The Ultrastructure of the Osteoclast and its Functional Implications

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Even though the osteoclast was first described about a century ago,[3,4] it was not until the early sixties that we gained some insight into its function. A great step was made when the results from ultrastructural[6,4] and microcinematographic[18, 19, 25] studies convincingly showed that the osteoclast is involved in bone resorption and that the ruffled border is the active site of that resorption. The discussion that ensued has continued ever since and has centered around two main points: (1) the physiological functions of the osteoclast in bone metabolism and (2) the mechanism by which the osteoclast participates in bone resorption.

In order for bone to be resorbed, both mineral and matrix components have to be dissolved. Most investigators agree that the mineral component has to be dissolved first, probably by the interaction with hydrogen ions,[5,2] before a breakdown of the organic components, the collagen and protein polysaccharides, can take place.[5] It is not clear whether the osteoclast is involved in the transport of mineral from the bone matrix and how the calcium phosphate reaches the extracellular space. The contribution of the cell to the breakdown of the organic matrix is not fully understood either: how much matrix is broken down outside the cell, how much inside the cell and where do the products go?

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Resorption has a dual function in the metabolism of bone. It occurs continuously in the process of morphogenesis and in the remodeling of adult bone. It is well established that in this slow and well integrated process of resorption and formation osteoclasts play a role in the breakdown of bone. The other function of bone resorption is the release of calcium in response to low calcium levels in the serum and body fluids. This is a fast process[5,3] and because an immediate response of numerous cells is thought to be required, the role of osteoclasts, so few in number, has been seriously disputed.[2]

Descriptive and histochemical studies at the ultrastructural level have contributed greatly to the understanding of the mechanism of action of the osteoclast. Furthermore, changes in its ultrastructure after exposure to hormones have provided information on its functional role in bone metabolism. The most recent findings in both of these areas will be reviewed and discussed in this paper.

The ultrastructural features that are characteristic for the osteoclast have been described in detail by numerous investigators[1,4, 20, 25, 64] The cytoplasm contains many nuclei, an abundance of mitochondria, sparse rough endoplasmic reticulum and many free ribosomes or clusters of ribosomes (Figs. 1 and 2). In addition, a variety of vacuoles may be present, as well as a ruffled border and an adjacent clear zone (Figs. 1 and 2). In this paper these features will be dealt with only in so far as more recent findings have

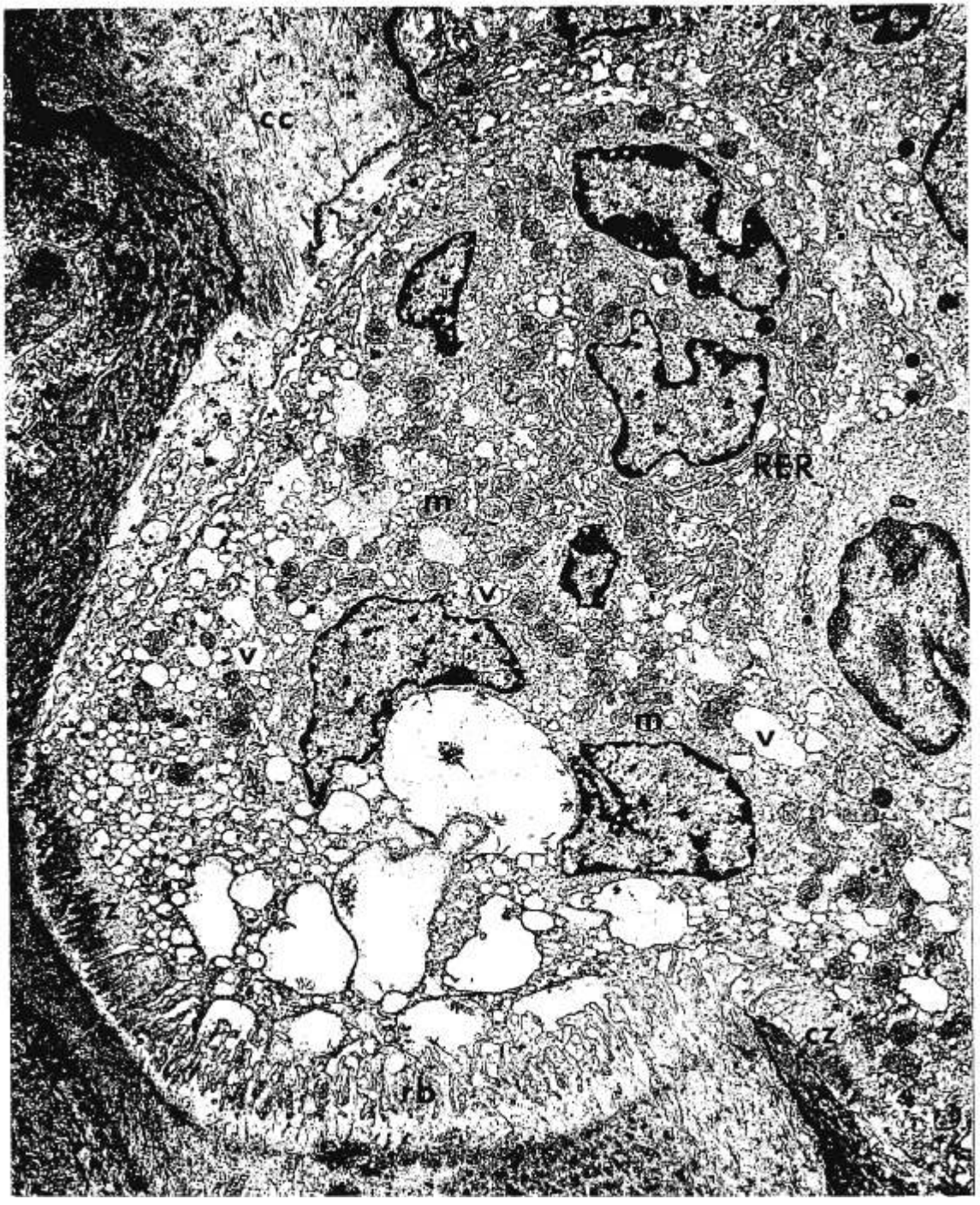

FIG. 1. Profile of an osteoclast. Notice the abundance of mitochondria (m), sparse rough endoplasmic reticulum (RER) and many free ribosomes or clusters of ribosomes distributed throughout the cytoplasm. Vacuoles (v), a ruffled border (rb) and clear zone (cz) are present in this profile. A lamina limitans (arrows) can be seen along a spicule of calcified cartilage (cc). ×4,600

contributed to newer insights into the functional activity of the cell.

Cells that are ultrastructurally indistinguishable from osteoclasts are involved in the breakdown of calcified cartilage and dentin. Some investigators name these cells according to the tissue being resorbed, *e.g.* chondroclasts or odontoclasts, respectively. Although minor differences may exist in the enzyme systems of these resorptive cells, based on the ultrastructural similarity one can assume that the major mechanisms are similar. Therefore, no distinction in types of "clasts" will be made in this review.

RUFFLED BORDER

The most outstanding feature of the osteoclast is the part of the plasma membrane that was originally called "brush border" or "striated border."[34] Scott and Pease[64] were the first to describe this area ultrastructurally. They renamed it "ruffled border," a term which seems the most appropriate and is in common usage today.

The ruffled border consists of a complicated system of folds in the cell membrane, resulting in finger-like projections of the cytoplasm which are separated by spaces (Fig. 2). There is a great variability in longitudinal extent of these projections[39] as well as in width. The spaces between these projections may also vary from very small and narrow channels to large and bulbous areas.[20, 39] The many folds of the membrane provide for a greatly increased surface area of the cell. The extent to which the membrane can be involved in the ruffles is well demonstrated in Figure 3 which represents a cross section through a ruffled border in a plane roughly parallel to the bone surface. The total area of projections and spaces that faces the bone varies considerably and may be very localized or spread out along the bone surface. Cross sections through osteoclasts do not always show a ruffled border. In cultures of fetal rat bones only 11 per cent of the osteoclast profiles showed this specific area.[28] This is mainly the result of the plane of section through the cells: even if all osteoclasts would have a ruffled border, only in a certain percentage of cell profiles would this area be present. It is also possible that some osteoclasts do not have a ruffled border. From serial sections through an entire cell, a 3-dimensional reconstruction of a cell could be made and the presence and distribution of the cell organelles determined. However, this is technically extremely difficult. For instance, one osteoclast with a diameter of 30μ would be represented by as many as 500 thin sections. A compromise was worked out by Lucht[39] who collected thin sections at intervals of 2.5μ. In this way meaningful information can be obtained on structures larger than 2.5μ, such as ruffed borders, clear zones and nuclei, but not on smaller structures. Using this technique he found that some osteoclasts do not have a ruffled border. Sometimes two ruffled borders can be seen in an osteoclast profile, far enough apart from each other to assume that these represent two separate areas in the cell.[39] The average area of ruffled border, including spaces, occupied 3.4 per cent of the cytoplasm in profiles of unstimulated osteoclasts sampled from the metaphysis of rat long bones.[33]

The surface of the bone opposing the ruffled border is frayed and no lamina limitans is present.[39, 60] It is well accepted that here resorption of bone is taking place. Between the bone and the ruffled border and between the cytoplasmic processes of the ruffled border, crystals can be seen[4, 7, 14, 20, 25, 26, 59, 64] (Fig. 4). Schenk[59] noticed crystals only when phosphate buffer was used in the fixative, and not with cacodylate buffer. Furthermore, when tissue is preserved using aqueous solutions and the sections are collected from the surface of water,

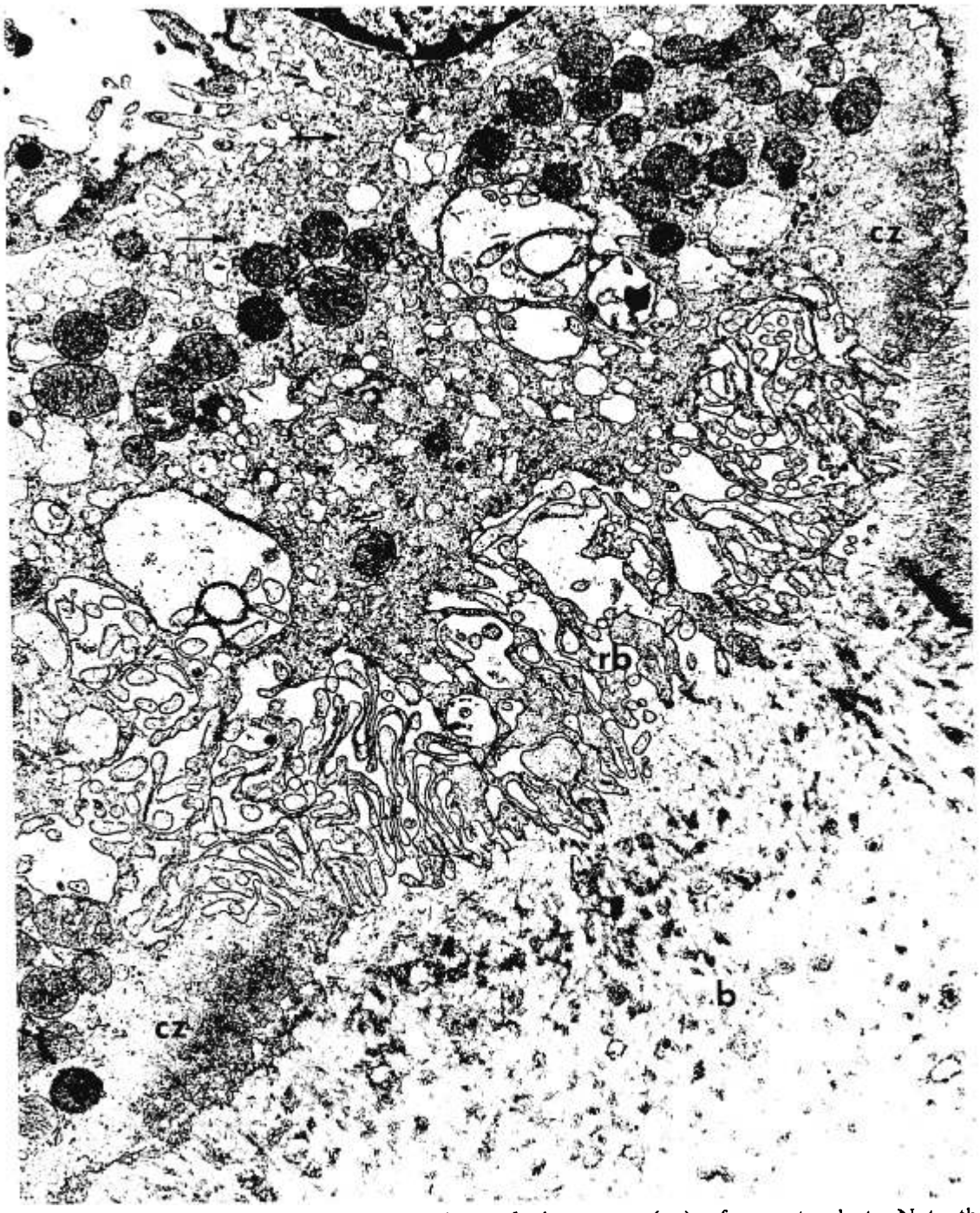

FIG. 2. Detail of the ruffled border (rb) and clear zone (cz) of an osteoclast. Note the abundance of mitochondria (m) and the clusters of free ribosomes (arrows). The bone (b) is demineralized. ×8,500

not only is relocation of calcium possible, but also a change of phase from amorphous to crystalline has to be considered. As a result no conclusions can be made about location or phase of the mineral using such standard techniques. Unmineralized col-

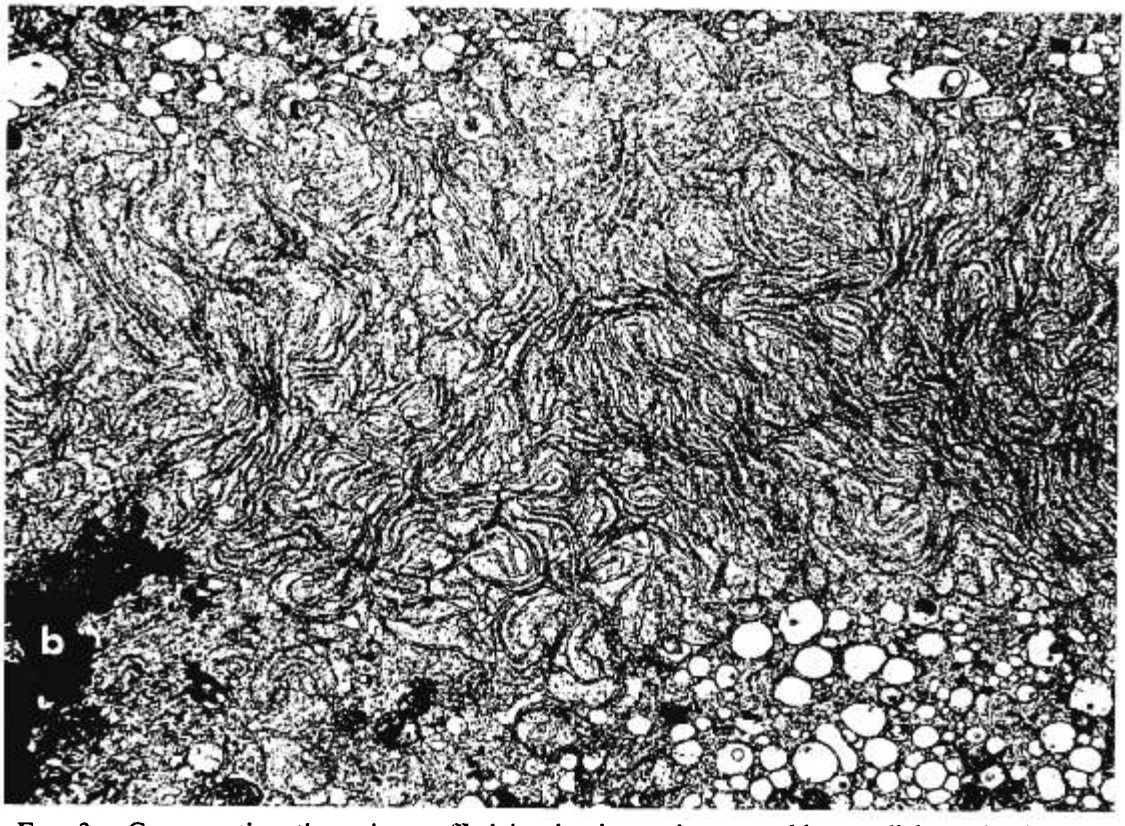

FIG. 3. Cross section through a ruffled border in a plane roughly parallel to the bone (b) surface. ×10,600

lagen fibers have been seen by some workers[7, 25, 26] but not by others[14, 20, 64] This difference is probably due to variations in techniques of preservation. Figure 4 shows some collagen fibers that are partly demineralized and others that are completely exposed. Since this tissue was processed in aqueous solutions, no conclusions about the degree of mineralization can be made. In sections that were decalcified by flotation on formic acid, Bonucci[4] noticed structures whose outline seemed similar to the inorganic crystals found in untreated sections, and named these "crystal ghosts." From their staining properties he concluded that these crystal ghosts consisted of proteoglycans that probably formed the organic component of the crystal and was usually found in the ground substance of the matrix.

The architecture of the ruffled border with its many projections provides an extended surface area ideally suited for intensive exchange between the cell and the extracellular space. However, exactly what is transported and in which direction is still not clear. Certainly, excretion of some agents is required to effectuate the dissolution of the bone matrix. However, unanswered questions remain concerning the nature and degree of lysis occurring extracellularly and the fate of the breakdown products. Are these products transported through the cell to the extracellular space and possibly altered en route, or are they allowed to reach the extracellular space directly through the space between cell and bone surface? Morphologically the ruffled border membrane is different from the rest of the plasma membrane. Kallio *et al.*[29] described a coating of the membrane with

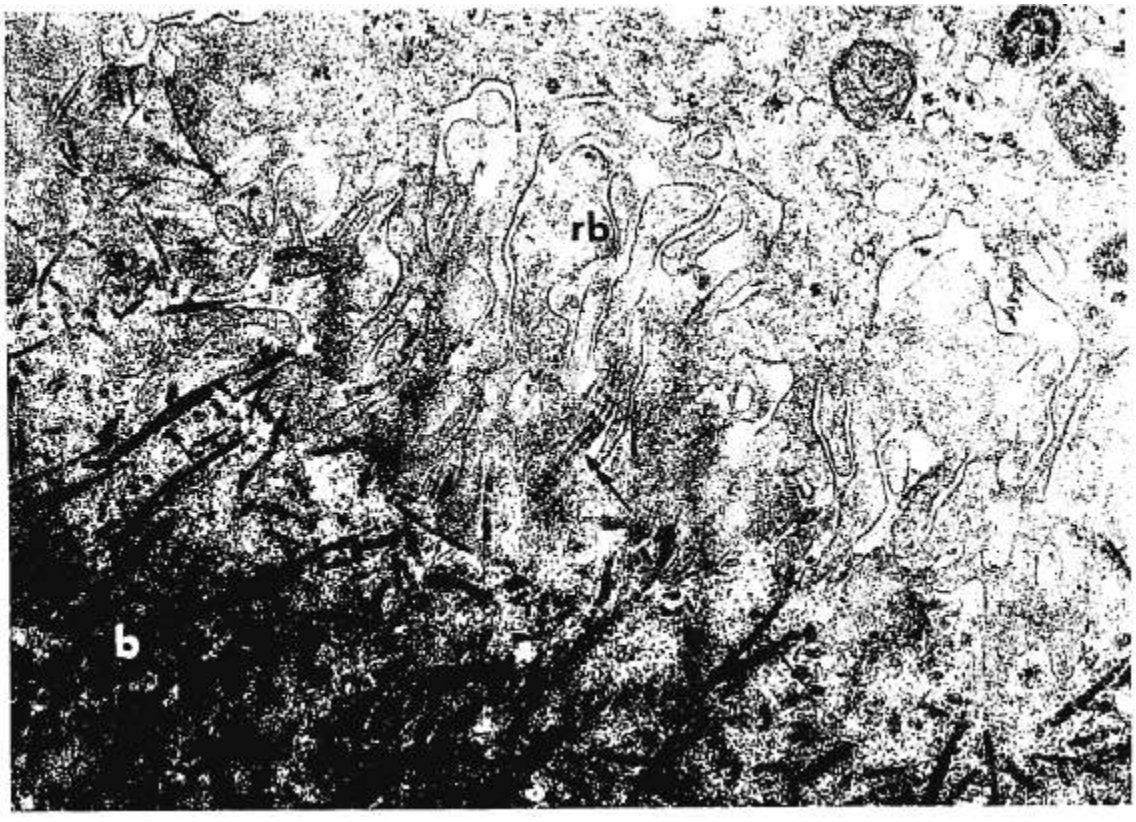

FIG. 4. Detail of the bone (b)—ruffled border (rb) interface. Some collagen fibers are partly demineralized (arrow) and others are completely exposed (double arrow). Bone crystals can be seen between the cytoplasmic processes. ×28,000

fine bristle-like structures, evenly spaced 200–250Å apart and projecting perpendicularly into the cytoplasm and speculated that this may represent sites of enzymatic activity related to the demineralization of bone.

There are many indications that the ruffled border membrane is the site of active secretion as well as uptake. By means of histochemistry, the reaction products of several enzymes have been demonstrated along the ruffled border membrane, and between the membrane and the bone, whereas these enzymes were not found at other sites along the cell membrane. ATPase activity was found in great quantity in this area, as well as in structures similar to lysosomes.[13, 23, 37] Part of the ATPase activity in the ruffled border appeared Mg^{++} dependent. This same type of enzyme activity was also found on the cell membrane of matrix producing cells, such as osteoblasts and fibroblasts. Göthlin[23] therefore suggested that this Mg^{++} dependent enzyme may be governing energy-consuming transport functions, as in the brush border of the kidney, whereas the Mg^{++} independent ATPase found in the ruffled border is of the same type as found in lysosomes. There is some controversy about the presence of Mg^{++} independent ATPase in the ruffled border. Doty[13] stated that the ATPase along the cell membrane was Mg^{++} dependent whereas Göthlin found that the bulk of the ATPase in the ruffled border region is Mg^{++} independent, as in lysosomes, and that the precipitate was only slightly diminished when Mg^{++} was omitted from the incubation medium.

Another enzyme demonstrated histo-

chemically in the ruffled border area is acid phosphatase. Here again the results obtained by different investigators are not compatible. Reaction product was found in the ruffled border channels by Lucht[37] and Schofield,[61] but not by Göthlin[21] and Thyberg.[68] Göthlin[21] suggested that differences may be explained by different species and tissues, but also by differences in techniques, as for instance the concentration of the decalcifying agent EDTA and the time exposed to it. Decalcification is needed since the Gomori technique for the demonstration of acid phosphatase does not work in mineralized bone.[37] From biochemical studies Ericsson and Göthlin[15] concluded that EDTA did not inhibit activity of acid phosphatase in unfixed tissues, but did cause partial release of the enzyme from the lysosomes, whereas in properly fixed tissues no release of acid phosphatase was observed. Hence, besides the concentration of EDTA, the degree of fixation in glutaraldehyde before decalcification and incubation with substrate seems to be a critical factor in the procedure. Thyberg[68] sometimes found reaction product on the lamina limitans of the bone matrix, but considered this non-specific. This controversy demonstrates how critical the histochemical procedures are and how cautiously one should evaluate the results.

Other indications for enzyme secretion are the release of collagenase[57, 58] and lysosomal acid hydrolases[70] into the medium of cultivated bones. It is likely that the enzymes originated mainly from osteoclasts and not so much from other cells present, since in these culture systems osteoclasts are morphologically the most differentiated and viable cells present. Interesting information has come from studies on the ia rat, an osteopetrotic strain that shows a defect in the bone resorptive process. It appears that the osteoclasts in these animals fail to resorb bone. Schofield *et al.*[61] noticed that these cells lacked ruffled borders. Histochemically the cells showed greater than normal lysosomal enzyme activity but enzyme release could not be demonstrated.[47, 61] Therefore it was concluded that the ruffled border is a prerequisite for lysosomal enzyme release.

When deciding whether uptake of material takes place by the ruffled border membrane one has to distinguish between ruffled border channels (*e.g.* extracellular space) and vacuoles inside the cell. An electron micrograph has limits in that it only represents a two dimensional profile of three dimensional structures. Hence structures that appear as a vacuole may well be in connection with extracellular space in another plane of section. This is well demonstrated by Lucht[38] who used lanthanum as an extracellular marker in bone tissue. The tracer was added to the fixative so that cell activity could be ruled out. In cross sections of osteoclasts the lanthanum was present in many vacuole-like profiles, some of which were located deep in the cytoplasm centrally to the ruffled border and behind the clear zone. The presence of the tracer identified these structures as deep invaginations of the cell membrane instead of vacuoles.

Indications for the uptake of material by the ruffled border membrane has been provided by several investigators. Tracers, such as peroxidase,[40] thorium dioxide[22, 67] and ferritin,[67] have been found between ruffled border and bone as well as in increasing amounts inside the cell. Uptake of ferritin as well as thorium dioxide[22, 67] was noticed also in other cell types and is therefore not specific for the ruffled border membrane. It has been suggested that other parts of the osteoclast membrane may also have endocytotic ability, such as the clear zone membrane[22] or even the entire cell membrane.[40, 67] Lucht[38] observed coated invaginations along the cell membrane and some small coated vacuoles nearby in the cytoplasm. Coated pits are generally considered the beginning of the formation of a pinocytotic vesicle.[11]

THE CLEAR ZONE

The clear zone has often been neglected or even completely overlooked in descriptions of the ultrastructure of the osteoclast. It seems however that this area is as much part of the osteoclast as is the ruffled border. Various names have been attached to this area, *e.g.* transitional zone,[38, 39, 64] ectoplasmic layer,[22, 62] granular zone,[7] clear zone[59] or contact zone.[46] "Clear zone" seems to be the term that is most widely accepted. This area of the cytoplasm is located adjacent to the ruffled border along the cell membrane at the bone surface. Along this zone the cell membrane is smooth and follows the contour of the bone. The bone surface is unaltered and the lamina limitans, if present, is intact.[46] In sections through osteoclasts the clear zone is always present whenever a ruffled border is included and can always be found on either side of the ruffled border.[28] If this information from two dimensional profiles is converted into a three dimensional representation one can infer that the clear zone encircles the ruffled border completely. Not infrequently cell profiles are seen with clear zones and no ruffled border. The ruffled border may be present in another plane of section, but it is also possible that clear zones exist without ruffled borders.

At low magnification the clear zone appears to consist of an amorphous, finely granular material devoid of any specific cell organelles such as mitochondria, endoplasmic reticulum or ribosomes. Vesicles are usually not present either, although sometimes a few small vesicles can be found. Serial sectioning would be needed to know whether or not these vesicles are in communication with the ruffled border or the extracellular space.

At higher magnification and with optimal preservation of the cells more detailed structure can be recognized. Malkani *et al.*[46] described parallel dark bands, or electron dense material in a loose network and Lucht[39] distinguished numerous thin filaments with a diameter of 50–100Å, which were closely packed and randomly oriented. King and Holtrop[31] noticed that the darker bands are directed perpendicular to the bone surface, usually ending in short processes that extend into indentations of the bone surface, and that in a favorable plane of section these dark bands consist of bundles of filaments with a diameter of 50–70Å (Fig. 5). They demonstrated that these filaments bind specifically to heavy meromyosin and hence can be considered actin-like. The fine granular material through which these bundles pass is morphologically very similar to the material in the cytoplasmic extensions of the ruffled border. King and Holtrop[31] have suggested that this amorphous material could represent g-actin, stored to be transformed into filamentous actin when needed.

The more detailed structure of the clear zone makes speculation of its function more meaningful. Actin-like filaments are found in many non-muscle cells but their function is not yet fully understood. King and Holtrop[31] pointed out the similarity between clear zones and microfilament concentrations in fibroblasts at the point of contact with another cell or with a substrate. Although a fully developed clear zone is much more extensive than the filamentous zones in fibroblasts, the areas are quite comparable in the early development of the clear zone. In fibroblasts these microfilament concentrations are thought to represent cell-cell or cell-substrate adhesions[24] and, by analogy, it has been proposed that the function of the clear zone may be adhesion to the bone surface.[31] The observation that the filament bundles in the clear zone often extend into little indentations of the bone surface, like fingers in a crack of a rock, certainly supports this idea. From microcinematography it is known that the ruffled border is highly motile so that an attachment of encircling clear zone to the bone would give support

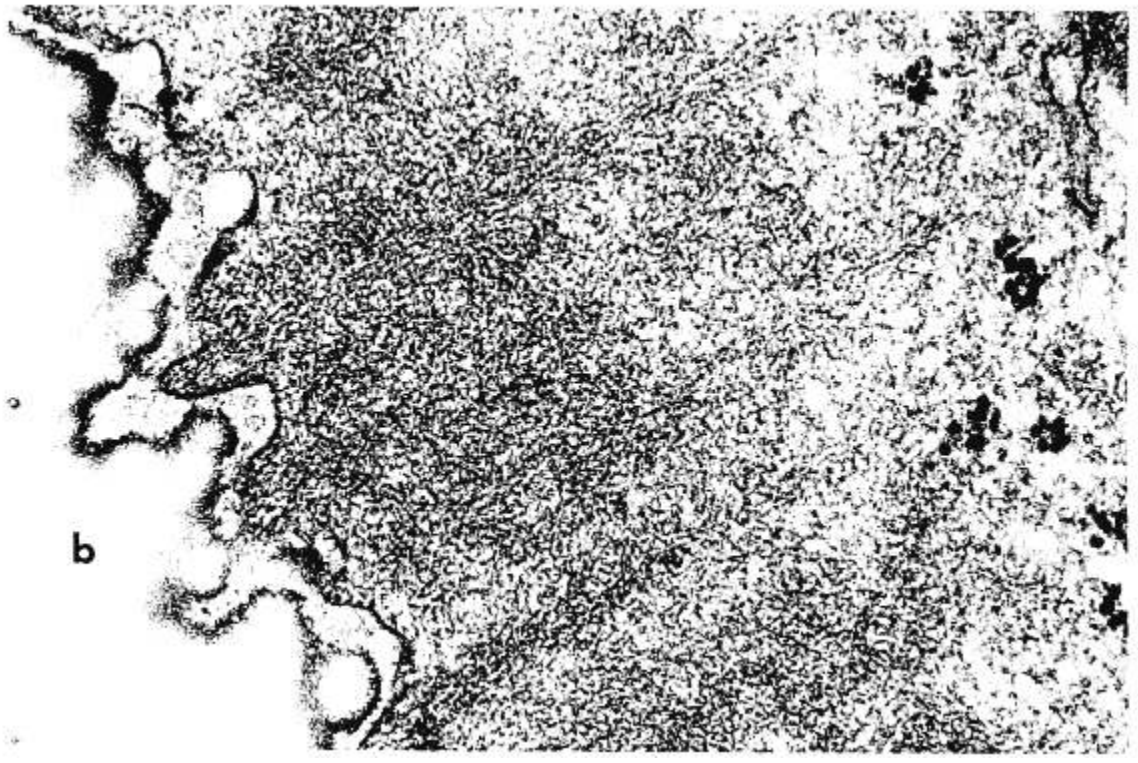

FIG. 5. Clear zone with bundles of filaments perpendicular to the bone surface. Note how these bundles end in short cell processes extending into indentations of the bone surface. The bone (b) is demineralized. (Micrograph courtesy of B. Landis.) ×80,750

and stability to the actively resorbing area.[39] Schenk[59] proposed that the attachment of the clear zone to the bone could be so tight that it would seal off the area under the ruffled border from the extracellular space, thus providing and maintaining a microenvironment in the zone of resorption. This would allow lysosomal enzymes, secreted by the cell into the resorption area under the ruffled border, to function at the site needed without seepage, and would maintain the supposedly acid environment thought to be necessary for removal of the mineral from the matrix. However, even with the best preservation, spaces can be seen with the electron microscope between the cell membrane and the bone surface. Moreover, when lanthanum is added to the fixative, this electron dense marker can be found between the clear zone and the bone and under the ruffled border.[38] It is, however, possible that the cells had retracted somewhat from the bone at the moment they came in contact with the fixative, so that the lanthanum could get in between the clear zone and the bone surface. In another study the extracellular marker thorium dioxide was injected and was found in the channels of the ruffled border after 9 hours.[22] During that time the osteoclasts studied could very well have moved to a piece of bone that had the marker attached to its surface. However, peroxidase was seen in the channels of the ruffled border as early as 5 minutes after injection[40] and this observation is so far the clearest indication that the clear zone does not form a seal but that indeed a space exists between clear zone and bone surface.

Malkani[46] sees the clear zone as an extension of the ruffled border and suggests that it can progressively develop into a

ruffled border. The amorphous fine granular material in the clear zone can indeed not be distinguished from the material within the cytoplasmic evaginations of the ruffled border.[29]

Osteoclasts are motile cells and after they have resorbed bone they will retreat from the bone surface, move to another site and start resorbing again. This can clearly be seen in time-lapse motion pictures of cultured bones.[18, 19, 25] It is not known what happens to the ruffled border and the clear zone when the cell detaches. We have recorded thousands of osteoclast profiles[28, 32, 33] and have not seen osteoclasts away from the bone surface showing ruffled border or clear zone. Malkani[46] reported that clear zones are visible in certain osteoclasts not in contact with calcified tissue and showed a micrograph of a disrupted cell with a clear zone split in two parts. This cell might have just left the bone surface.

The way the osteoclast detaches itself from the bone may well be so fast that it has not been captured or has not been recognized in electron micrographs.

VACUOLES

Numerous vacuoles, vesicles and cytoplasmic bodies are found in the osteoclast. Many descriptions are given of these structures and a variety of names are attached to them, reflecting the lack of understanding about their origin, contents, function and fate.

Scott[62] noted a great variety in number of vacuoles in the cytoplasm of different osteoclasts and concluded that the cell goes through cyclic variations of activity.

The largest and most numerous vacuoles, vesicles and bodies are found close to the ruffled border,[39] suggesting a functional relationship to the ruffled border. Of course, many of these vacuole-like profiles may be in contact with extracellular space in another plane of section and hence not be true vacuoles.[38]

The term vacuole is usually reserved for the larger membrane bounded bodies that appear largely empty.[38, 62] They measure between 0.03μ and 5μ in diameter.[38] Their membrane, like the ruffled border membrane, has a diameter of roughly 90Å[38, 68] and is coated[12, 29, 38, 67, 68] Coated vesicles are generally the result of invagination of part of the cell membrane and are believed to be specialized for the cellular uptake of protein. These can then fuse to form larger vacuoles usually referred to as phagosomes.[11] Studies with the uptake of tracers demonstrate that at least some of these vacuoles can be considered phagosomes. Peroxidase, injected intravenously into young rats[40] was located after 5 minutes around the osteoclast between the ruffled border and the bone and also inside some cytoplasmic vacuoles. After 40 minutes the tracer was still found in a high concentration subjacent to the ruffled border, but also in many vacuoles throughout the cell. Ferritin added to cultures of bone metaphysis was found mainly in coated vesicles after 1–2 hours and appeared in increasing amount in vacuoles inside the cell after 4–6 hours.[67]

The mechanism of uptake by the ruffled border membrane is not known. Pinocytotic vesicles have not been observed. Possibly the very end of a ruffled border channel pinches off to form a vacuole. The fate of the ingested material is not known either. Thorium dioxide was located in the channels of the ruffled border as well as in vacuoles and cytoplasmic bodies at 9 hours,[22] one day and four days[67] after intravenous injection, but no indication of excretion of the tracer was found. It is surprising that after that length of time tracer was still found in the channels of the ruffled border. However, osteoclasts do not stay at one site on the bone surface for very long.[18, 19] They move around to other sites on the bone where tracer may still have been present the entire length of the experiment.

Even though the evidence for endocytosis at the ruffled border is convincing, the question still remains whether hydroxyapatite crystals, freed from the bone matrix, are taken up by the osteoclast, transported through the cell and secreted into extracellular space. Another question concerns whether the components of the bone matrix are taken up and internally digested before being excreted. Electron micrographs suggest that crystals are taken up by the cell (Fig. 4).[16, 29, 62] Lucht[38] pointed out that vacuoles containing crystals never contain the extracellular marker lanthanum, and hence can be considered true vacuoles. However, Göthlin[22] did not notice crystals in vacuoles that had taken up thorium dioxide from the extracellular space, but these vacuoles may have been invaginations instead of true vacuoles. These studies were done using conventional techniques for preparing the tissue for electron microscopy, and relocation of calcium during the procedure may have been a factor. Conclusive evidence would have to come from a study using non-aqueous techniques.

Vacuoles may be involved in exocytosis as well as endocytosis. In bone cultures stimulated to resorb by the addition of parathyroid hormone to the medium, increased secretion of six lysosomal hydrolases was demonstrated.[70] Lucht[38] distinguished vacuoles with a coating and vacuoles without. Coated vacuoles are presumably involved in the uptake of proteins. The vacuoles without a coating may be involved in the secretion of products, such as lysosomal enzymes, at the ruffled border site.

A variety of membrane bounded structures other than vacuoles can be found in the cytoplasm of osteoclasts. These are smaller than vacuoles, 0.02–3μ in diameter, and have an internal structure or a stainable matrix of varying density.[38] A clear distinction from vacuoles cannot be made and intermediate forms can be found.[38, 62] The many appearances promoted a variety of names, such as: vesicles containing electron opaque material,[59] specific granules,[62] dense bodies,[17] dense granules,[16] cytoplasmic dense bodies,[22, 68] cytoplasmic bodies[38] and lysosomes.[78] Lucht[38] distinguished (a) light cytoplasmic bodies, (b) dense cytoplasmic bodies, (c) coated cytoplasmic bodies, and (d) cytoplasmic bodies with inclusions. It is clear from this list that the function of these cytoplasmic bodies is not well understood.

Some bodies and small uncoated vacuoles[38] seem to have an association with the Golgi apparatus and it has been suggested that these may be lysosomes[38, 59, 62] Acid phosphatase, which is indicative for lysosomes, has been demonstrated histochemically inside cytoplasmic bodies,[6, 13, 21, 37, 68, 71] as well as in the Golgi apparatus[13, 21, 37] and in vacuoles.[21, 37, 68] Another lysosomal enzyme found in bodies in the cytoplasm is aryl sulfatase.[68] In addition to lysosomal acid hydrolases, osteoclasts also contain neutral hydrolytic enzymes in coated and in uncoated small vacuoles close to the ruffled border[12] and a Mg^{++} independent ATPase[12, 23] in bodies morphologically indistinguishable from lysosomes.

Lysosomes may follow one of two pathways: (1) fusion with phagosomes and/or (2) secretion out of the cell. Fusion with phagosomes results in the formation of secondary lysosomes in which ingested material is digested inside the cell.[11] We have seen earlier that the cell is capable of endocytosis of exogenous markers such as peroxidase, lanthanum and ferritin and consequently probably matrix components as well. An indication that internal digestion indeed takes place is the presence of residual bodies in the osteoclast.[38, 62] These are membrane bounded acid phosphatase negative structures containing electron dense material of various shapes that probably represent indigestible products.

Lysosomal enzymes may also be secreted into the space between the ruffled border and bone. Release of lysosomal acid hydrolases[70] as well as collagenase[57, 58, 65] into the medium of cultured bones after admin-

istration of parathyroid hormone indicates that at least part of the degradation of bone matrix occurs outside the cell. Furthermore, reaction product to various lysosomal enzymes has been demonstrated histochemically between the ruffled border and the bone surface.[37, 61] Secretion of lysosomal enzymes seems to be a prerequisite for bone resorption. In ia rats, an osteopetrotic strain characterized by a lack of bone resorption, accumulation of lysosomal enzymes can be found only inside the cell and is absent outside the cell in the ruffled border area.[47, 61] It has been suggested that lysosomal enzymes cannot be secreted because ruffled borders are not formed, and that consequently bone resorption is impaired.[61]

The many studies on the phagocytic and secretory system of the osteoclast suggest that bone mineral and bone matrix are separated and the components partially degraded outside the cell, followed by phagocytosis and further digestion inside the cell.

OTHER CELL ORGANELLES

It has always been assumed that the osteoclast is a multinuclear cell and the existence of mononuclear osteoclasts capable of resorbing bone has been doubted. Serial sectioning at the light microscopic level has not clarified this issue. Utilizing the technique of interval serial sectioning whereby ultrathin sections were obtained at intervals of 2.5μ, Lucht[39] was able to distinguish cells with the characteristic cytoplasmic features of osteoclasts containing only one nucleus. These cells did not have a ruffled border and he suggested that they were small inactive osteoclasts. It is also possible that these cells were preosteoclasts as described by Scott[63] and Luk *et al.*[44, 45]

There are numerous mitochondria in the osteoclast whose function is poorly understood. Occasionally electron dense granules, consisting of clusters of finer particles, have been observed in these mitochondria. These were found using conventional fixation techniques[1, 10, 20] and also after fixation in anhydrous organic solvents.[35] Lucht[43] noticed these granules in osteoclasts from rats treated with high doses of parathyroid extract and provided evidence by means of X-ray analysis that they contained calcium and probably phosphorus. These observations have led to the theory that mitochondria may be functioning as sites of storage of calcium that may be transported from the bone surface to the extracellular fluid.[36]

Multiple centrioles have been reported usually located in a cluster close to the Golgi complex.[8, 41, 48] They have the same structure as those in other cell types. It has been suggested that there is one pair of centrioles for each nucleus in the osteoclast[48] and that these are derived from a pooling of centrioles from mononuclear precursors.[41]

Microtubules are present in the cytoplasm of the osteoclast with no apparent preferential orientation,[28, 41] although Lucht[41] noticed that they are sometimes especially numerous in regions close to the centrioles. When microtubules are disaggregated by the drug colchicine, bone resorption induced in cultured bones by parathyroid hormone is inhibited.[54] Osteoclasts in such cultures showed an accumulation of filaments with a diameter of 100Å that are thought to represent assemblies of microtubules.[28] These data suggest that microtubules are somehow involved in the resorptive function of the cell. They may play a role in the secretion of lysosomal enzymes, as has been suggested in human leukocytes.[81] On the other hand, the frequency of ruffled borders in osteoclast profiles in cultured bones that were stimulated by parathyroid hormone, decreased significantly after one hour of colchicine treatment to 19 per cent as compared to 68 per cent in the controls.[28] This effect was very similar to the effect of treatment with calcitonin.[28] This may indicate that microtubules

are involved in the formation of the ruffled border. Whether this effect is primary or secondary is not at all understood.

THE INFLUENCE OF HORMONES ON OSTEOCLASTS

Descriptive Studies

It has been known for some time that parathyroid hormone (PTH) increases the number of osteoclasts after about 18 hours.[3, 69] More recent studies indicate that the hormone may increase individual cell activity more rapidly than the increase in cell number. In a careful autoradiographic study[3] enhanced nuclear RNA synthesis could be demonstrated as soon as 90 minutes after exposure to parathyroid extract. This was followed by increased production of cytoplasmic RNA reaching a maximum after 7–12 hours. Moreover, the transmembrane potential of osteoclasts in culture was shown to change rapidly in response to PTH and was followed over a longer period by an increased rate of RNA synthesis.[49]

Cameron[9] was the first to investigate the effect of PTH on the ultrastructure of osteoclasts. He injected rats repeatedly with very high doses of parathyroid extract but found that the osteoclasts were little different from those in normal animals. The main change was swelling of the mitochondria, which could have been attributed to damage of the cells by the high doses of the hormone. In another quite similar study[43] rats were injected with high doses of parathyroid extract in one to three doses over a period of 12 hours, and the osteoclasts were examined 12–24 hours after the first injection. The most obvious change seemed to be a stimulation of the macrophage-like function and the lysosomal system. Large vacuoles, positive for acid phosphatase, were seen containing phagocytosed cells, some of which resembled osteoblasts and osteocytes. In addition, an increase was noted in small coated cytoplasmic bodies and large bodies with dense inclusions. Another change was the occurrence of granules containing calcium in the mitochondria. Occasionally membranes of the endoplasmic reticulum were fused, the significance of which is not understood. The ruffled borders did not differ in extent or appearance from those in control osteoclasts. In an electron microscopic cytochemical study, acid phosphatase and adenosine triphosphatase activity were both increased 6 hours after injection of rats with parathyroid extract.[13] However, acid phosphatase was not increased in isolated osteoclasts 13–21 hours after exposure to parathyroid extract.[71] Moreover, no morphological changes were noted in osteoclasts after exposure to PTH for 8 hours in rabbits[51] or 15–16 hours in the deciduous teeth of puppies.[17] In all these studies intact animals were used and hence endogenous sources of PTH and calcitonin were not eliminated. Weisbrode[77] examined the bone cells in rats that were thyroparathyroidectomized. High doses of parathyroid extract were given twice daily for a period of 7 days and during that time the rats were fed a low calcium, vitamin D-deficient diet. In another very similar experiment, the rats received, in addition to the parathyroid extract, high doses of vitamin D_3 daily.[75] In both experiments osteoclasts seemed more numerous, but no ultrastructural changes were noticed.

The inability of many investigators to demonstrate consistent morphological changes in the osteoclast after exposure to PTH could be explained by the various methods applied. Impure extracts of bovine parathyroid gland, with variable specific activity were used; changes in blood calcium were usually not measured to insure biological activity of the hormone; experiments were terminated usually between 12 and 48 hours after injection of PTH, and these time intervals are very long in relation to the rapid changes in blood calcium.

Calcitonin is an inhibitor of bone resorption both *in vitro*[5] and *in vivo* (reviewed by Hirsch[27]). The effects of this hormone on the ultrastructure of osteoclasts *in vitro* as well as *in vivo* have been studied by several investigators. Kallio[30] cultured calvaria of 6-day-old mice in a system with a high intrinsic resorption. Ultrastructural changes seemed well underway at 15 minutes after adding salmon calcitonin to the medium and only one out of 10 cell profiles showed a ruffled border. In cultures that were not treated with the hormone all osteoclast profiles exhibited a ruffled border. At one hour after administration of the hormone the effects seemed maximal: no cell profiles showed ruffled borders, the membrane had lost its cytoplasmic coat, and former ruffled border areas now resembled clear zones. At 24 hours the osteoclasts seemed also smaller and many were separated from the bone.[30]

In vivo studies on the effect of calcitonin on osteoclasts are compatible with the *in vitro* findings. In rats, in which porcine calcitonin was administered continuously for 12 hours up to 4 weeks, osteoclasts were often separated from the bone and ruffled borders were lacking.[79, 80] Short term effects of calcitonin on osteoclasts[42] were seen at 1½ hours after injection of the hormone in rats and were maintained after 12 hours. A total of 100 osteoclast profiles from calcitonin-treated animals were examined and none showed ruffled borders, whereas in control osteoclasts the majority showed ruffled borders. Instead, intermediate forms between the typical ruffled border and clear zone were noticed. Vacuoles seemed to be diminished in number, and no bone crystals were found within these structures. However, autophagic vacuoles seemed more numerous. No acid phosphatase activity could be demonstrated between the osteoclast and the bone.

The effect of calcitonin on rabbits seems very similar: a loss of ruffled borders was noticed that seemed to start after 15 minutes of injection of the hormone and seemed maximal after 30–60 minutes.[50]

In all these *in vivo* studies the thyroids and parathyroids were left intact, so that the effects on osteoclasts were not necessarily the effects of the hormone proper. However, similar results were obtained in thyroparathyroidectomized rats that were stimulated to resorb bone by administration of large doses of vitamin D_3 orally: the osteoclasts in the calcitonin treated animals lacked ruffled borders, and also lacked clear zones.[73] No differences were found in vesicles or mitochondria.

The action of vitamin D on bone is not fully understood. Administration of the active metabolite $1,25(OH)_2D_3$ to vitamin D-deficient rats on a low calcium diet increased their serum calcium concentration, implying bone mineral mobilization.[66] In tissue culture $1,25(OH)_2D_3$ is a potent stimulator of osteoclastic bone resorption.[55, 56] The ultrastructure of osteoclasts was studied in thyroparathyroidectomized rats after receiving normal and high doses of vitamin D_3 orally for 7 days.[74, 76] Osteoclasts seemed more numerous after high doses of vitamin D_3, but no alterations were found in the ultrastructural features of the cell, nor in ATPase activity.

Morphometric Studies

The inconsistency of results between various investigators with respect to the effects of hormones on osteoclasts can readily be attributed to the evaluation of the tissue by means of descriptive electron microscopy. Information is derived from cell profiles which represent only a minute portion of the cell and do not represent the appearance and contents of an entire cell (Fig. 6). Within one population of cells, cell profiles will show a great variability dependent on the distribution of the cell contents in the cytoplasm and the plane of section. Even if all cells in a population

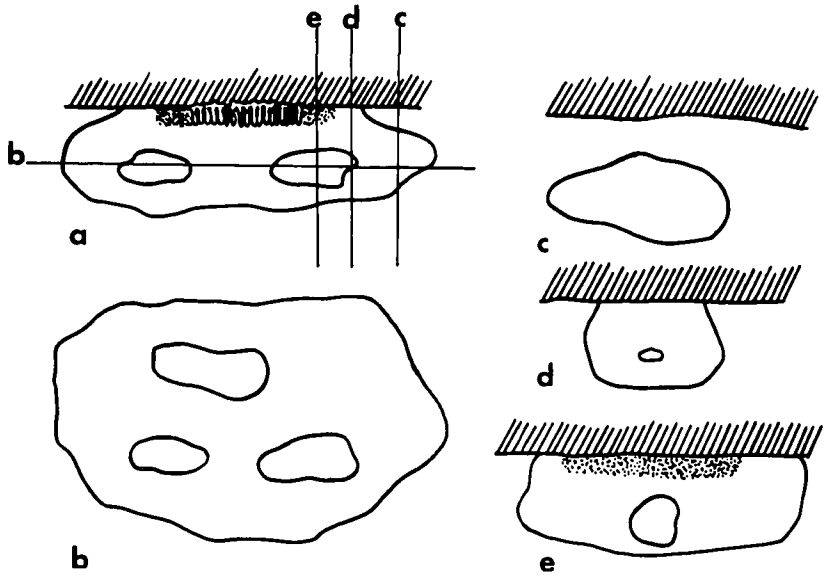

FIG. 6. Diagram illustrating the variability in cell profiles as a result of the plane of sectioning through an osteoclast. An osteoclast on the bone surface, with a ruffled border and clear zone present, may be represented in a section by any of the profiles a, b, c, d or e.

were identical, a variability would show up in the cell profiles. The normal biological variability between cells in a population also causes a variability in cell profiles, which is superimposed on the variability due to the plane of sectioning. In addition, viewing tissue in the electron microscope is time consuming, and osteoclasts are sparsely distributed, so that only few osteoclast profiles can be observed in a given time. From this it may become clear that subtle differences between two populations of cells as represented in their cell profiles are easily masked by the great variability in profiles within either population. In order to deal with these problems, careful random sampling of cell profiles and morphometric methods have to be employed. The variability of cell profiles within one population will then be expressed in the variance of the mean (standard deviation or standard error) and the actual differences between two populations under different circumstances can be assessed by statistical tests.

A quantitative method was applied in a study of PTH on the ultrastructure of osteoclasts in cultured bones.[28] In randomly selected sections of bone the number of osteoclast profiles showing a ruffled border or a clear zone were registered. A larger cell organelle will be represented more frequently in randomly selected cell profiles than a smaller cell organelle, so that the frequency of finding a cell organelle in cell profiles is directly related to its size. It was found that treatment of long bones from rat embryos with purified parathyroid hormone for 48 hours increased the frequency of ruffled borders in osteoclast profiles significantly to 64 per cent as compared to 11 per cent in profiles of untreated osteoclasts. Cell profiles with clear zones were found in 79 per cent of the treated cells as compared to 55 per cent of the controls. Thus, PTH increased the size of ruffled borders as well as clear zones.

In a subsequent study,[32] the areas occupied by cytoplasm, ruffled borders and clear zones were measured. The mean area in randomly selected profiles of objects of a certain type has a direct relationship to the mean volume of those objects.[72] In other words, if the mean area of profiles of cells or cell organelles in one population is larger than in another population, this is also true for the mean volume of these cells or cell organelles. In cultured long bones from 19-day-old fetal rats treated with parathyroid hor-

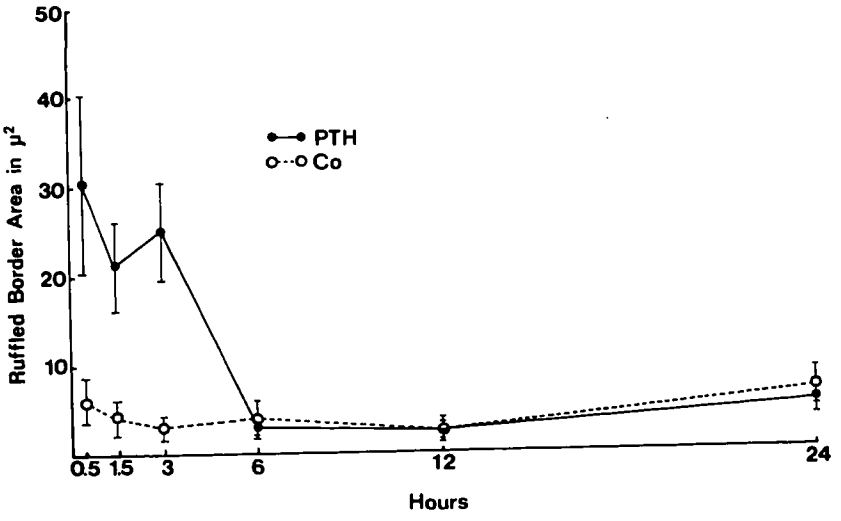

FIG. 7. Size of the ruffled borders in osteoclast profiles at various time intervals after injection of parathyroid hormone in thyroidparathyroid - ectomized rats. Each point represents the mean ruffled border area of 24-26 cell profiles with the standard errors indicated by vertical bars.

mone for various periods, a significant increase in cell and clear zone sizes, as compared to controls, could be demonstrated after three hours, and a significant increase in ruffled border sizes after 6 hours. This correlated well with a significant increase in the release of calcium into the medium by 6 hours.[32] The effects of parathyroid hormone on osteoclasts *in vivo* were seen even sooner[33]: PTH given to thyroparathyroidectomized rats increased the ruffled border and clear zone area significantly as early as 30 minutes (Figs. 7 and 8) and the cytoplasmic area at 1.5 hours (Fig. 9). These findings suggest that osteoclasts can modulate very rapidly from a resting phase to an active phase.

In another study[28] the effects of salmon calcitonin on osteoclasts were examined using cultured long bones from 19-day-old rat fetuses that were stimulated to resorb by PTH for 48 hours. The frequency of the presence of ruffled borders and clear zones in osteoclast profiles was determined in randomly selected sections of the cultured bones. In osteoclasts pretreated with PTH, 68 per cent of the profiles showed ruffled borders. One hour after addition of calci-

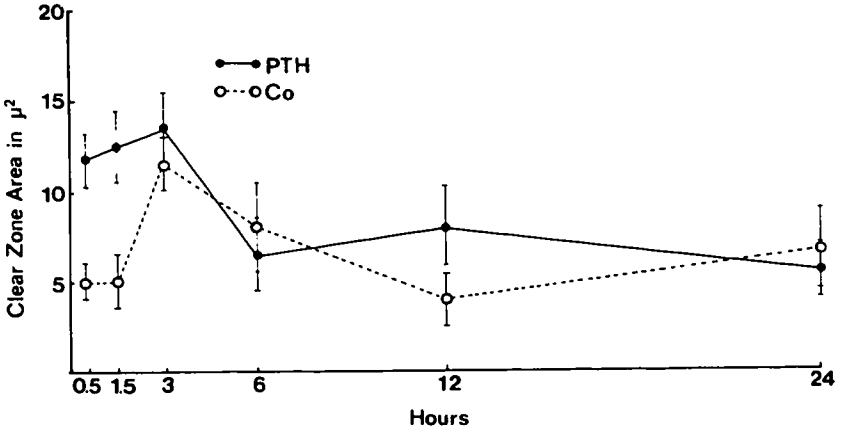

FIG. 8. Size of the clear zones in osteoclast profiles in the same cell profiles as represented in Fig. 7.

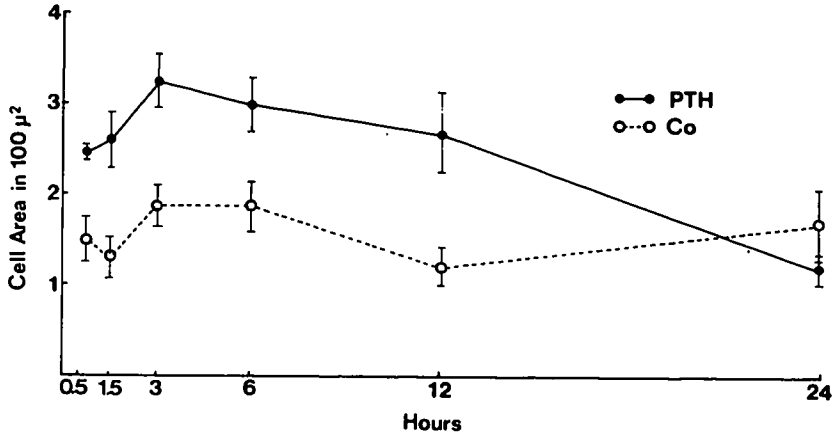

FIG. 9. Size of cytoplasmic area in osteoclast profiles in the same cell profiles as represented in Figs. 7 and 8.

tonin to the medium, only 29 per cent of the profiles showed ruffled borders, whereas osteoclasts that had not been stimulated by PTH showed a frequency of 11 per cent ruffled borders. After four hours of calcitonin treatment the frequency of ruffled borders had decreased to 10 per cent, the same value as found in unstimulated, untreated osteoclasts, as opposed to 58 per cent in osteoclasts stimulated by PTH. The frequency of clear zones in profiles of osteoclasts at the two time points was 77 per cent and 78 per cent in PTH stimulated cells, and 58 per cent and 57 per cent in unstimulated cells. After one hour treatment of stimulated cells with calcitonin the frequency was 69 per cent and after four hours this decreased even further to 49 per cent. This indicates that not only ruffled borders, but also clear zones become smaller after exposure to calcitonin. Hence, ruffled borders do not transform into clear zones as was suggested by Kallio.[30] These data suggest that PTH causes a modulation of osteoclasts from a resting phase to an active phase and that calcitonin reverses this process.

These results indicate that the osteoclast may have a significant function in the control of calcium levels in the body fluids by means of a rapid increase in cell activity rather than an increase in cell number. It should be pointed out that these morphometric studies were done using young, growing rats and hence do not necessarily reflect the mechanism of calcium regulation in adult animals in which much fewer osteoclasts are present.

SUMMARY

Recent findings on the ultrastructure of the osteoclast indicate that special attention should be given to the ruffled border, clear zone, and the vacuoles and vesicles of the cell and their significance for the mechanism of breakdown of bone matrix. The ruffled border is seen as an extensive area of cell surface where secretion of enzymes as well as uptake of matrix components takes place. The clear zone encircles the ruffled border completely and thus forms an integral part of the resorbing apparatus. Vacuoles and vesicles are thought to secrete enzymes as well as take up extracellular material and possibly digest or transport these products in the cell. The changes that occur in the ultrastructure of the osteoclast after exposure to parathyroid hormone and calcitonin indicate an important role of the osteoclast in bone metabolism. The cell can increase its activity very rapidly in response to parathyroid hormone, and decrease its activity in response to calcitonin.

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