Calc. Tiss. Res. 16, 13—36 (1974)

The Organic-Inorganic Relationships in Bone Matrix Undergoing Osteoclastic Resorption

E. Bonucci

I° Istituto di Anatomia Patologica, University of Rome

Received October 18, 1973, accepted January 1, 1974

The organic-inorganic relationships in bone matrix undergoing osteoclastic resorption have been studied in rat tibial diaphyses using electron microscope techniques in an attempt to identify the steps of the resorption process. Results suggest that bone resorption occurs in two phases: the first, an extracellular phase, leads to bone matrix fragmentation and partial dissolution, and the second, an intracellular phase, to complete digestion of the breakdown products of the bone matrix. The first component of the bone matrix to be attacked by the osteoclast is the ground substance. This induces the release of the crystals lying between, and on, the collagen fibrils; any crystals lying within fibrils are released later, when the fibrils break up. As this stage proceeds, the collagen fibrils retain their normal intrinsic texture, but gradually loose their lateral aggregation, appearing as individual fibrils (some of them uncovered by crystals), mixed with fragments of fibrils and many free crystals. The loosened but otherwise structurally normal collagen fibrils, and their fragments, are strongly argyrophilic. Complete dissolution of the disaggregated fibrils occurs outside the cell, both in the resorption zone and in the initial portion of the channels of the ruffled border. The free crystals present in the resorption zone and those phagocytosed in cytoplasmic vacuoles are organic-inorganic structures, whose organic component (the crystal ghost) is, at least in part, of proteoglycan nature. Dissolution of inorganic material occurs within the cytoplasmic vacuoles of the osteoclasts. Results are viewed in relation to the process of bone resorption and, as far as crystal ghosts are concerned, to that of bone calcification. A tentative summary of the various steps involved in the mechanism of bone resorption is given.

Key words: Osteoclast — Bone — Resorption — Crystals — Organic-inorganic relationships.

Introduction

Although electron microscopy has yielded a great deal of information about the fine structure of the osteoclast [63, 23, 27, 30, 15, 31, 60, 62, 61, 36, 40, 17, 22, 28, 37, 41–3, 70, 44], a great deal remains to be discovered about the physiological processes operative during bone matrix resorption and degradation [69, 17, 29]. One of the main unresolved problems is which component of the matrix is first attacked and removed by osteoclasts.

Working from the finding that collagen fibrils deprived of some or all of their inorganic crystals can be observed near and between the cytoplasmic infoldings which form the 'ruffled' border of the osteoclast [30, 67, 15, 31, 16], it has been suggested that osteoclasts may first remove the inorganic substance, exposing decalcified collagen fibrils which can then be digested. Other investigations have failed to reveal decalcified collagen fibrils near the ruffled border; instead, free crystals are observed [63, 23, 27] and it appears possible that the organic sub-

For reprints: Dr. E. Bonucci, I° Istituto di Anatomia Patologica, Policlinico Umberto I°, Viale Regina Elena 324, 00161 Rome, Italy.

stance may be removed by osteoclasts before the removal of the inorganic substance.

Clearly, the mechanism of osteoclastic bone resorption cannot be fully understood as long as the process of bone matrix degradation is not understood in detail. Electron microscope investigations centered on the fine structure of the zone of osteoclastic resorption and on the organic-inorganic relationships within it can help to solve this problem. An electron microscope investigation has therefore been carried out on the periosteal bone of rat tibiae. The main aim was to study the fine structure of bone matrix undergoing osteoclastic resorption, the organic-inorganic relationships in it, and the fate of its components during osteoclastic activity.

Material and Methods

Small specimens of tibial diaphysis of three-day-old white rats were fixed for 1 h in cold 1% osmium tetroxide buffered to pH 7.2 with phosphate or cacodylate buffer. A few specimens were also fixed for 2 h in 4% formalin (prepared from paraformaldehyde) buffered at pH 7.2 with phosphate buffer; these specimens were then post-fixed for 1 h in 1% OsO_4 prepared as above. After fixation, all the specimens were briefly washed in the buffer and rinsed 3 times in distilled water. They were then dehydrated with acetone and embedded in Araldite.

Sections 0,5–1 μ thick were cut with a Porter-Blum ultramicrotome and were stained with Azure II-Methylene blue with the aim of localizing the areas undergoing osteoclastic resorption. Once an osteoclast was detected, the block was trimmed, the tissue area was reduced, and ultra-thin sections including the osteoclast were collected for electron microscope observations. To prevent decalcification, care was taken to collect the ultra-thin sections as soon as possible after they had been cut. Usually, they remained floating on distilled water for less than 3 min.

Because of the presence of central cores of calcified cartilage in the metaphyseal trabeculae, only the periosteal side of each specimen was chosen to ensure that the osteoclast examined was not reabsorbing bone and calcified cartilage at the same time. About one hundred osteoclasts were studied.

Ultra-thin sections were examined under the electron microscope (a) without any treatment, so as to observe the fine structure of the untreated mineral substance; (b) after "staining" with uranyl acetate and lead citrate, to study the relationships between mineral substance and organic components, especially those of the osteoclast; (c) after decalcification by a 30-min flotation of the sections on a 2% solution of formic acid, to study the structure of the resorption area after removal of the inorganic material; (d) after decalcification as in (c) and post-staining with uranium and lead, to study the structure of the unmasked organic matrix in the areas of resorption; (e) after decalcification as in (c) and post-staining with silver nitrate — methenamine (SNM) to ascertain whether argyrophilic structures were present in the same areas; (f) after decalcification as in (c) and post-staining with 1% phosphotungstic acid (PTA) in 0.1 N hydrochloric acid [46, 47], to determine whether proteoglycan material was present.

Selected area electron diffraction of the resorption zone was performed in untreated sections, in sections decalcified by formic acid and unstained, and in sections decalcified by formic acid and stained with uranyl acetate and lead citrate. Selected area electron diffraction was carried out at 80 kV and at a magnification of 22000 in a Siemens Elmiskop 1A electron microscope equipped with a circular field-limiting diaphragm of 50 μ. Calibration was obtained by selected area electron diffraction of magnesium oxide crystals.

To avoid acid-metal interaction and contamination of the ultrathin sections on the grids, both decalcification and staining were carried out on free floating sections [45].

Results

Because the fine structure of the osteoclast has already been described in detail [63, 23, 27, 30, 15, 31, 62, 36, 17], only the results relevant to the breakdown and resorption of the bone matrix are reported.

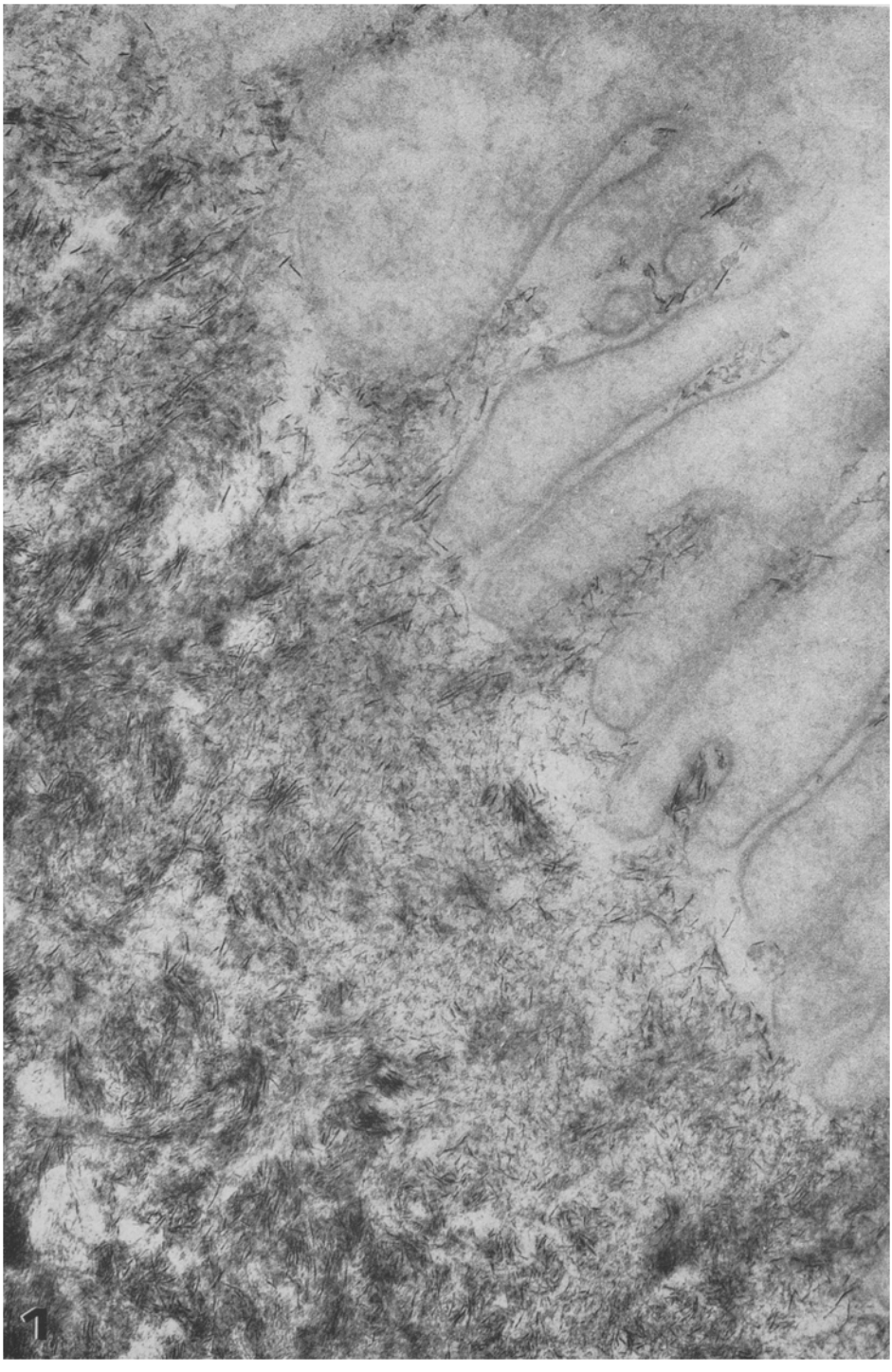
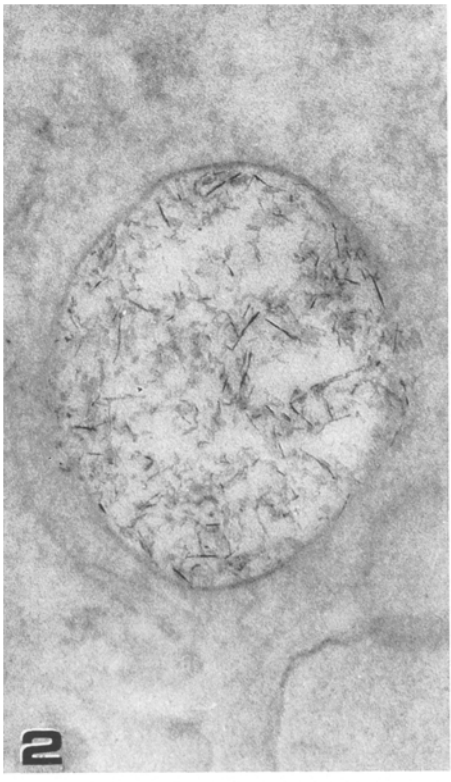
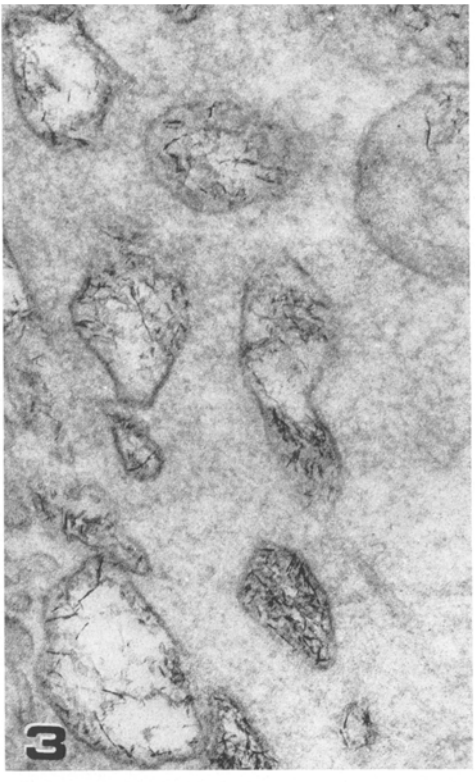
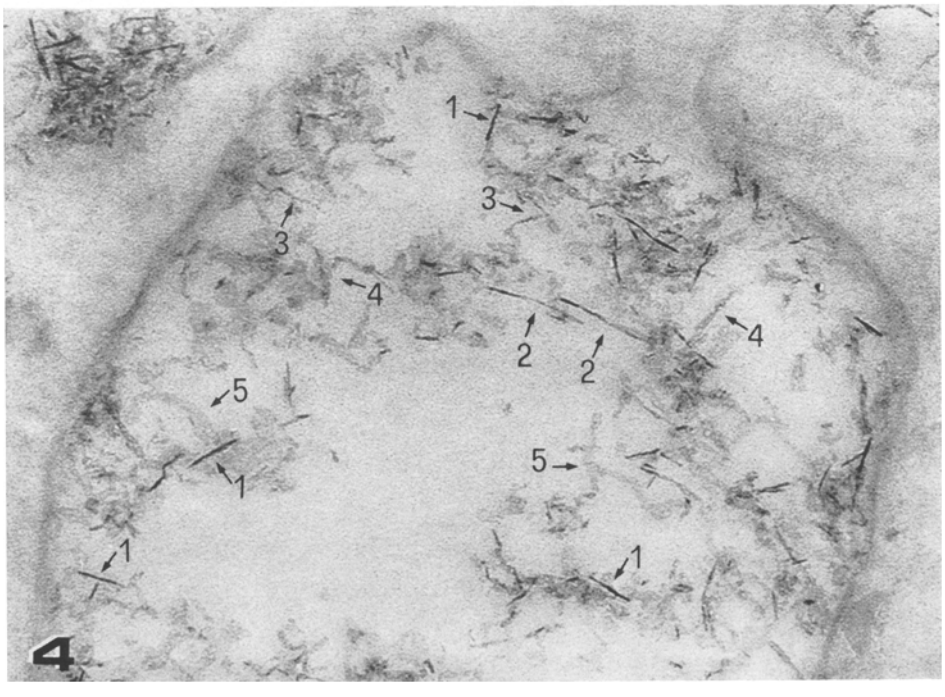


Fig. 1. Detail of the resorption zone and ruffled border of an osteoclast. The zone of resorption consists of loosened crystals distributed at random. Many are contained within channels of the ruffled border. Between the crystals a granular and amorphous material can be seen. Unstained, × 96000


2


3


1
3
3
4
2
2
4
5
1
5
1
1
4

The fine structure of bone matrix, of bone matrix undergoing resorption, of collagen fibrils, and of inorganic crystals is not affected by the type of fixation (simple fixation with OsO_4 or double fixation with formalin and OsO_4) nor by the buffers (phosphate or cacodylate) used for preparing the fixative solutions.

a) Untreated Sections

The most striking finding at the initial stage of osteoclastic resorption is the formation of a small area of fragmented bone. As the activity of the osteoclast increases, this area grows in size until there is a large zone of disrupted bone between the osteoclast and the normal bone matrix (Fig. 1). The thickness of this zone is irregular, a fact which probably depends on differences between levels of activity in the various parts of the osteoclastic cytoplasm and on differences in the duration of this activity. The main characteristic of this zone is the presence of free, randomly oriented inorganic crystals about 25 Å thick. They vary considerably in length, and are not easily measurable because of their random orientation. This resorption zone also contains granular and amorphous material whose intrinsic electron density is lower than that of crystals.

Free crystals are also found between the processes of the ruffled border (Fig. 1) and within cytoplasmic vacuoles (Figs. 2, 4) of the osteoclasts. These vacuoles vary in size, the largest measuring up to 2 μ in diameter. Some of them seem to be empty, while others contain variable amounts of crystals which have the same morphology as those outside the cell. The vacuoles also contain other structures of variable morphology (Figs. 2, 4). Some of them have almost the same structure as the crystals, but with a lower electron density. Others not only have a lower electron density than the crystals, but are irregular in shape and of variable thickness, which is greater than that of the crystals. On the whole, many of these structures look like damaged or modified crystals (Fig. 4).

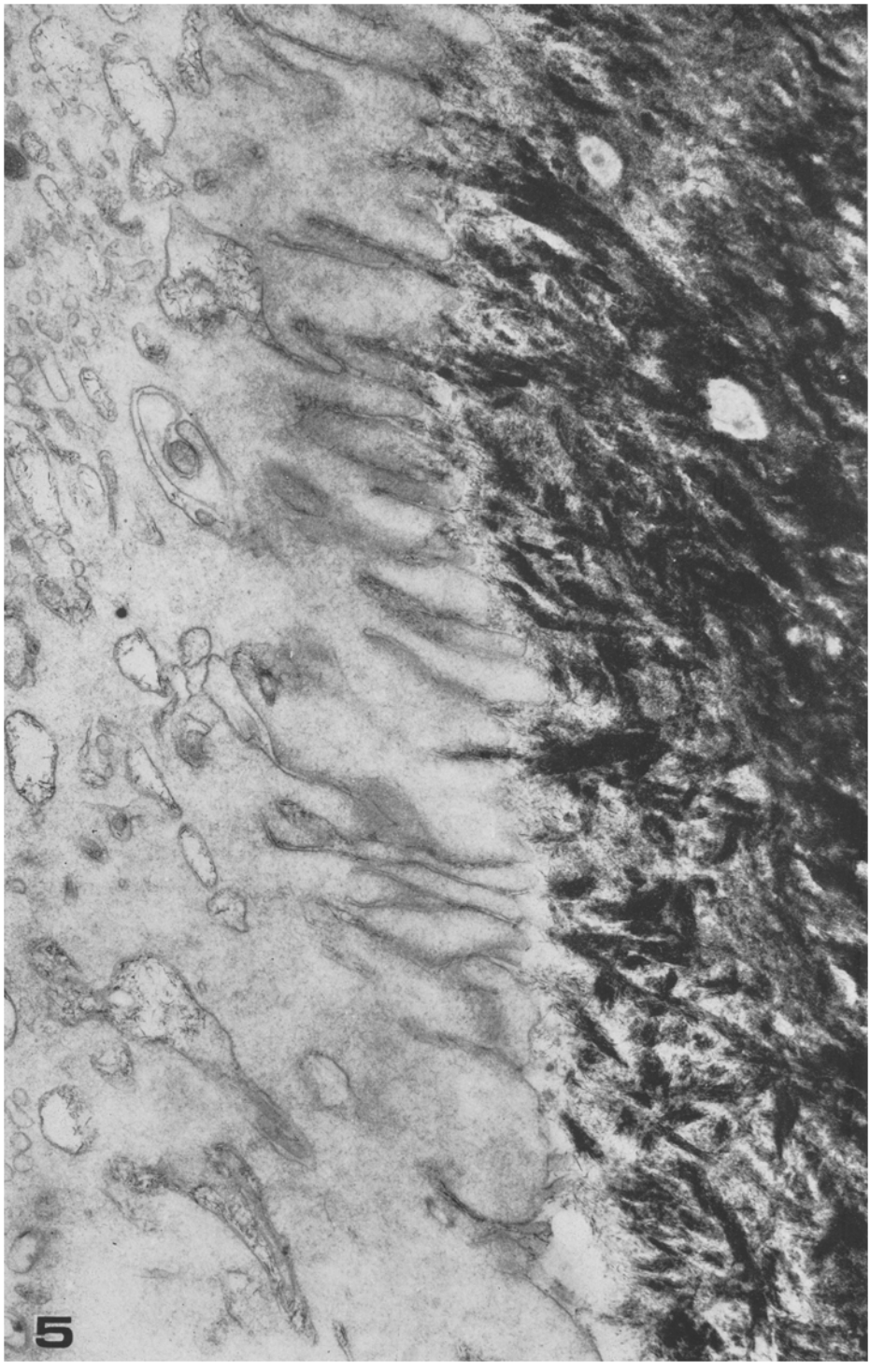
Calcified collagen fibrils are often recognizable between the free crystals of the zone of resorption. These fibrils appear either unchanged, or they are fragmented, the fragments sometimes being closely surrounded by the infoldings of the ruffled border. Fibrils are never present in cytoplasmic vacuoles.

Electron diffractograms of the resorption zone (Fig. 19) are characterized by very faint diffraction rings. Although these rings indicate the presence of crystal-

Fig. 2. Cytoplasmic vacuole containing phagocytosed crystals and irregular structures whose intrinsic electron density is lower than that of crystals. Unstained, × 96000

Fig. 3. Cytoplasmic vacuoles containing phagocytosed crystals and irregular structures (lower left vacuole) similar to those shown in Fig. 2. Both crystals and irregular structures are unchanged after staining. Uranyl acetate and lead citrate, × 85000

Fig. 4. Detail of a cytoplasmic vacuole containing phagocytosed crystals and irregular structures like those shown in Figs. 2 and 3. Arrows *1* point to apparently normal crystals; arrows *2* point to crystals of irregular electron density; arrows *3* show structures which are similar to, but have an electron density lower than normal crystals; arrows *4* point to structures which are similar to, but have an electron density lower, and a thickness greater, than normal crystals; arrows *5* point to structures similar to those shown by arrows *4*, but irregular in shape. This sequence of structures could show different phases of progressive crystal dissolution. Unstained, × 145000


5

line structures in the selected area, their limits are not clear and distinct making measurement of reflexions impossible.

b) Sections Treated with Uranium and Lead

Staining with uranyl acetate and lead citrate increases the electron density of the bone matrix undergoing resorption, but does not significantly change its fine structure (Figs. 3, 5, 6, 7). The zone of resorption contains numerous free crystals similar to those described in the preceding section (Fig. 5). The same crystals are found between the infoldings of the ruffled border (Figs. 5, 6) and within the cytoplasmic vacuoles (Figs. 3, 5).

This staining procedure clearly shows that collagen fibrils and their fragments are present in the zone of resorption (Figs. 5, 6, 7). A few of them sometimes appear to be completely lacking in inorganic crystals, so that their periodic banding is clearly recognizable. More often, they are partially or completely covered with crystals (Fig. 6) which mask the periodic pattern in an irregular way. Fragments of collagen fibrils are sometimes present in the initial, but not in the inner portion of the channels of the ruffled border (Fig. 7). They are never found in cytoplasmic vacuoles.

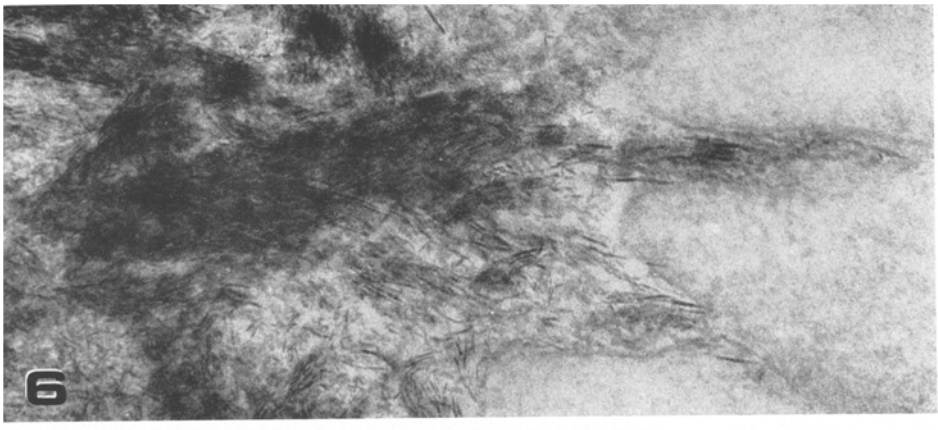
c) Sections Decalcified by Flotation on Formic Acid

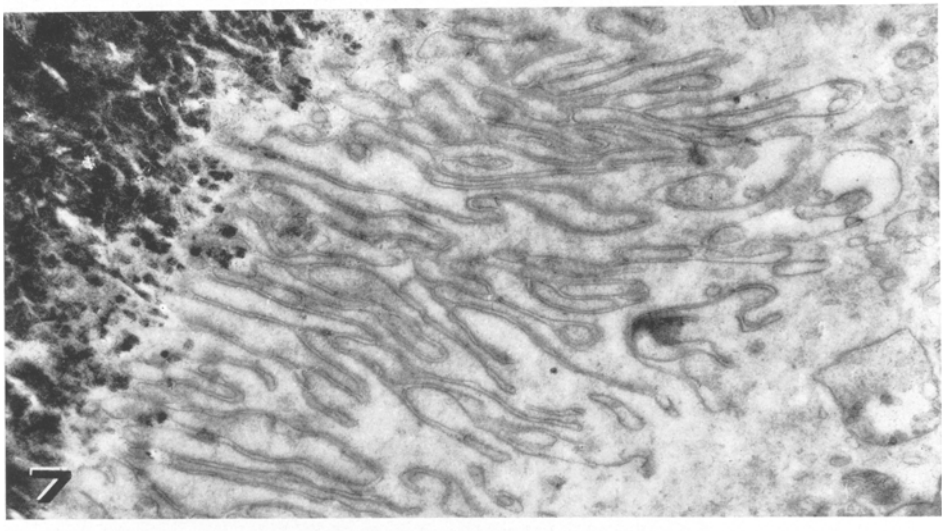
In these sections inorganic crystals are no longer visible in the zone of resorption, in the adjacent normal bone matrix, between the infoldings of the ruffled border, or in cytoplasmic vacuoles (Fig. 8). All the areas previously occupied by inorganic substance, especially the zone of resorption and the bone matrix, now appear as areas of low electron density in which no ultrastructural details are visible. Electron diffractograms of the decalcified zones are of the "amorphous" type; only one shaded and rather large diffraction ring, probably due to the embedding medium, is visible.

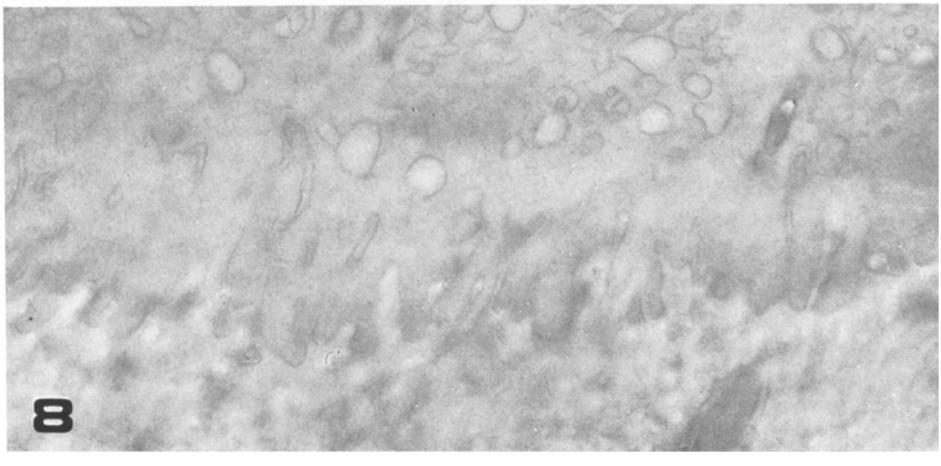
d) Sections Decalcified by Flotation on Formic Acid and Stained with Uranium and Lead

The zone of resorption is easily recognizable after this treatment, because it stains more deeply than the adjacent bone matrix not yet attacked by the osteoclast (Fig. 9). This normal matrix consists of a compact collagenous network, whose fibrils are almost unstained by uranium and lead (Fig. 9). The zone of resorption consists of elongated structures about 30 Å thick, whose length cannot be measured with any accuracy because of their irregular orientation (Figs. 10, 11). On the whole, these structures are strikingly similar to the inorganic crystals found in untreated sections and described in Section *a*. However, because these

Fig. 5. Part of the cytoplasm of an osteoclast and of the bone matrix undergoing resorption after staining with uranium and lead. The resorption zone contains free crystals and fragments of calcified collagen fibrils. Two osteocyte processes are visible in the bone matrix not yet attacked by the osteoclast (upper right corner). The ruffled border, with crystals within its channels, and cytoplasmic vacuoles containing phagocytosed crystals, are clearly visible. Stained with uranyl acetate and lead citrate, × 27000


6


7


8

structures are found in decalcified sections, they cannot be inorganic crystals. They will therefore be referred to as *crystal ghosts*.

The zone of resorption contains not only crystal ghosts, but also collagen fibrils and their fragments (Figs. 12, 13). A few of these fibrils show a clearly recognizable periodic banding but more often, they are masked by elongated structures whose morphology is similar to that of crystal ghosts. In areas where only a few of these elongate structures are present, segments of collagen fibrils are left uncovered so that their period is to some extent visible (Fig. 12). In other cases, the fibrils seem to be swollen and have a disaggregated appearance (Fig. 13). Crystal ghosts are often found closely adjacent to these fibrils (Fig. 13). Finally, where the collagen fibrils are oriented at right angles to the osteoclast surface, the fibrils are often found abruptly broken off. Then, crystal ghosts lying at right angles to the osteoclast surface bulge out from the tip of the fibrils as if they had been produced by disaggregation of the end of the fibrils (see also Section *f* and Fig. 17).

Crystal ghosts are present not only in the resorption zone, but also between the infoldings of the ruffled border and within the cytoplasmic vacuoles of the osteoclast (Figs. 14, 15). In all of these areas their morphology is like that of inorganic crystals. In the cytoplasmic vacuoles they are mixed with other structures which, although like crystals in appearance, are more irregular and less sharply outlined (Fig. 14). These structures correspond perfectly to those observed in cytoplasmic vacuoles in untreated sections (see Section *a*).

Electron diffractograms of the zones which contain crystal ghosts are again of the "amorphous" type. They show a large diffraction ring with a diffuse outline, probably due to the embedding plastic (Fig. 19).

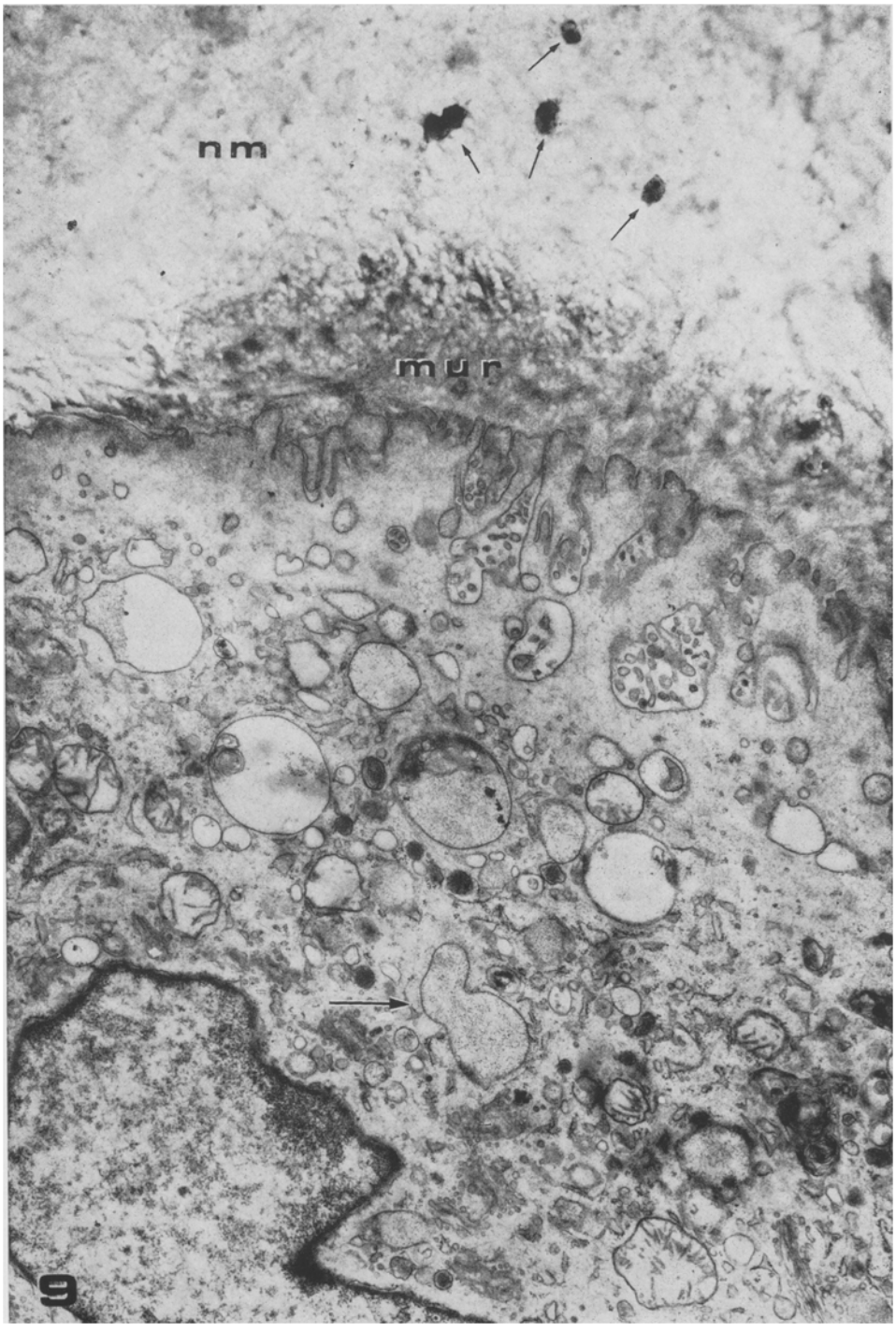
e) Sections Decalcified by Flotation on Formic Acid and Stained with Silver Nitrate-methenamine (SNM)

After formic acid decalcification and SNM staining, the zone of resorption is characterized by the presence of a layer of deeply stained collagen fibrils (Fig. 16). This layer is sharply marked off from the normal matrix which contains relatively few stained collagen fibrils. In the zone of resorption, the argyrophilic collagen fibrils placed near the normal bone matrix are closely bunched and are often distributed with their periodic banding in register. The argyrophilic fibrils

Fig. 6. Detail of the resorption zone shown in Fig. 5. Many free crystals are present, some of them within channels of the ruffled border. A fragment of collagen fibril is also present; its fine structure is masked by crystals lying over it. Uranyl acetate and lead citrate, × 92000

Fig. 7. Part of the resorption zone in contact with the ruffled border of an osteoclast. Note the cross-sectioned collagen fibrils in the resorption zone and outer portion of the ruffled border and the lack of them in the internal portion of the ruffled border and in cytoplasmic vacuoles. Stained with uranyl acetate and lead citrate, × 20000

Fig. 8. Detail of the resorption zone (below) and of an osteoclast (above) in a section decalcified by flotation on formic acid and unstained. No crystals are visible and the previously calcified zones now appear as areas of lower electron density. Decalcified and unstained, × 19000


nm
mur
9

near the ruffled border of the osteoclast are, by contrast, dissociated, fragmented and separated by seemingly empty spaces. A few argyrophilic fibrils are present between the infoldings of the ruffled border. None is found within the cytoplasmic vacuoles of the osteoclast.

After this method of decalcification and staining no crystal ghosts are visible.

f) Sections Decalcified by Flotation on Formic Acid and Stained with Phosphotungstic Acid at Low pH (PTA)

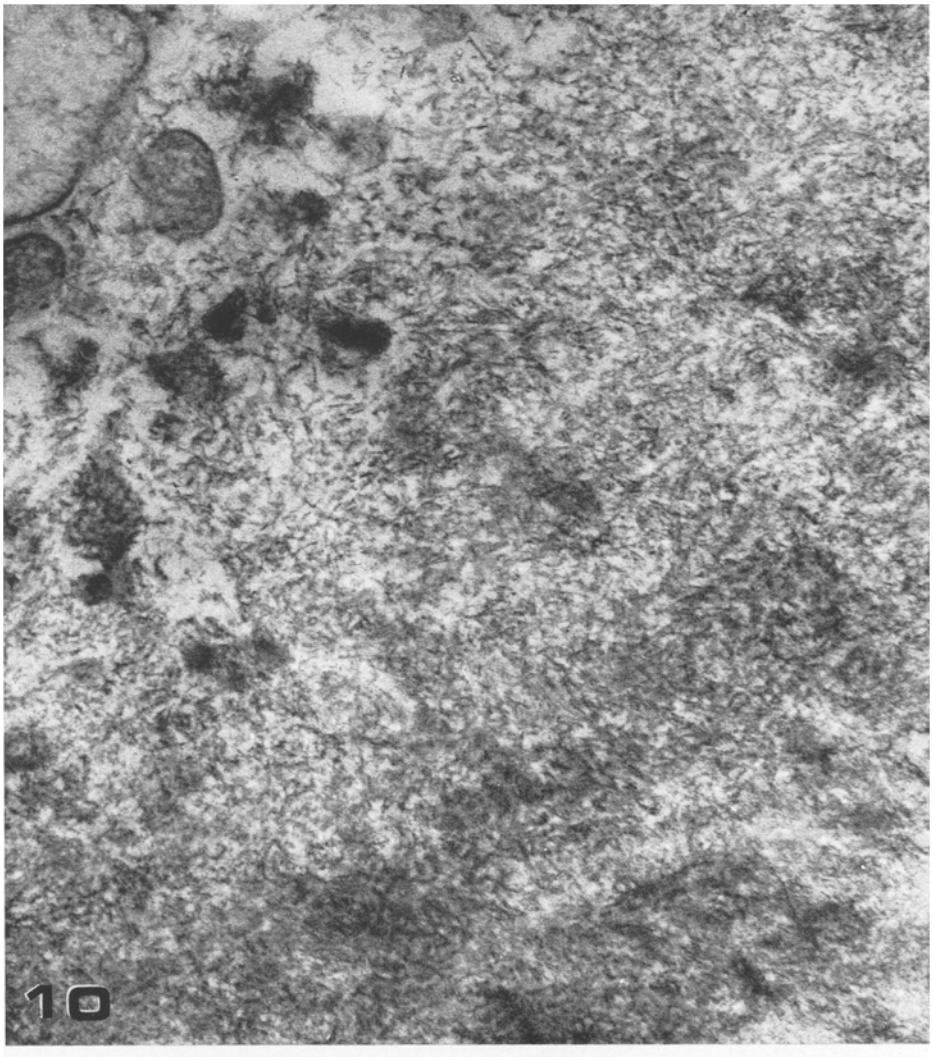
This technique of decalcification and staining gives results not unlike those described in Section *d*. The zone of resorption contains crystal ghosts together with dissociated collagen fibrils and fragments of fibrils (Fig. 17). The periodic banding of these collagen fibrils is not so clearly evident as might be expected after PTA staining. In many areas it is partly obscured by elongated, filament-like structures which look like crystal ghosts. Where the collagen fibrils lie at right angles to the osteoclast surface, crystal ghosts are found at the free end of the fibrils, as if they were about to become detached from their disaggregating tip (Fig. 17).

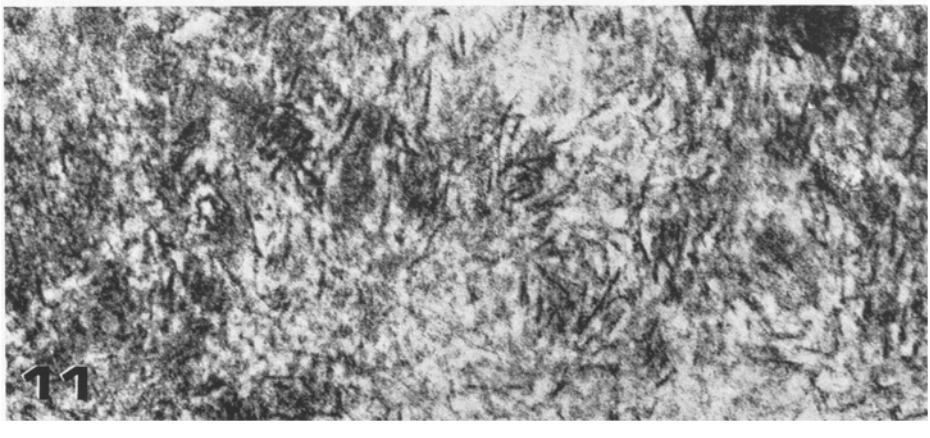
As previously described in Section *d*, crystal ghosts are also present between the infoldings of the ruffled border and within the cytoplasmic vacuoles of the osteoclast. After PTA staining they are less sharply outlined than after staining with uranium and lead.

Discussion

The organic-inorganic relationships in the normal bone matrix, especially the relationship between the inorganic crystals and the components of the collagen fibrils, are not yet fully known [11, 12, 17]. The crystals, for instance, have been described by some authors as being localized between, on, or within collagen fibrils; others have described them as being between and on fibrils; still others have localized them between, on and within fibrils (cf. Bonucci, 1971a). This lack of certainty about organic-inorganic relationships in the normal bone matrix is a major source of doubt and difficulty when the organic-inorganic relationships in the same matrix during osteoclastic resorption are under discussion. A discussion of the main focus of interest here — the effects of osteoclastic activity on each of the various components of the bone matrix — has been extended at various points to include the formulation of hypotheses and drawing of inferences as to the nature of bone calcification.

Fig. 9. Section decalcified with formic acid and stained with uranium and lead. Part of an osteoclast is shown. Its ruffled border is in contact with an area of bone matrix undergoing resorption (*mur*). This is more deeply stained than the normal matrix (*nm*) which consists of almost unstained, closely aggregated and packed collagen fibrils. On the left, this matrix is directly in contact with a zone of the osteoclast cytoplasm lacking the ruffled border. Cross sections of osteocytes processes (arrows) are visible in the normal matrix. Many vacuoles are present in the cytoplasm. One of them (arrow), from another section, is indicated at higher magnification in Fig. 15. Below this vacuole part of the Golgi apparatus is visible. A nucleus (lower left corner), and slightly swollen mitochondria are also shown. Decalcified and stained with uranyl acetate and lead citrate, × 15000


10


11

The present investigation has yielded results similar to those reported by other authors [63, 23, 27] as far as the fine structure of the zone of resorption in untreated sections is concerned. Between the osteoclast on one side and the normal bone matrix on the other, many free crystals are found, which have apparently been produced by the breakdown of the calcified matrix. Crystals of the same sort are also found between the infoldings of the ruffled border and within cytoplasmic vacuoles of the osteoclast; a clear indication that they are phagocytosed while being detached from the bone matrix [23, 27, 30, 31].

The fine structure of crystals is not affected by the type of fixation (simple OsO_4, or formalin followed by OsO_4) nor by the buffer (phosphate or cacodylate) used in fixation. The morphology of crystals found in the resorption zone does not seem to be affected by osteoclast activity, as shown by the fact that they are similar to crystals usually described in normal bone matrix, calcified cartilage, and dentine [59, 63, 67, 15, 4, 19, 8, 9, 11, 12, 32, 17]. They are mixed with intrinsically electron-dense granular and amorphous material, which probably derives from the breakdown of the granular or "amorphous" mineral substance which has been described as being closely associated with the collagen period in bone [50, 5, 26].

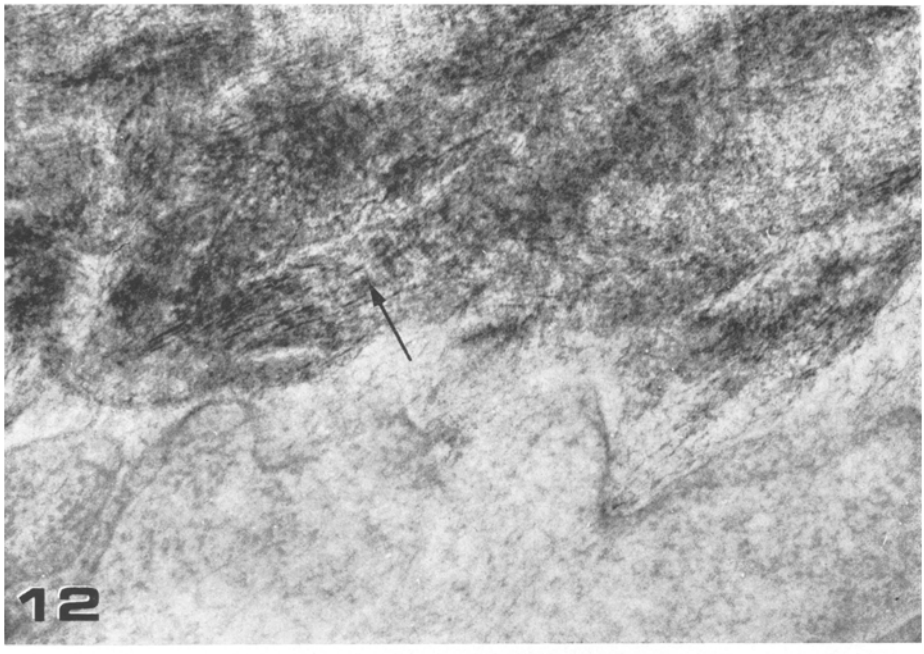
The crystals contained in the cytoplasmic vacuoles of the osteoclast are found with other intrinsically electron-dense structures. These resemble crystals to some extent, but are thicker, irregularly-shaped, and less electron dense than normal crystals. These structures seem to derive, at least in part, from the gradual dissolution of crystals, an interpretation supported by their intrinsic electron density and by the resemblance between some of them and true crystals.

A definitive explanation cannot be given for any of these findings at this stage, but they seem to suggest that although inorganic crystals become detached from the bone matrix, they undergo no great changes until, by a process of pinocytosis, they reach the segregated medium of the cytoplasmic vacuoles, where they are gradually solubilized. This seems to lead to a loss of their sharp outline and to their transformation into irregular structures of lower electron density which gradually become completely solubilized.

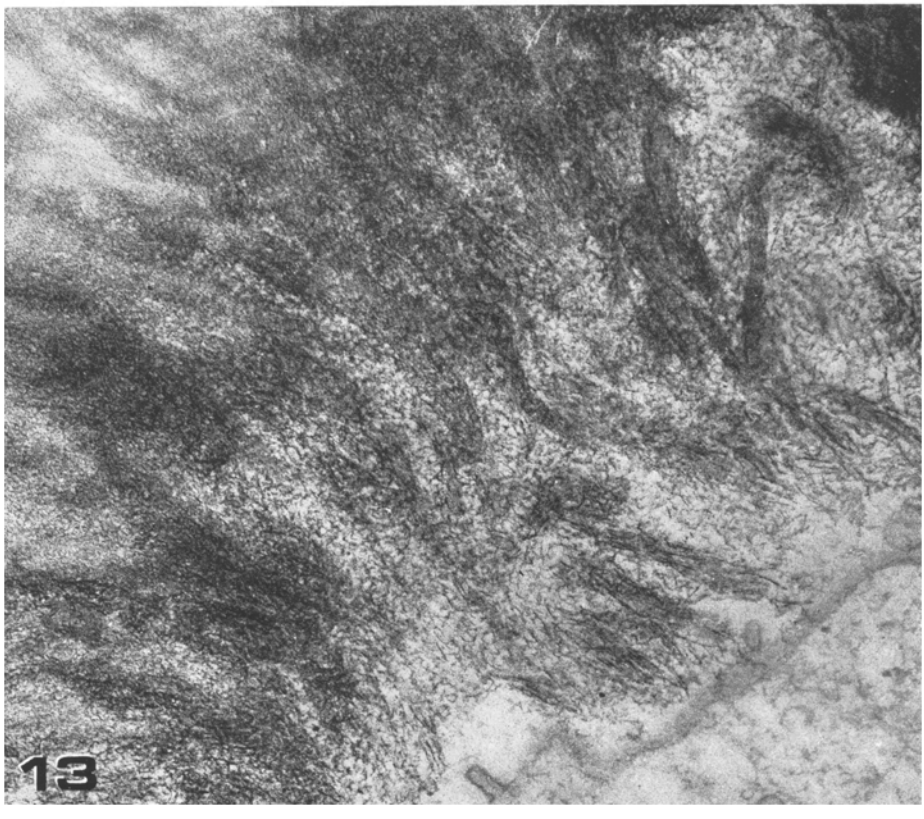
It has been suggested that the inorganic substance could be at least partly solubilized outside the cell by acid or chelating substance [35, 68, 49], but the presence of apparently normal crystals in the zone of resorption suggests that rather than being dissolved, the crystals are dislodged from the bone matrix [30, 15, 31, 17]. The present results suggest that the solubilization of the inorganic crystals does not occur outside the cell. They are probably solubilized in the cytoplasmic vacuoles of the osteoclast. The structure and properties

Fig. 10. Detail of resorption zone after decalcification and uranium and lead staining. Note the presence of elongated structures (crystal ghosts) whose morphology is similar to that of the crystals found in untreated sections and in undecalcified sections stained with uranium and lead. A small portion of the ruffled border is visible at the upper left corner. Decalcified and stained with uranyl acetate and lead citrate, × 90000

Fig. 11. Detail of crystal ghosts. Decalcified and stained with uranyl acetate and lead citrate, × 145000



12



13

of these vacuoles are like those of secondary lysosomes [18, 25, 40, 22, 43] and they very probably have a low pH value [73]. It is therefore likely that they both induce the solubilization of the inorganic substance and, at the same time, promote the digestion of the organic matrix by acid hydrolases.

One result of osteoclastic activity is that the organic matrix of the zone of resorption is highly disorganized and dissociated. This can be clearly observed in stained sections, especially those stained with SNM. Thus, collagen fibrils sited in bone matrix not yet attacked by osteoclast appear laterally aggregated, with many of them lying in register, while those sited in the zone of resorption appear as separate, unconnected structures. The first stain feebly; the second show high affinity for uranium and lead, and for silver nitrate, a property they share with collagen fibrils in the osteoid border prior to their calcification (Bonucci, unpublished results). Argyrophilic fibrils increases in number in the uterus during pregnancy as a result of the splitting of collagen bundles [24]; so too, the argyrophilia of collagen fibrils in the zone of resorption may be due to the disaggregation induced by osteoclastic activity. This disaggregation could be caused by the digestion of the interfibrillar ground substance leading to disruption of interfibrillar bonds. If this is so, the argyrophilia of these collagen fibrils would be indirect evidence that the osteoclastic dissociation of bone matrix begins with the degradation of the interfibrillar ground substance (see also Hancox and Boothroyd, 1961; Scherft, 1968). This seems to occur simultaneously with, but probably largely independently of, the breakdown of fibrils, as shown by the fact that many argyrophilic but otherwise normal collagen fibrils are left behind in the zone of resorption. Even so, the digestion of the interfibrillar ground substance (that unmasked by inorganic substance) seems to be an indispensable precondition for collagen degradation because it produces dissociation of laterally aggregated collagen fibrils and, presumably, greatly increases the penetrability and activity of enzymes in the matrix. It may well also increase collagen solubility. It has, in fact, been shown that the removal of ground substance by treatment of connective tissues with α-amylase or EDTA allows insoluble collagen to be dispersed in dilute acids [21, 65] and to be digested by collagenase [54].

The dissociation of the disaggregated collagen fibrils seems to reach completion outside the cell. No collagen fibrils or segments of fibrils have ever been found within cytoplasmic vacuoles (not even after staining of sections). Besides crystals,

Fig. 12. Part of resorption zone, showing a collagen fibril (arrow) partly covered by crystal ghosts. Note that its cross-banding is intact. Pictures like this suggest that in many cases the crystals and their ghosts lie between the fibrils and on their surface (i.e., are components of the ground substance), so that they can be loosened and released without damaging the fibril texture. Part of the osteoclastic cytoplasm is apparent below. Decalcified and stained with uranyl acetate and lead citrate, × 92000

Fig. 13. Part of an osteoclast (lower right corner), of the matrix not yet attacked by the osteoclast (upper left corner), and of the resorption zone (in between). Besides crystal ghosts, the zone of resorption contains disaggregated collagen fibrils and segments of fibrils, some of which (those near the osteoclast ruffled border) seem about to undergo complete dissociation. Decalcified and stained with uranyl acetate and lead citrate, × 70000

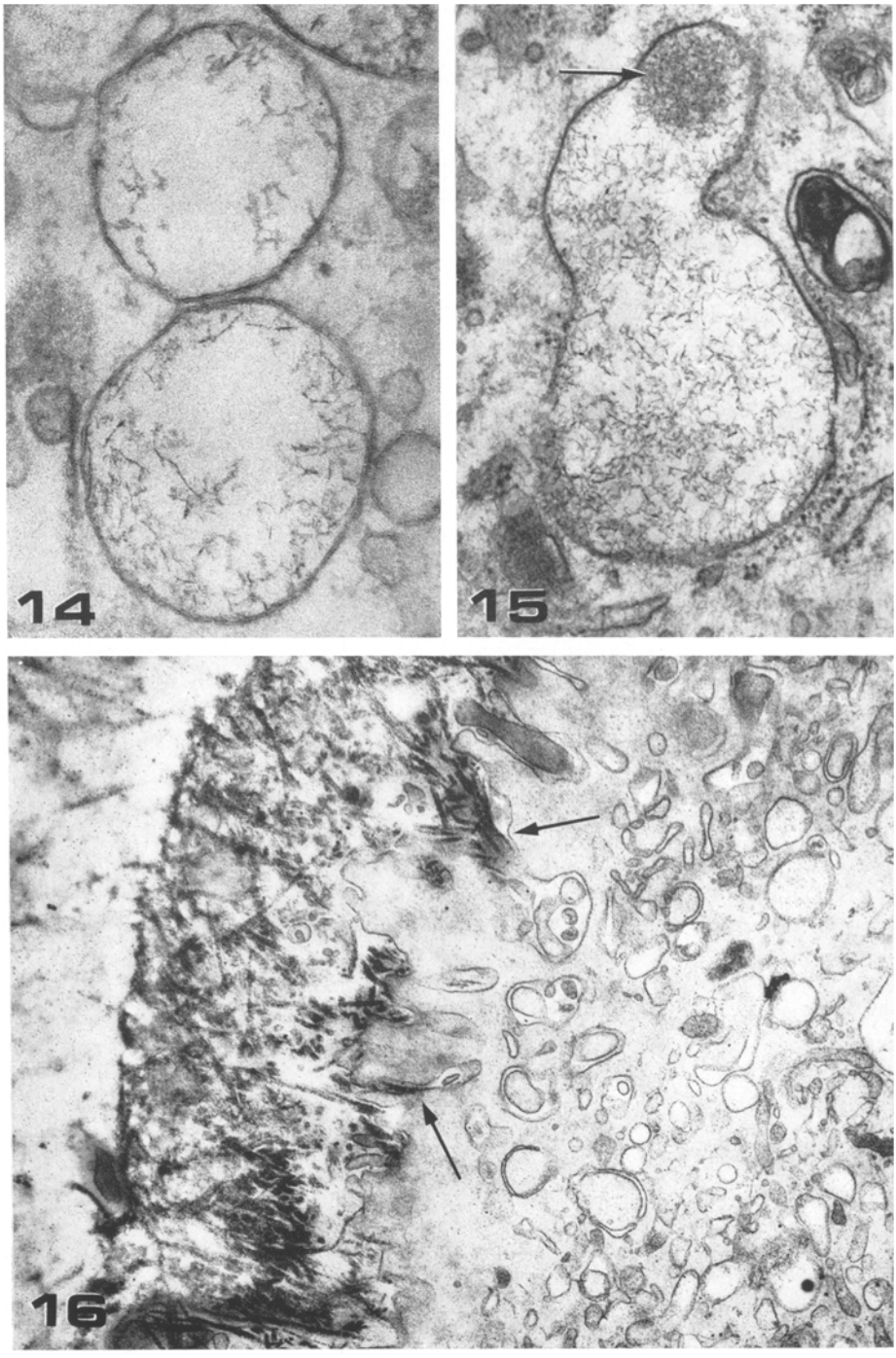


Fig. 14. Cytoplasmic vacuoles containing crystal ghosts. Compare with the undecalcified vacuoles shown in Figs. 2, 3 and 4. Decalcified and stained with uranyl acetate and lead citrate, × 105000

cytoplasmic vacuoles contain only irregular structures which might well be considered organic fragments of the bone matrix but which, because of their intrinsic, although slight, electron density, very probably consist of inorganic substance. It could be speculated that the collagen fibrils are digested extracellularly in the resorption zone and outer portion of the ruffled border and that the products of this digestion, now unrecognizable as collagenous structures, are accumulated in cytoplasmic vacuoles.

The sections stained after decalcification seem to yield particularly useful results. After the ultrathin sections have been floated on formic acid solution, all the inorganic substance is solubilized and removed; both the matrix not yet attacked by the osteoclast and the resorption zone now appear as electron transparent areas. No crystals are recognizable in these areas, nor are they found between the infoldings of the ruffled border or in the cytoplasmic vacuoles of the osteoclast. Moreover, the faint diffraction rings found in diffractograms obtained from the resorption zone in untreated sections completely disappear after decalcification. With all of the inorganic substance removed, it might be supposed that the only structures which would be found after staining these decalcified areas would be organic structures. They would presumably be collagen fibrils in the normal bone matrix, and fibrils and segments of fibrils in the resorption zone. But staining decalcified sections with uranyl acetate and lead citrate, and with PTA shows that the zone of resorption contains not only the expected whole and fragmented collagen fibrils but also elongated structures whose morphology is like that of untreated inorganic crystals. The same structures are found between the infoldings of the ruffled border and within the cytoplasmic vacuoles of the osteoclast. It must be underlined that these structures are not visible in decalcified and unstained sections, and that they do not give evidence of crystallinity by electron diffraction; i.e., they are not inorganic crystals left in incompletely decalcified areas. However, they closely resemble inorganic crystals and for this reason they have been called "crystal ghosts".

Crystal ghosts have been previously described in calcifying cartilage, bone, and calcified soft tissues [8, 3, 9, 1, 64, 2, 11, 12, 66, 14, 13]. They have been considered organic structures and the conclusion has been reached that in cartilage and bone, as well as in other calcified tissues, the crystals are organic-inorganic structures whose organic component, the crystal ghosts, is of proteoglycan and, perhaps, lipidic nature [8, 9, 11, 12, 14, 13]. Although it cannot be excluded that the formation of the crystal ghosts might partly depend on adsorption of organic

Fig. 15. Cytoplasmic vacuole containing crystal ghosts and a dense material (arrow) which could represent the content of a primary lysosome (cfr. Scott, 1967, and Cohn and Fedorko, 1969). Compare with the undecalcified vacuoles shown in Figs. 2, 3 and 4, and with Fig. 9. Decalcified and stained with uranyl acetate and lead citrate, × 68000

Fig. 16. Detail of an osteoclast (right), of the bone matrix not yet attacked by the osteoclast (left), and of the resorption zone (in between). The resorption zone contains dissociated, argyrophilic collagen fibrils, some of which (arrows) are contained within infoldings of the ruffled border. None of them are visible in cytoplasmic vacuoles. The normal bone matrix (left) is almost unstained. Decalcified and stained with SNM, × 19000

Fig. 17. Detail of collagen fibrils undergoing resorption. They run perpendicular to the surface of the osteoclast (whose ruffled border is partly visible below). Crystal ghosts project outwards from the tip of the fibrils as if they were about to become detached from them. Decalcified and stained with PTA, × 100000

material to the crystal surface during fixation, the evidence suggests that they are intrinsic components of the crystals, that they are exposed to the external environment to some degree, and that they can partly be removed without doing great damage to the crystal appearance [13].

Staining with PTA shows that the crystal ghosts found in the present investigation likewise consist, at least partly, of proteoglycans. Crystal ghosts are, in fact, stained by PTA at low pH. This technique, when applied to plastic embedded material, is now considered to reveal glycoproteic complexes [51, 46, 47, 55, 7, 6, 33, 53]. Further confirmation is provided by the close parallel between the results of this technique and those obtainable with other specific histochemical [47, 56, 58, 52, 53, 57, 14] and autoradiographic [71, 72] methods.

The fact that the crystal ghosts found in the resorption zone are stained by PTA not only implicitly confirms that they are organic structures, but goes to show that they consist of proteoglycan material. It is thought that most bone proteoglycans are contained in the interfibrillar ground substance, and it may therefore be suggested, in line with previous investigations on calcifying cartilage and bone [9, 11, 12], that the crystal ghosts form part of this substance. In fact, the present results show that most crystal ghosts lie between collagen

fibrils and on their surface. Proteoglycans are not only contained in the interfibrillar ground substance, but are also strictly associated with, and found within, collagen fibrils [34, 54, 48, 53, 21, 39, 38], so it is not unlikely that crystal ghosts may be sited within fibrils too. In some cases crystal ghosts have, in fact, been observed to bulge out from the tips of collagen fibrils undergoing osteoclastic resorption as if they were intrinsic components of these fibrils.

In investigations concerning organic-inorganic relationships during bone and cartilage calcification, Bonucci [8–13] has suggested that crystal ghosts may play a fundamental role in determining and regulating the binding of the inorganic substance, that both bone and cartilage crystals may be organic-inorganic structures, and that both bone and cartilage calcification may be a multistep process which, once begun by structures of cellular origin (calcifying globules), may then proceed in the ground substance lying between and on the surface of collagen fibrils, until the interior of the fibrils is more or less completely involved. This view has been partly confirmed by the present results, which show that bone crystals are organic-inorganic structures, that their organic component is at least partly proteoglycan in nature, and that it is mainly sited in the ground substance and secondarily within collagen fibrils.

If this view is correct, the organic-inorganic relationships in normal bone matrix mainly depend on the amount and siting of proteoglycans in the ground substance of the matrix. A further consequence of this view is that crystals will mostly be sited between collagen fibrils and on their surface when plenty of ground substance is present, that is, when collagen fibrils are not closely bunched, as in cartilage and primary bone, and will mostly be sited within collagen fibrils when little ground substance is present (when collagen fibrils are closely bunched, as in secondary bone). Any osteoclastic dissociation of the ground substance consequent to digestion of its uncalcified components would lead to the release of crystals in both cases, but especially in the former. The complete release of all crystals would follow the total dissolution of collagen fibrils.

The presence in the resorption zone of swollen, seemingly disaggregating collagen fibrils suggests a direct dissociating effect of the osteoclast on the fibrils. However, it might be supposed that the same effect could be produced by the hydrolytic activity of the formic acid solution used for decalcification. This type of artifact is almost always produced when bone specimens are decalcified before embedding. However, it does not occur when decalcification is performed on floating sections, perhaps because the fibres are stabilized by the embedding medium. In the present case, the effect of formic acid is greater in the normal compact bone matrix than in the resorption zone. This may be due to the fact that, because of its compactness, the normal bone matrix is less easily penetrated by the embedding medium than the dissociated resorption zone and, consequently, its collagen fibrils are less "protected" by the embedding medium and more exposed to formic acid than those in the resorption zone. Evidently, the fibrils of the normal matrix would appear more dissociated than those in the resorption zone if the disaggregated aspect of the latter depends on the hydrolytic activity of formic acid. On the contrary, the normal bone matrix appears to be formed by a compact network of closely aggregated, undissociated, faintly stainable

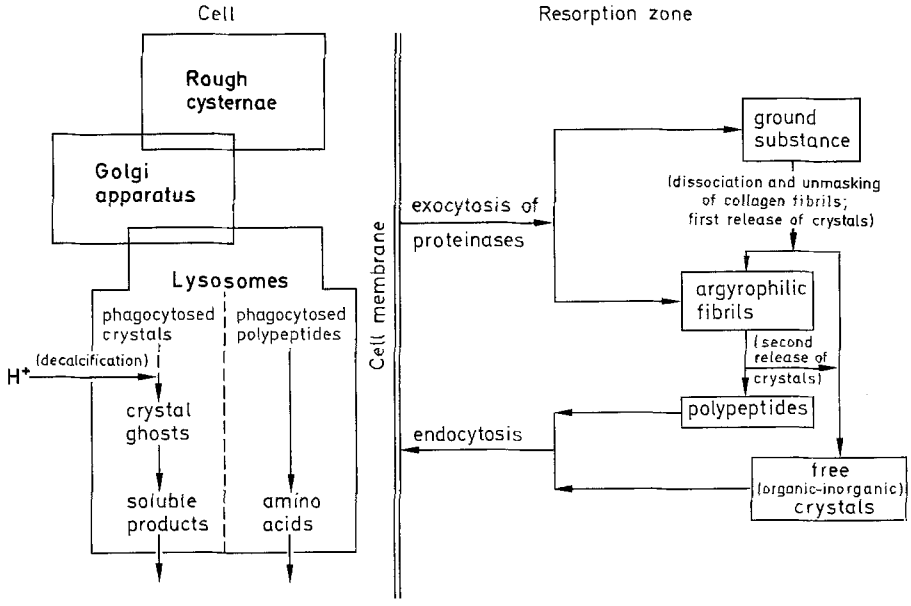


Fig. 18. Scheme of the presumed steps of the process of osteoclastic bone resorption

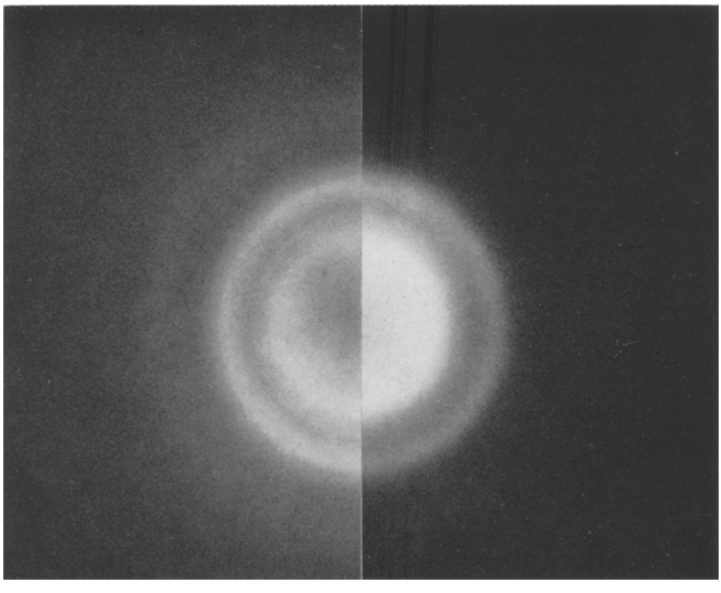


Fig. 19. Left: electron diffractogram pertinent to a zone of resorption in an untreated section. Very faint diffraction rings, suggesting the presence of crystalline structures, are visible. Right: electron diffractogram of "amorphous" type pertinent to a zone of resorption in a section decalcified with formic acid and stained with uranium and lead

fibrils (cf. Fig. 9), thus confirming that collagen fibril dissociation in the resorption zone is a direct effect of the osteoclastic activity.

The presence of "decalcified" collagen fibrils in the resorption zone, a finding occasionally observed in this study but frequently reported in another type of bone by Hancox and Boothroyd [30, 31], does not necessarily mean that the inorganic substance is solubilized outside the cell. It could mean that no crystals were originally or ever contained within these fibrils, so that they appear decalcified when the inorganic material once belonging to the ground substance around and on them has been removed by osteoclastic activity.

By bringing together the results of the present investigation and those of previous studies on bone and collagen resorption [68, 73], and on lysosomal enzymes in skeletal tissues [20], the main steps of the mechanism regulating osteoclastic bone resorption have been tentatively summarized in Fig. 18. Here the osteoclastic bone resorption is shown as a two-phase process; the first, extracellular phase leads to fragmentation of the calcified matrix, and the second, intracellular phase leads to digestion of the phagocytosed fragments. The first components of the bone matrix to be dissociated by osteoclastic activity are very probably those of the ground substance which are not masked by inorganic material and are therefore exposed to the osteoclastic enzymes. This has two effects. First, crystals placed between and on collagen fibrils (i.e., crystals related to components of the ground substance) become detached (first crystal release); second, the aggregated collagen fibrils are disaggregated, rendered argyrophilic and partly unmasked. The released crystals are organic-inorganic structures, whose organic component (the crystal ghost) consists, at least partly, of proteoglycan material and may well be a substrate for binding inorganic substance during the calcification process. The argyrophilic collagen fibrils are further digested and reduced to small fragments and then to very small components. The intrafibrillar crystals, where present, may be released at this stage (second crystal release). They, too, are organic-inorganic structures, whose organic component probably derives from intrafibrillar proteoglycans.

Not only the released crystals, but the uncalcified products of collagen fibril and ground substance degradation too are phagocytosed by the osteoclast. The final stage of digestion occurs within the osteoclast's cytoplasmic vacuoles (secondary lysosomes). The inorganic substance of the crystals is solubilized at this site, probably as a result of its low pH value, and the crystal ghosts are therefore released at this point. These can then be digested together with, and in the same way as the other phagocytosed organic components of the bone matrix.

Acknowledgments. This investigation has been supported by grants from Italian National Research Council. The author is grateful to Dr. Lyle C. Dearden (University of California, Irvine) for valuable criticism of the manuscript and to miss Giuliana Silvestrini and Mr. Lucio Virgilii for their skilled technical assistance.

References

1. Appleton, J.: Ultrastructural observations on early cartilage calcification. The use of chromium sulphate in decalcification. Calc. Tiss. Res. **5**, 270–276 (1970)
2. Appleton, J.: Ultrastructural observations on the inorganic/organic relationships in early cartilage calcification. Calc. Tiss. Res. **7**, 307–317 (1971)

3. Appleton, J., Balckwood, H. J. J.: Ultrastructural observations on early mineralization in cartilage. J. Bone Jt. Surg. **51B**, 385 (1969)
4. Ascenzi, A.: The relationship between mineralization and bone matrix. In: Bone and tooth (ed. H. J. J. Blackwood), p. 231–243. Oxford: Pergamon Press 1964
5. Ascenzi, A., Bonucci, E., Steve Bocciarelli, D.: An electron microscope study of osteon calcification. J. Ultrastruct. Res. **12**, 287–303 (1965)
6. Babai, F., Bernhard, W.: Détection cytochimique par l'acide phosphotungstique de certains polysaccharides sur coupes à congélation ultrafines. J. Ultrastruct. Res. **37**, 601–617 (1971)
7. Bernard, G. W., Pease, D. C.: An electron microscopic study of initial intramembranous osteogenesis. Amer. J. Anat. **125**, 271–290 (1969)
8. Bonucci, E.: Fine structure of early cartilage calcification. J. Ultrastruct. Res. **20**, 33–50 (1967)
9. Bonucci, E.: Further investigation on the organic/inorganic relationships in calcifying cartilage. Calc. Tiss. Res. **3**, 38–54 (1969)
10. Bonucci, E.: Fine structure and histochemistry of "calcifying globules" in epiphyseal cartilage. Z. Zellforsch. **103**, 192–217 (1970)
11. Bonucci, E.: The locus of initial calcification in cartilage and bone. Clin. Orthop. **78**, 108–139 (1971a)
12. Bonucci, E.: Problemi attuali attinenti all'istochimica di talune matrici calcificanti normali e patologiche. Riv. Istochim. Norm. Patol. **17**, 153–234 (1971b)
13. Bonucci, E.: Organic-inorganic relationships in calcified organic matrices. In: Colloque international sur la physicochimie et la cristallographie des apatites d'interet biologique (ed. G. Montel), (in press)
14. Bonucci, E., Derenzini, M., Marinozzi, V.: The organic-inorganic relationship in calcified mitochondria. J. Cell Biol. **59**, 185–211 (1973)
15. Cameron, D. A.: The fine structure of bone and calcified cartilage. Clin. Orthop. **26**, 199–228 (1963)
16. Cameron, D. A.: The ultrastructural basis of resorption. Calc. Tiss. Res. **4**, 279–280 (1969)
17. Cameron, D. A.: The ultrastructure of bone. In: The biochemistry and Physiology of bone, 2nd Ed., vol. I (ed. G. H. Bourne), p. 191–236. New York and London: Academic Press 1972
18. Cohn, Z. A., Fedorko, M. E.: The formation and fate of lysosomes. In: Lysosomes in biology and pathology, vol. I (ed. J. T. Dingle, H. B. Fell), p. 43–63. Amsterdam: North Holland 1969
19. Decker, J. D.: An electron microscopic investigation of osteogenesis in the embryonic chick. Amer. J. Anat. **118**, 591–614 (1966)
20. Dingle, J. T.: Lysosomal enzymes in skeletal tissues. In: Hard tissue growth, repair and remineralization, Ciba Found. Symp. 11, p. 295–311. Amsterdam: Elsevier-Excerpta Medica-North-Holland 1973
21. Dixon, J. S., Hunter, J. A. A., Steven, F. S.: An electron microscopic study of the effect of crude bacterial α-amylase and ethylenediaminetetraacetic acid on human tendon. J. Ultrastruct. Res. **38**, 466–472 (1972)
22. Doty, S. B., Schofield, B. H.: Electron microscopic localization of hydrolytic enzymes in osteoclasts. Histochem. J. **4**, 245–258 (1972)
23. Dudley, H. R., Spiro, D.: The fine structure of bone cells. J. biophys. biochem. Cytol. **11**, 627–649 (1961)
24. Fainstat, T.: Extracellular studies of uterus I. Disappearance of the discrete collagen bundles in endometrial stroma during various reproduction states in the rat. Amer. J. Anat. **112**, 337–369 (1963)
25. Freilich, L. S.: Ultrastructure and acid phosphatase cytochemistry of odontoclasts: effects of parathyroid extract. J. Dent. Res. **50**, 1047–1055 (1971)
26. Glimcher, M. J., Krane, S. M.: The organization and structure of bone, and the mechanism of calcification. In: Treatise on collagen, vol. II B, Biology of collagen (ed. B. S. Gould), p. 67–251. London and New York: Academic Press 1968
27. Gonzales, F., Karnovsky, M. J.: Electron microscopy of osteoclasts in healing fractures of rat bone. J. biophys. biochem. Cytol. **9**, 299–316 (1961)

28. Göthlin, G., Ericsson, J. L. E.: Observations on the mode of uptake of thorium dioxide particles by osteoclasts in fracture callus. Calc. Tiss. Res. **10**, 216–222 (1972)
29. Hancox, N. M.: Biology of bone. Cambridge: Cambridge University Press 1972
30. Hancox, N. M., Boothroyd, B.: Motion picture and electron microscope studies on the embryonic avian osteoclast. J. biophys. biochem. Cytol. **11**, 651–661 (1961)
31. Hancox, N. M., Boothroyd, B.: Structure-function relationships in the osteoclast. In: Mechanisms of hard tissue destruction (ed. R. F. Sognnaes), p. 497–514. Washington: American Association for the Advancement of Science 1963
32. Höhling, H. J., Kreilos, R., Neubauer, G., Boyde, A.: Electron microscopy and electron microscopical measurements of collagen mineralization in hard tissues. Z. Zellforsch. **122**, 36–52 (1971)
33. Iglesias, J. R., Bernier, R., Simard, R.: Ultracryotomy: a routine procedure. J. Ultrastruct. Res. **36**, 271–289 (1971)
34. Jackson, D. S., Bentley, J. P.: Collagen-glycosaminoglycans interactions. In: Treatise on Collagen, vol. II A: Biology of collagen (ed. B. S. Gould), p. 189–214. London and New York: Academic Press 1968
35. Jenkins, G. N., Dawes, C.: The possible role of chelation in decalcification of biological systems. In: Mechanism of hard tissue destruction (ed. R. F. Sognnaes), p. 637–662. Washington: American Association for the Advancement of Science 1963
36. Kallio, D. M., Garant, P. R., Minkin, C.: Evidence of coated membranes in the ruffled border of the osteoclast. J. Ultrastruct. Res. **37**, 169–177 (1971)
37. Knese, K.-H.: Osteoklasten, Chondroklasten, Mineraloklasten, Kollagenoklasten. Acta anat. (Basel) **83**, 275–288 (1972)
38. Kobayashi, T. K., Pedrini, V.: Proteoglycans-collagen interactions in human costal cartilage. Biochim. biophys. Acta (Amst.) **303**, 148–160 (1973)
39. Lowther, D. A., Natarajan, M.: The influence of glycoprotein on collagen fibril formation in the presence of chondroitin sulphate proteoglycan. Biochem. J. **127**, 607–608 (1972)
40. Lucht, U.: Acid phosphatase of osteoclasts demonstrated by electron microscopic histochemistry. Histochemie **28**, 103–117 (1971)
41. Lucht, U.: Absorption of peroxidase by osteoclasts as studied by electron microscope histochemistry. Histochemie **29**, 274–286 (1972a)
42. Lucht, U.: Osteoclasts and their relationship to bone as studied by electron microscopy. Z. Zellforsch. **135**, 211–228 (1972b)
43. Lucht, U.: Cytoplasmic vacuoles and bodies of the osteoclast. An electron microscope study. Z. Zellforsch. **135**, 229–244 (1972c)
44. Malkani, K., Luxembourger, M.-M., Rebel, A.: Cytoplasmic modifications at the contact zone of osteoclasts and calcified tissue in the diaphyseal growing plate of foetal guinea-pig tibia. Calc. Tiss. Res. **11**, 258–264 (1973)
45. Marinozzi, V.: Cytochimie ultrastructurale du nucléole-RNA et protéines intranucléolaires. J. Ultrastruct. Res. **10**, 433–456 (1964)
46. Marinozzi, V.: Réaction de l'acide phosphotungstique avec la mucin et les glycoprotéines des plasmamembranes. J. Microscopie **6**, 68a (1967)
47. Marinozzi, V.: Phosphotungstic acid (PTA) as a stain for polysaccharides and glycoproteins in electron microscopy. In: Electron microscopy 1968, vol. II (ed. D. S. Bocciarelli), p. 55–56. Rome: Tipografia Poliglotta Vaticana 1968
48. Mathews, M. B.: The interactions of proteoglycans and collagen. Model systems. In: Chemistry and molecular biology of the intercellular matrix, vol. II (ed. E. A. Balazs), p. 1155–1169. London and New York: Academic Press 1970
49. Nisbet, J. A., Helliwell, S., Nordin, B. E. C.: Relation of lactic and citric acid metabolism to bone resorption in tissue culture. Clin. Orthop. **70**, 220–230 (1970)
50. Nylen, M. U., Scott, D. B., Mosley, V. M.: Mineralization of turkey leg tendon. II. Collagen-mineral relations revealed by electron and X-ray microscopy. In: Calcification in biological systems (ed. R. F. Sognnaes), p. 129–142. Washington: American Association for the Advancement of Science 1960
51. Pease, D. C.: Polysaccharides associated with the exterior surface of epithelial cells: kidney, intestine, brain. J. Ultrastruct. Res. **15**, 555–588 (1966)

52. Pease, D. C.: Phosphotungstic acid as a specific electron stain for complex carbohydrates. J. Histochem. Cytochem. **18**, 455–458 (1970)
53. Pease, D. C., Bouteille, M.: The tridimensional ultrastructure of native collagenous fibrils, cytochemical evidence for a carbohydrate matrix. J. Ultrastruct. Res. **35**, 339–358 (1971)
54. Quintarelli, G., Dellovo, M. C., Balduini, C., Castellani, A. A.: The effects of alpha amylase on collagen-proteoglycans and collagen-glycoprotein complexes in connective tissue matrices. Histochemie **18**, 373–375 (1969)
55. Rambourg, A.: Détection des glycoprotéines en microscopie électronique par l'acide phosphotungstique à bas pH. In: Electron microscopy 1968, vol. II (ed. D. S. Bocciarelli), p. 57–58. Rome: Tipografia Poliglotta Vaticana 1968
56. Rambourg, A.: Localisation ultrastructurale et nature du matériel coloré au niveau de la surface cellulaire par le mélange chromique-phosphotungstique. J. Microscopie **8**, 325–342 (1969)
57. Rambourg, A.: Morphological and histochemical aspects of glycoproteins at the surface of animal cells. Int. Rev. Cytol. **31**, 57–114 (1971)
58. Rambourg, A., Hernandez, W., Leblond, C. P.: Detection of complex carbohydrates in the Golgi apparatus of rat cells. J. Cell Biol. **40**, 395–414 (1969)
59. Robinson, R. A., Cameron, D. A.: Electron microscopy of cartilage and bone matrix at the distal epiphyseal line of the femur in the newborn infant. J. biophys. biochem. Cytol. **2** (Suppl.), 253–260 (1956)
60. Schenk, R. K., Spiro, D., Wiener, J.: Cartilage resorption in the tibial epiphyseal plate of growing rats. J. Cell Biol. **34**, 275–291 (1967)
61. Scherft, J. P.: The resorption of the organic matrix of calcified cartilage as seen with the electron microscope. Calc. Tiss. Res. **2** (suppl.), 96–96B (1968)
62. Scott, B. L.: The occurrence of specific cytoplasmic granules in the osteoclast. J. Ultrastruct. Res. **19**, 417–431 (1967)
63. Scott, B. L., Pease, D. C.: Electron microscopy of the epiphyseal apparatus. Anat. Rec. **126**, 465–495 (1956)
64. Smith, J. W.: The disposition of proteinpolysaccharide in the epiphyseal plate cartilage of the young rabbit. J. Cell Sci. **6**, 843–864 (1970)
65. Steven, F. S.: The Nishihara technique for the solubilization of collagen. Application to the preparation of soluble collagens from normal and rheumatoid connective tissue. Ann. Rheum. Dis. **23**, 300–301 (1964)
66. Sundström, B., Takuma, S.: A further contribution on the ultrastructure of calcifying cartilage. J. Ultrastruct. Res. **36**, 419–424 (1971)
67. Takuma, S.: Electron microscopy of the developing cartilaginous epiphysis. Arch. Oral Biol. **2**, 111–119 (1960)
68. Vaes, G.: On the mechanism of bone resorption. J. Cell Biol. **39**, 676–697 (1968)
69. Vaughan, J. M.: The physiology of bone. Oxford: Clarendon Press 1970
70. Walker, D. G.: Enzymatic and electron microscopic analysis of isolated osteoclasts. Calc. Tiss. Res. **9**, 296–309 (1972)
71. Weinstock, A.: Elaboration of enamel and dentin matrix glycoproteins. In: The biochemistry and physiology of bone, 2nd ed., vol. II (ed. G. H. Bourne), p. 121–154. New York and London: Academic Press 1972
72. Weinstock, A., Leblond, C. P.: Elaboration of the matrix glycoprotein of enamel by the secretory ameloblasts of the rat incisor as revealed by radioautography after galactose-^{3}H injection. J. Cell Biol. **51**, 26–51 (1971)
73. Woessner, J. F., Jr.: Biological mechanisms of collagen resorption. In: Treatise on collagen, vol. II (ed. B. S. Gould), p. 253–330. London and New York: Academic Press 1968