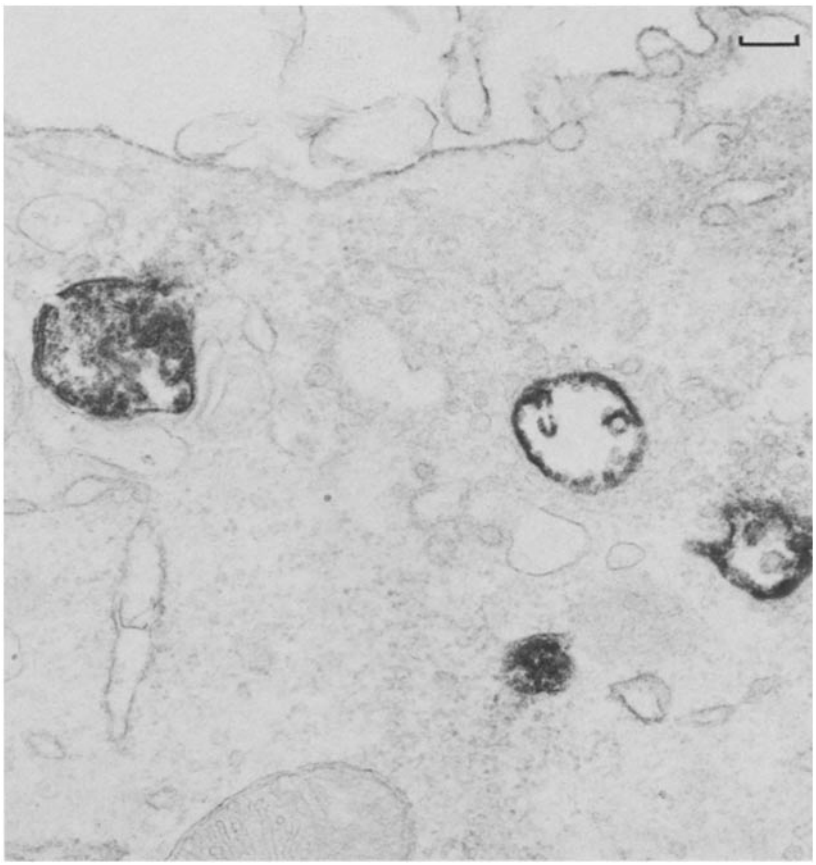


FIGURE 6 The plasma membrane of the osteoclast opposite the ruffled border and facing the bone marrow compartment does not stain for the 100-kD antigen. Adjacent secondary lysosomes are stained and constitute a positive control. PLP fixation. Bar, 200 nm. × 35,000.

has been observed at the plasma membrane of the sealing zone (Fig. 5) or facing the bone marrow compartment (Fig. 6).

Some, but not all, of the large profiles located just behind the ruffled border, apparently within the cytoplasm of the osteoclast, were stained by immunoperoxidase along their luminal side (Fig. 5). A continuity was frequently observed between pockets of the ruffled border membrane and large ampullar vacuoles very deep into the cytoplasm of the cell (Figs. 1 and 5; see also Fig. 9) suggesting that stained closed profiles were part of the complex geometry of the ruffled border plasma membrane, and not intracellular vacuoles. Some typical secondary lysosomes or multivesicular bodies, usually located deeper in the cytoplasm or at the pole opposite the ruffled border were also stained (Fig. 6).

Endoplasmic reticulum and Golgi membranes were not stained in formaldehyde–glutaraldehyde–fixed tissues but were occasionally labeled in PLP-fixed tissues. The very numerous coated and uncoated vesicles located around the Golgi complexes and close to the ruffled border membrane were still unlabeled (Fig. 8 *a*). This lack of reaction product could not be interpreted as a lack of access of the antibody to the antigen due to limited permeability since, in adjacent sections, Golgi cisternae and some Golgi vesicles were immunolabeled with antibodies directed against the mannose-6-phosphate receptor (Fig. 8 *b*) or against Golgi membranes (Fig. 8 *c*).

Controls for the specificity of this localization were obtained by processing the tissues in the same manner but omitting the incubation with the primary antibody or by replacing the primary antibody by non-immune rabbit IgG. In both cases (Figs. 4 *b* and 7 *b*), no reaction product was observed at the ruffled border plasma membrane or at the level of lysosomal membranes in other cells.

These observations demonstrate that the plasma membrane of the osteoclast contains assymetrically distributed integral membrane proteins as indicated by the specific concentration of the lysosomal 100-kD antigen at the ruffled border. They also demonstrate that the plasma membrane at the ruffled border and the limiting membrane of secondary lysosomes share some common properties: both contain the 100-kD antigen and limit acidic compartments.

Cytochemical Localization of Lysosomal Enzymes

We then proceeded to localize the lysosomal enzymes β-glycerophosphatase and arylsulfatase in the osteoclasts, in order to compare their distribution with that of the 100-kD antigen. We have been able to conserve most of the enzymatic activity in bone cells by sharply decreasing the decalcification time (see Materials and Methods). Using this improved procedure, the osteoclasts demonstrated a high density of organelles positive for both β-glycerophosphatase and arylsulfatase. Both enzymes had an identical distribution and will therefore be described together.

A prominent and characteristic feature of osteoclasts is the presence of very numerous Golgi complexes surrounding each of their many nuclei. All these Golgi complexes were strongly positive for both arylsulfatase (Fig. 1) and β-glycerophosphatase (Fig. 9 *e*). Unlike in other cells, most Golgi cisternae were stained (Fig. 9, *c* and *e*). Very numerous small coated or uncoated vesicles containing lysosomal enzymes could be observed at the periphery of the Golgi cisternae (Figs. 1 and 9). Hence, lysosomal enzymes could be observed along the entire secretory pathway: large areas of the endoplasmic reticulum, including the nuclear envelope, most Golgi stacks, and

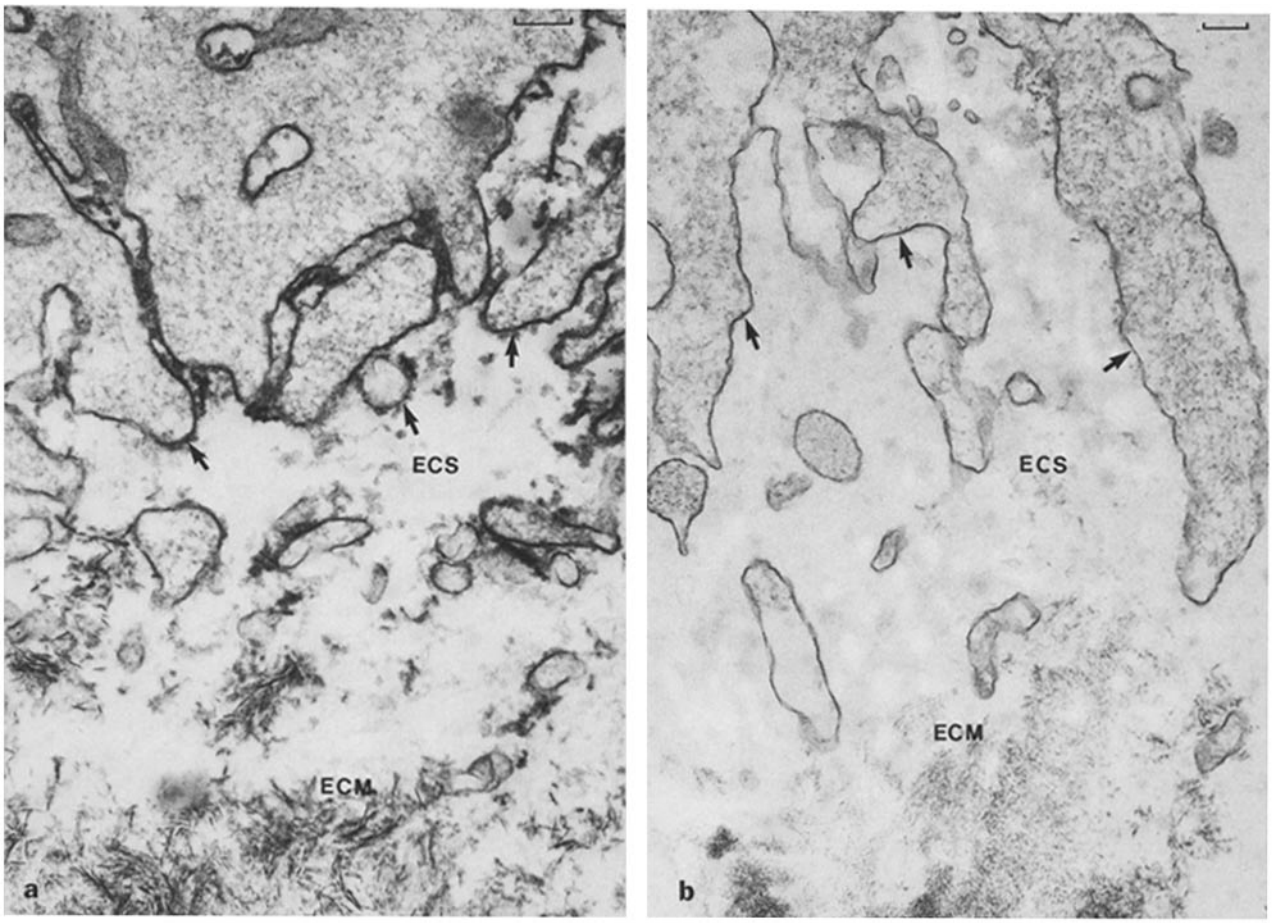


FIGURE 7 Immunolocalization of the 100-kD membrane protein in an osteoclast, glutaraldehyde–formaldehyde fixation. (*a*) Sections incubated with the specific antibodies: the reaction product is localized to the external surface of the ruffled border plasma membrane of this osteoclast (arrows). (*b*) Control sections incubated with non-immune IgG fail to show any reaction product at the same site (arrows). *ECS*, extracellular space; *ECM*, extracellular bone matrix. Bars, 200 nm. (a) × 55,000; (*b*) × 40,000.

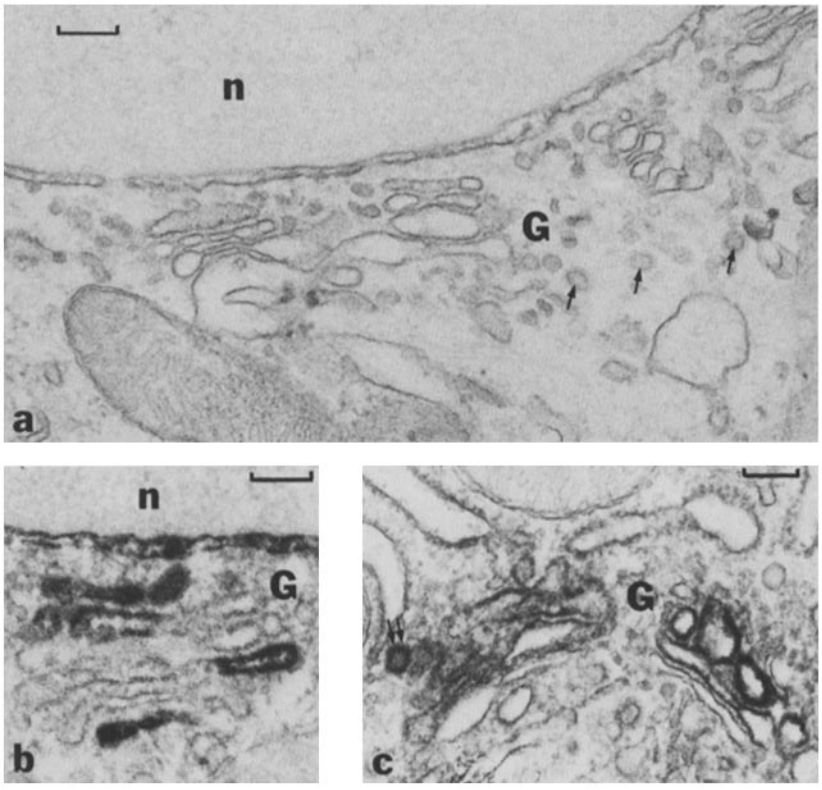


FIGURE 8 Immunolabeling of Golgi complexes in osteoclasts, PLP fixation. (*a*) 100-kD protein: no reaction product in Golgi saccules (*G*) and vesicles; arrows point to coated vesicles. (*b*) Mannose-6-phosphate receptor. (*c*) 135-kD Golgi protein: staining of the Golgi saccules (*G*) and some peri-Golgi vesicles (double arrow indicates coated vesicle) demonstrates the permeability of the sections to the antibodies. Bars, 200 nm. × 35,000.

most of the small vesicles surrounding the Golgi cisternae were strongly stained for lysosomal enzymes (Figs. 1 and 9), a pattern suggestive of a secretory process.

The deep invaginations of the ruffled border plasma membrane delimited extracellular channels that often were in close proximity to the Golgi stacks located on the resorbing side of the nuclei (Figs. 1 and 9*d*). In these areas, as well as all along the ruffled border, numerous small lysosomal enzyme–rich vesicles, similar to those observed around the Golgi complexes, could be seen. These vesicles appeared to fuse with the membrane of numerous closed profiles close to the ruffled border and positive reaction product could be localized along the luminal side of the membranes at sites where the small lysosomes were in continuity with the luminal space (Fig. 10*a*). Vesicles of similar size and location were not stained for the 100-kD antigen (Fig. 10*b*). As observed by all investigators (11, 19, 20, 24, 45), it is not possible with cytochemical methods to demonstrate enzymes in the large vacuoles of the ruffled border complex and in the extracellular bone–resorbing compartment, despite the very short decalcification time used in this study. However, some particles of unknown nature (50–100 nm in size) were stained both for arylsulfatase (Fig. 9*d*) and for β-glycerophosphatase (Fig. 10*a*) in the extracellular space. Despite the lack of demonstrable extracellular reaction product, which we attribute either to the large excess of endogenous substrate in the resorbing lacuna or to enzyme inhibition by the reaction product, we interpret these cytochemical results as indicating that the osteoclast is essentially engaged in the synthesis and secretion of lysosomal enzymes into the bone-resorbing compartment. These secretory products are probably carried from the Golgi area to the ruffled border area via primary lysosomes, in which the 100-kD antigen could not be demonstrated.

DISCUSSION

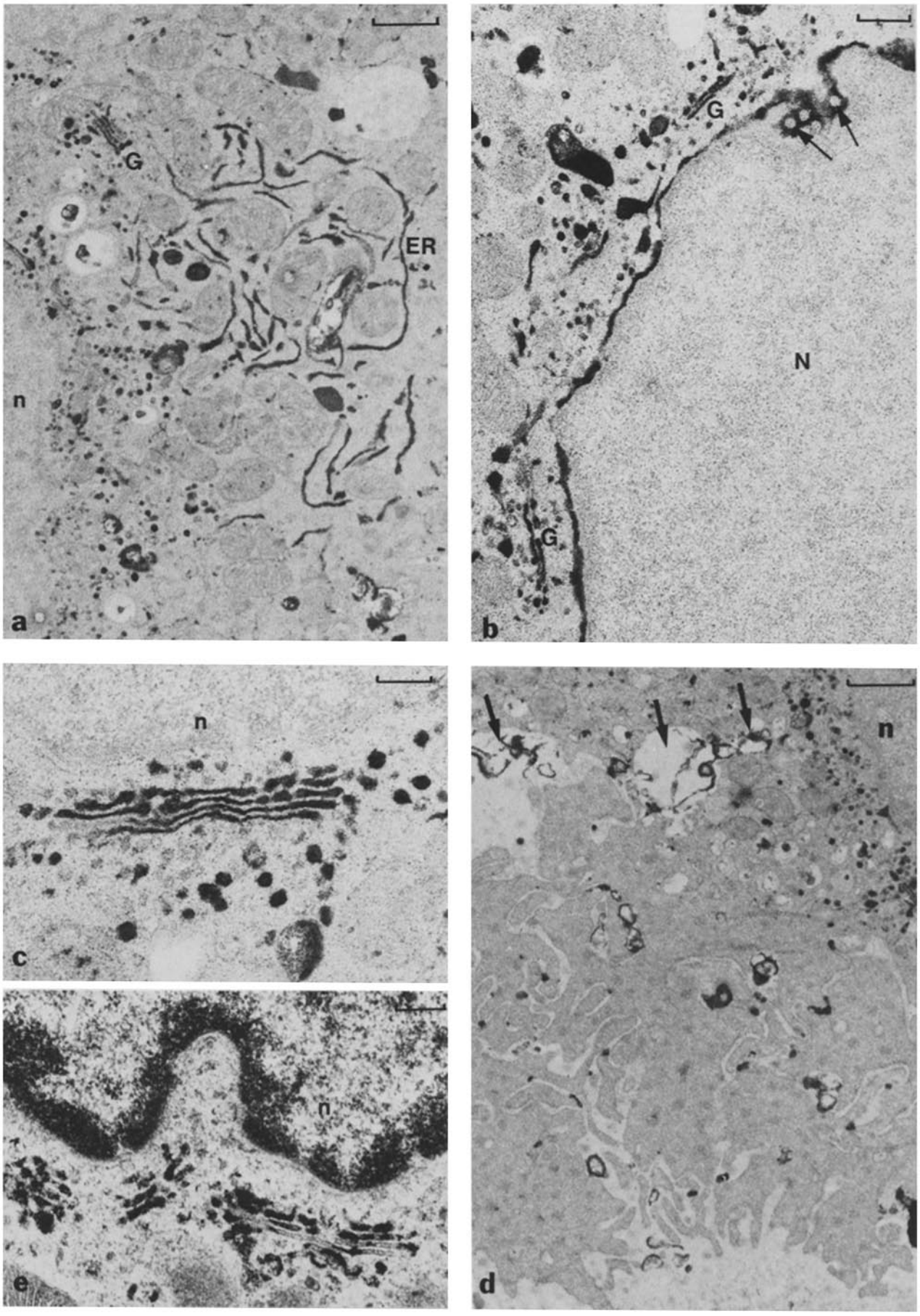
The observations made in this study can be summarized as follows. First, the bone-resorbing lacuna is actively acidified by its limiting cell, the osteoclast. Next, the ruffled border membrane of the osteoclast facing this extracellular acidic compartment expresses a 100-kD protein comprising epitopes related or identical to those present in the limiting membrane of secondary lysosomes, whose content is also acidified. Thirdly, lysosomal enzymes, which are synthesized by the osteoclast (44) are transported via primary lysosomes, which apparently lack the 100-kD antigen, and are secreted into the bone-resorbing lacuna. Thus, bone resorption by osteoclasts involves both the acidification of, and the secretion of lysosomal enzymes into, the resorbing lacuna.

Evidence for acidification of the bone-resorbing lacuna relies upon acridine orange experiments. Acridine orange is a fluorescent dye which behaves as a weak base whose unprotonated form diffuses freely through intact biological membranes and is sequestered in the protonated form in closed acidic compartments (10, 29). When living cells are incubated in medium containing acridine orange they accumulate this compound mostly in lysosomes and endosomes, therefore showing a punctate pattern corresponding to individual organelles when viewed under fluorescent light (29, 35). Using this methodology, we were able to observe in situ the cells attached to the endosteal surface of bones and showed that osteoclasts demonstrated large and uniform orange fluorescent disks superimposed on their whole area. Since most of the osteoclasts, once isolated from their bone substratum, were no longer associated with a large acridine orange brilliant disk, it appears that most, if not all, large acidic compartments associated in situ with these cells are indeed extracellular.

Some difficulty in interpreting these results is, however, related with the fact that 5–10% of the freshly isolated osteoclasts demonstrated an extreme accumulation of acridine orange–stained vacuoles, reminiscent in intensity, but generally not in pattern (see Fig. 2), of the in situ observations. Nevertheless, these cells represented only a small fraction of the otherwise viable isolated osteoclasts (which contained acidic vacuoles) whereas all osteoclasts observed in situ were associated with a uniformly fluorescent disk. The bright isolated osteoclasts could represent a subpopulation of cells at a different functional state at the time of isolation, or cells in which the ruffled border membrane had been internalized as a result of the isolation procedure. We altogether believe that the data obtained with acridine orange provide a direct experimental evidence that the resorbing lacuna is acidic, as previously postulated by Neuman et al. (31) and Vaes (44). Moreover, since our results show that this compartment is rapidly returned to acidic pH upon removal of ammonium chloride, we conclude that acidification is an active process, performed by the limiting osteoclast.

We next examined the possibility that the membrane limiting this acidic extracellular resorbing compartment, i.e. the ruffled border, could share some properties with the limiting membrane of lysosomes, which carries ATP-dependent proton pumps responsible for the acidification of lysosomal content (32, 37). Our results show that the ruffled border plasma membrane has a different protein composition than the rest of the osteoclast plasma membrane and specifically contains high concentrations of a membrane antigen otherwise present in the membranes of lysosomes and other related organelles. Reggio et al. (35) have shown that the antibody to this 100-kD antigen cross-reacts on protein transfers from SDS gels on nitrocellulose sheets with a H^+,K^+ ATPase purified from pig gastric mucosae (34) but not with other membrane ATPases of similar molecular weight, namely dog kidney Na^+, K^+ ATPase and rabbit muscle Ca^{++} ATPase. Hence, the 100-kD proton pump ATPase from gastric parietal cells (34) and the 100-kD lysosomal membrane protein share common antigenic determinants. In addition, these two proteins are localized in membranes limiting acidic compartments (35, 42) as extended here in a quite different biological

FIGURE 9 Distribution of arylsulfatase (*a–d*) and β-glycerophosphatase (e) in osteoclasts. The lysosomal enzymes are localized throughout the entire biosynthetic pathway, from the endoplasmic reticulum (*ER*) (a), including the perinuclear envelope (*b*), most stacks of the Golgi complexes (*G*), and numerous small vesicles surrounding the Golgi complexes, interpreted as primary lysosomes (c and e). Arrows in *b* point to negatively stained nuclear pores. Field at *d* shows the deep infoldings of the ruffled border plasma membrane delimiting extracellular spaces (arrows) close to the nuclei (*n*), Golgi complexes, and associated vesicles. Bars, (a and *d*) 800 nm; (*b*) 400 nm; (c and e) 200 nm. (a and *d*) × 15,000; (*b*) × 25,000; (c and e) × 45,000.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system. These similarities led Reggio et al. (35) to suggest that, despite important pharmacological differences between proton ATPases in stomach and acidified organelles (1, 7, 12, 16, 17, 40, 41) the 100-kD protein could be a component of the lysosomal proton pump involved in proton transfer across membranes.

These observations have a number of functional implications which might lead to a better understanding of the mechanisms of bone resorption. First, our results demonstrate that the osteoclast is polarized and exhibits distinct plasmalemmal domains, with an equivalent of the apical secretory pole (the ruffled border, where the 100-kD membrane protein is concentrated), an equivalent of a junctional complex (the sealing zone) rich in actin filaments (21), and a basolateral-like domain. The 100-kD membrane protein was not demonstrable in the two latter domains. Other instances of membrane polarization in cells devoid of tight junctions exemplify that lateral diffusion of proteins may be prevented in the absence of junctional complexes. Among these are the erythroblast at the time of nuclear expulsion (38) and adherent macrophages (47), a cell type possibly related with the osteoclast. We propose that the sealing zone, where actin filaments are anchored to the membrane and the cell is attached to its substratum (25), could play such a restrictive role. Future studies using specific antibodies against antigens known to be distributed in the basolateral membrane of epithelial cells, such as the Na^+,K^+ ATPase (22) would be of major interest to reinforce this hypothesis.

Second, the existence of distinct plasmalemmal domains is associated with a concentration of post-Golgi transport vesicles towards the ruffled border membrane, indicating a polarized secretion of lysosomal enzymes. We, like others (11, 19, 20, 34, 45), have not been able to demonstrate high concentrations of enzymes in the extracellular space using cytochemical methods, with the exception of some membrane staining and particles of unknown nature. These difficulties in demonstrating extracellular lysosomal enzymes might be due to the large excess of endogenous substrate in the bone-resorbing compartment, preventing the use of the exogenous substrate added during the procedure, or to enzyme inhibition by the reaction product. The use of antibodies to lysosomal enzymes might help resolve this question. There is, however, little doubt that these enzymes are directionally secreted by the osteoclast into the resorbing lacuna (44, 45).

Since the limiting membrane of the resorbing compartment, i.e. the ruffled border plasma membrane, contains a 100-kD lysosomal membrane protein and limits an acidic compartment where both lysosomal enzymes and their substrate are found, the resorbing lacuna can be considered as the functional equivalent of a secondary lysosome. Hence, one is led to the view that bone resorption could occur entirely within an extracellular lysosome-like compartment, acidified and equipped with lysosomal enzymes directionally secreted by the osteoclast. The low pH prevailing in the bone-resorbing compartment would be sufficient to dissolve the mineral phase of bone matrix (21, 30, 44) and would provide optimal conditions for the degradative action of acid hydrolases on the organic phase of bone matrix (44). Considering the extracellular bone-resorbing compartment as the functional equivalent of a secondary lysosome where digestion of bone matrix could be complete, residues from this extracellular digestion could diffuse through cell membranes as through lysosomes and would not require endocytosis for further digestion inside the osteoclasts.

We would like to stress that osteoclasts therefore resemble other acid-secreting cells like gastric parietal cells and specific cells of the urinary epithelia. First, their apical surface is extensively ruffled, thus providing a considerable surface to support a large number of proton pumps. As in these cells (17), reversible vesicular insertion of proton pumps at the secretory pole might modulate acid secretion by osteoclasts (4). Second, acid-secreting gastric and urinary cells also contain numerous mitochondria and very high carbonic anhydrase activity in the cytosol (1, 26) as does the osteoclast (2, 14, 15, 43). Inhibition of carbonic anhydrase activity prevents bone resorption in organ culture (28) and affects calcium metabolism in the intact animal (46). Genetic deficiency in carbonic anhydrase activity has recently been shown to be associated with a particular form of osteopetrosis, combining impaired osteoclastic resorption, and renal tubular acidosis (39). Thus, in osteoclasts, as well as the other cells, proton pumps would be supplied with ATP by the abundant mitochondria and with protons by carbonic anhydrase.

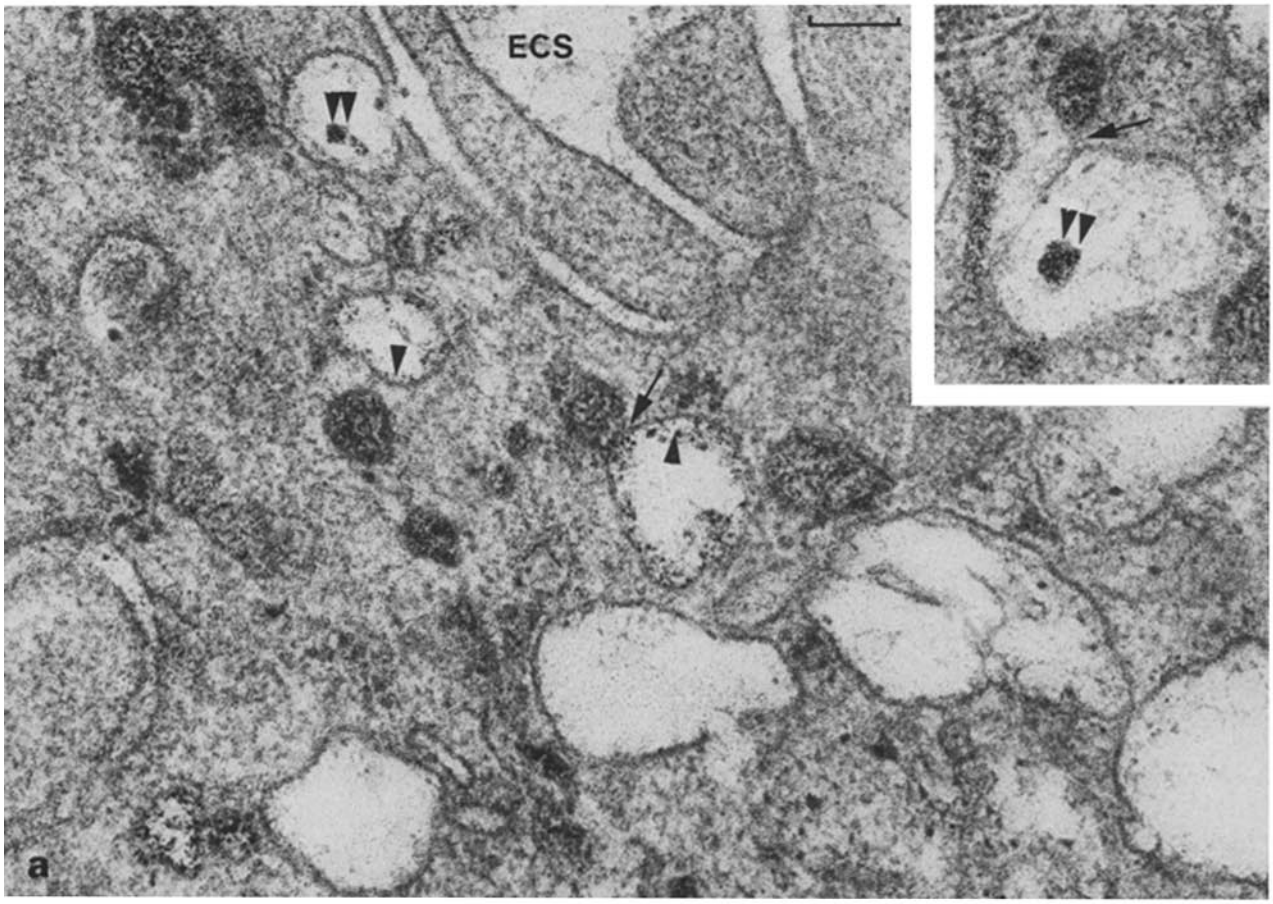
In conclusion, we propose that the osteoclasts should be regarded as a polarized secretory cell which secretes protons and acid hydrolases into the bone-resorbing compartment, leading to the extracellular digestion of the mineral and organic phase of bone matrix. The 100-kD lysosomal membrane protein found here to be specifically concentrated at the ruffled border membrane of osteoclasts might therefore play a critical role in the bone-resorbing process.

The authors are most grateful to Dontscho Kerjaschki, William Brown, and Marilyn G. Farquhar for helpful suggestions and discussions, and for providing them with antibodies; to Christine Roy for expert technical help; and to Barbara Devlin for typing the manuscript.

This work was supported by grant DE-04724 from the National Institutes of Health.

Received for publication 12 April 1985, and in revised form 1 August 1985.

FIGURE 10 Comparative distributions of β-glycerophosphatase (a) and the 100-kD lysosomal membrane protein (b) along the ruffled border. In a, note numerous small intracellular vesicles (primary lysosomes) some of which fuse with invaginations of the ruffled border (arrows). β-Glycerophosphatase, secreted in the extracellular space, is either found along the cell membrane (single arrowhead) or in extracellular granules (inset, double arrowheads). b shows that the 100-kD lysosomal membrane protein is localized at the luminal side of the ruffled border membrane (double arrows) but cannot be found at the limiting membranes of primary lysosomes (single arrows). (a) Glutaraldehyde fixation; (b) glutaraldehyde–formaldehyde fixation. Bars, 200 nm. × 60,000 and 80,000, respectively.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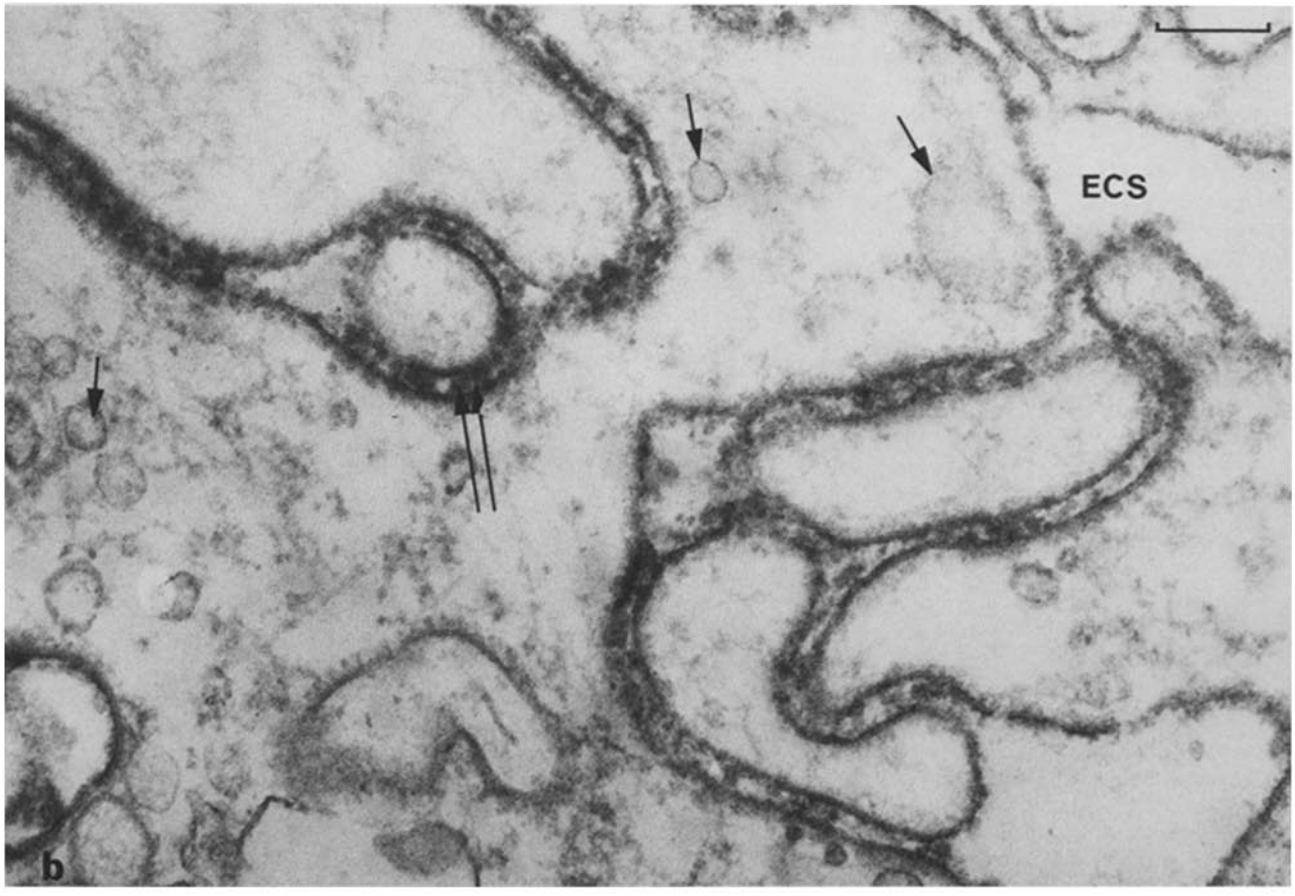
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