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Bone Changes in Hemodialyzed Uremic Subjects

Comparative Light and Electron Microscope Investigations*

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Summary. Needle biopsies from the iliac crest of 40 uremic patients treated with hemodialysis have been compared by light and electron microscopy. The most obvious bone changes were represented by an increased amount of osteoid tissue (osteomalacic changes) and by enhanced bone resorption. The osteomalacic changes were chiefly characterized by the presence of thick osteoid borders whose collagen fibrils were often completely uncalcified. In a few cases, small roundish aggregates of crystals were irregularly present through the osteoid matrix; some of them were closely related to roundish, electron-dense bodies surrounded by a membrane.

The increased rate of bone resorption, which was often comparable to that which occurs in the most severe cases of primary hyperparathyroidism, was due to both osteoclastic activity and osteocytic osteolysis. Electron microscopy showed that the enlargement and irregularity of the osteocytic lacunae were not always due to osteocytic osteolysis; the same effect might be due to defective calcification of the lacunar wall. The advantages of comparing the same specimens under the light and electron microscopes are discussed.

Key words: Renal osteodystrophy — Renal Osteomalacia — Secondary Renal Hyperparathyroidism.

Chronic renal failure causes skeletal changes consisting mainly of a combined picture of osteomalacia and hyperparathyroidism. These changes, which are usually collected under the denomination of renal or azotaemic osteodystrophy (Stanbury, 1957), have been extensively studied with qualitative and quantitative histology (Binswanger et al., 1971; Duursma et al., 1972; Ellis and Peart, 1973; Garner and Ball, 1966; Ingham et al., 1973; Ireland et al., 1969; Krempien et al., 1972b, 1973; Mankin, 1974; Olah, 1973; Ritz et al., 1973a, Stanbury, 1968; Tatler et al., 1973) and with electron microscopy (Bonucci et al., 1975; Maschio et al., 1974).

Similar skeletal changes have been reported in uremic subjects periodically treated with hemodialysis (Binswanger et al., 1973; Bishop et al., 1972; Bonomini and Bortolotti, 1975; Delling, 1972; Katz et al., 1969; Krempien et al., 1972a; Nichols et al., 1972; Parfitt et al., 1972; Ritz et al., 1973a, b; Woods et al., 1972). However, comparative histologic examination of bone from dialyzed and nondialyzed uremic subjects have produced conflicting results (Huffer et al., 1975), so that further investigations seem advisable. Moreover, to our knowledge electron microscope investigations on renal osteodystrophy of dialyzed subjects

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have not yet been carried out, while they could be very useful for fully understanding the morphological changes which occur in bone during maintenance hemodialysis. For these reasons, a comparative histological and electron microscope investigation has been carried out on needle biopsies from the iliac crest of 40 uremic patients treated with hemodialysis.

Material and Methods

Needle biopsies were taken from the iliac crest of 40 patients with chronic renal failure submitted three times every week to hemodialysis. They had not been treated with vitamin D and received unrestricted diet. None of the patients had been dialyzed for less than 2 months; most of them had been treated for 2–6 years. The metabolic and hormonal disorders found in these patients have been reported elsewhere (Mioni et al., 1976).

The bone specimens were immediately fixed in 4% paraformaldehyde buffered to pH 7.2 with phosphate or cacodylate buffers. They were successively reduced to small fragments, post-fixed with 1% osmium tetroxide buffered as above, and embedded in Araldite without decalcification.

Semithin sections (1 μ thick) were examined under the light microscope after staining with Azure II–Methylene blue. To evaluate the presence and extent of calcified areas, semithin sections were stained with Alyzarin red S or with von Kossa method, in which case counterstaining with Methylene blue was associated.

Ultrathin sections (about 700 Å thick) were examined under the electron microscope unstained and after staining with uranyl acetate and lead citrate.

Results

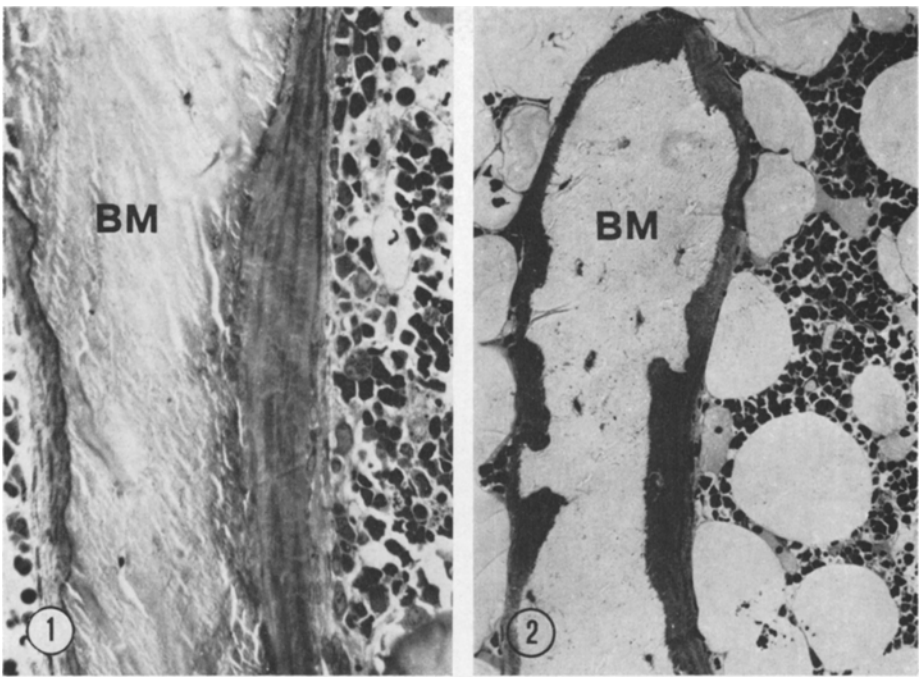
All of the patients showed osteomalacic changes and increased bone resorption. The severity of these pathological changes was greater in young than in old patients and was to some extent proportional to the duration of the dialytic therapy.

The Osteomalacic Picture

Under the optical microscope the osteomalacic changes where easily recognizable not only in sections stained with von Kossa method, but also in those stain with Azure II-Methylene blue (Figs. 1, 2). In both cases, the uncalcified osteoid tissue appeared blue, while the calcified matrix was black in the first case and unstained (white-grayish) in the second. Alyzarin red S was less efficient than von Kossa method in staining the calcified areas, probably because of the presence and relative impermeability of the embedding medium.

In all of the patients, and chiefly in the youngest and in those submitted to long-duration dialysis, severe osteomalacic changes were present. Although quantitative determination were not made, these changes were shown by the presence of thick layers of osteoid tissue bordering the osseous trabeculae and sometimes filling Howship's lacunae (Figs. 1, 2). This tissue was from 3 to 6 times thicker than in normal subjects and occasionally a whole trabecula consisted only of uncalcified osteoid matrix. A lamellar arrangement of its collagen fibrils was sometimes visible (Fig. 1); more often, the fibrils were irregularly oriented and the osteoid tissue resembled the so-called "woven" bone.

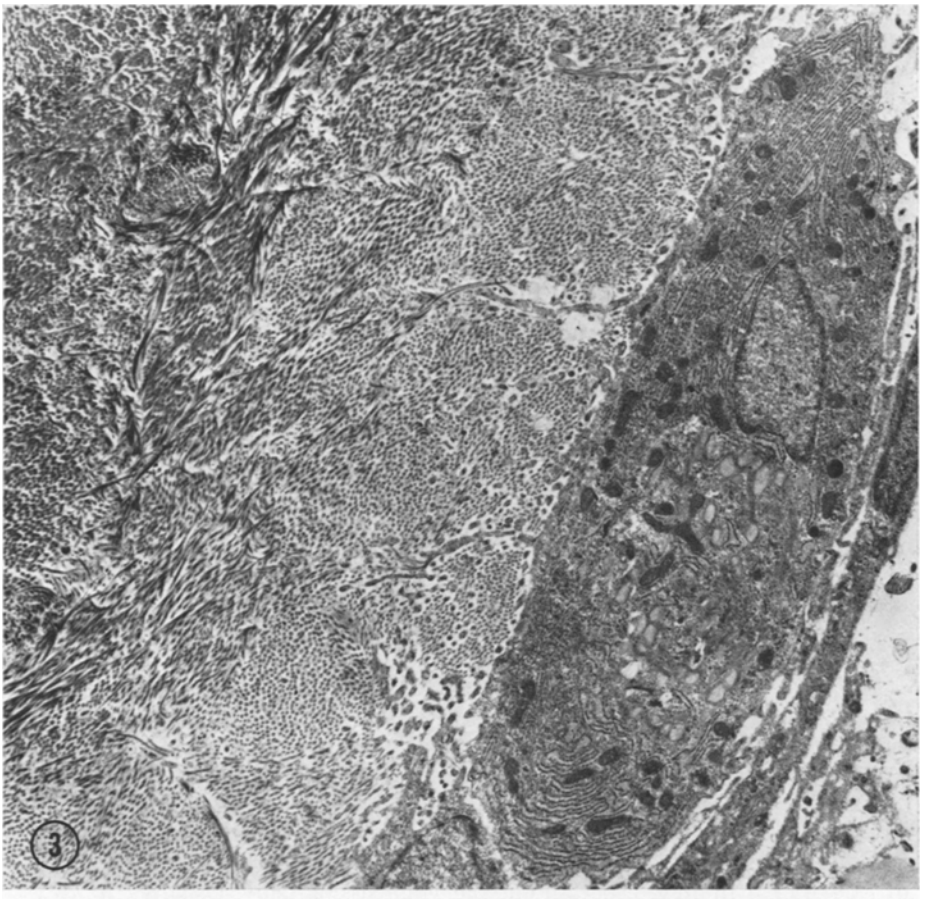
The osteoid tissue was frequently in contact with elongated, apparently inactive osteoblasts. However, the presence of large and plump osteoblasts having wide Golgi areas and apparently actively synthesizing the osteoid matrix was not



Figs. 1 and 2. Details of undecalcified bone trabeculae in sections stained with Azure II-Methylene blue; *BM* bone matrix. (1) Trabecula surrounded by thick, deeply stained osteoid tissue which has a lamellar arrangement on the right; ×350. (2) Osteoid tissue completely surrounding the calcified matrix of a trabecula whose border is irregular because of preexisting Howship's lacunae; ×190

an exceptional finding, especially in the youngest patients. Usually, the osteoid matrix appeared completely uncalcified and the calcification front was undetectable. However, in some cases small areas of calcification were recognizable, chiefly around roundish cells looking like osteocytes. Osteocyte-like cells were irregularly present through the uncalcified osteoid tissue.

Under the electron microscope, the osteoid tissue appeared to consist of loosely arranged collagen fibrils (Fig. 3) which were either collected in lamellae or, very frequently, were irregularly oriented. The single fibrils did not appear abnormal: their mean thickness was of about 700 Å and their periodic banding of about 650 Å. They were in contact either with elongated, fusiform cells or with roundish osteoblasts. The former showed a fusate nucleus and a reduced cytoplasm which contained very few rough ergastoplasmic cysternae, an apparent but not widely developed Golgi apparatus, a few mitochondria and lysosome-like bodies, and small bundles of very fine filaments often oriented parallel to the cell membrane. The roundish osteoblasts, on the contrary, showed a very developed rough ergastoplasmic reticulum, a wide Golgi apparatus, numerous and often swollen mitochondria, and elongated lysosome-like bodies containing either amorphous or filamentous electron-dense material. These osteoblasts often showed many cytoplasmic processes irregularly penetrating through the osteoid tissue.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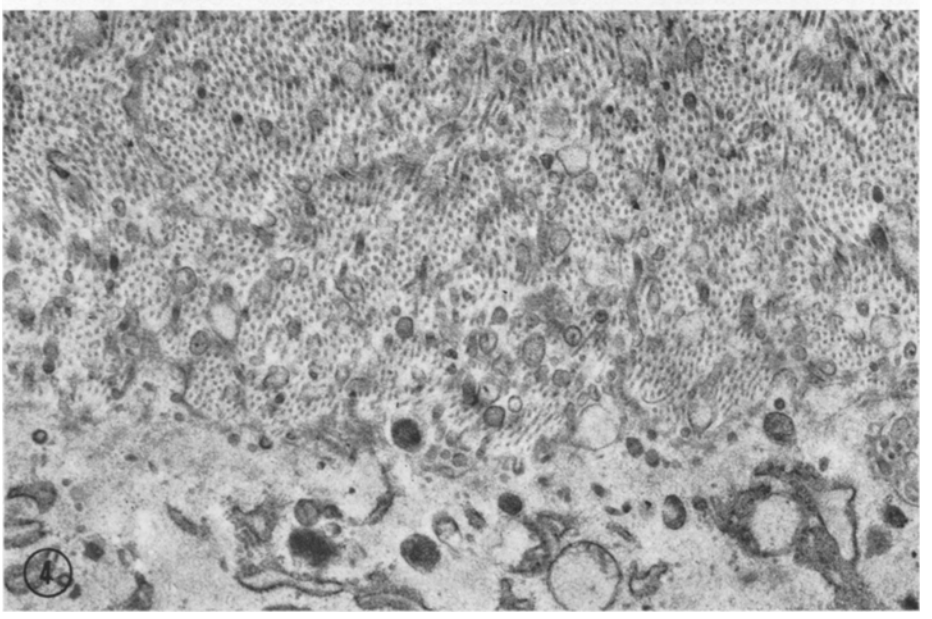
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Near these processes and between the fibrils, roundish electron-dense bodies were recognicable (Fig. 4), many of which were surrounded by a trilaminar membrane enclosing homogeneous, amorphous matrix. These bodies were irregularly distributed: there was plenty of them near some osteoblasts, and none near other osteoblasts. A few of these bodies contained thin, needle-shaped crystals having intrinsic electron density.

A few of the cells placed in contact with the osteoid tissue and having osteoblastic characteristics unexpectedly contained intracytoplasmic clusters of inorganic crystals (Fig. 5). Like active osteoblasts, these cells had a developed cytoplasm, aboundant rough ergastoplasmic reticulum, many ribosomes and wide Golgi areas. However, they contained also roundish clusters of thin, needle-like and intrinsically electron-dense structures similar to the crystals present in the calcified areas and in cytoplasmic vacuoles of the osteoclasts. Most of these clusters completely obscured the underlying structures; the smallest of them were contained within mitochondria.

Cells of variable shape were present between the collagen fibrils of the osteoid tissue (Fig. 6). Although the matrix around them was not calcified, these cells very closely resembled osteocytes. However, in respect to osteocytes thay had a relatively greater, sometimes indented nucleus with a clearly recognizable nucleolus, and a wider cytoplasm which, notwithstanding, contained few cytoplasmic organelles (Fig. 6). These cells had many cytoplasmic processes which irregularly penetrated through the uncalcified osteoid matrix and between the collagen fibrils. A very close contact was sometimes established between adjacent cells by interdigitation of their cytoplasmic processes.

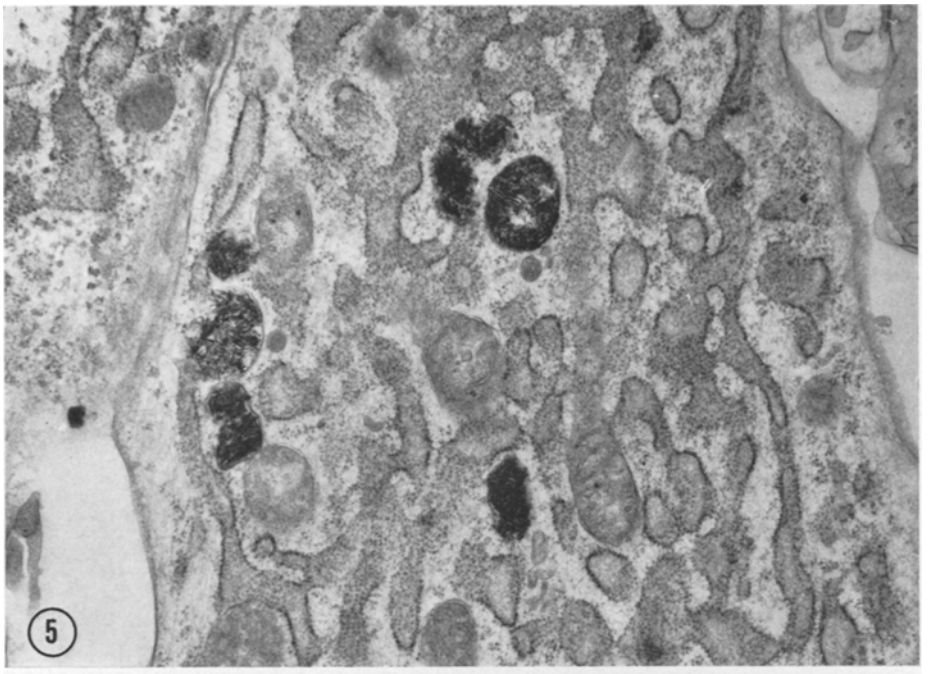
The limit between the calcified bone matrix and the osteoid tissue was usually rather sharp. A part of the cases in which scattered foci of calcification were present in the osteoid matrix (see below), the calcified matrix ended rather abruptly against completely uncalcified, randomly oriented collagen fibrils (Fig. 6). In some cases, single collagen fibrils and thin bundles of fibrils protruded like hairs of a brush from the calcified matrix towards the osteoblasts (Fig. 7). This picture was observed in areas recognizable as borders of Howship's lacunae.

Most of the osteoid tissue was completely uncalcified. However, in some cases, and independently on the age of the patients, it showed scattered areas of calcification (Fig. 8). Almost invariably, these areas were found near the fully calcified bone matrix and their concentration decreased going from this zone towards the osteoblasts.

The calcified areas consisted of very small, roundish clusters of thin, elongated crystals which completely obscured the underlying matrix (Fig. 8). In unstained

Fig. 3. Electron micrograph showing an osteoblast in contact with osteoid tissue. Note the developed Golgi apparatus of the osteoblast, the numerous mitochondria and the aboundant rough ergastoplasmic reticulum. The collagen fibrils of the osteoid matrix are loosely arranged and rather irregularly oriented. Uranyl acetate and lead citrate, $\times 7{,}000$

Fig. 4. Detail of the junction of an osteoblast (partly visible below) with the osteoid tissue. Between the collagen fibrils of the osteoid matrix many roundish structures are visible, some of which are cross-sections of cytoplasmic processes, others (the most electron-dense) are matrix vesicles. Lysosome-like bodies are present in the cytoplasm of the osteoblast. Uranyl acetate and lead citrate, $\times 13{,}000$



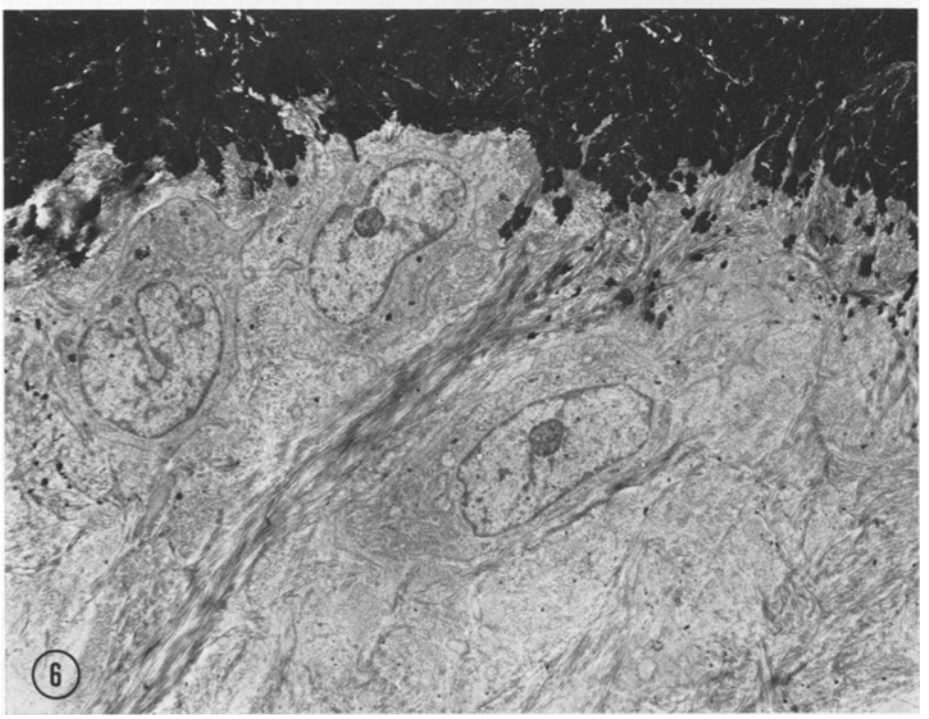


Fig. 5. Detail of on osteoblast-like cell containing clusters of inorganic crystals. Uranyl acetate and lead citrate, × 18,000

Fig. 6. Limit between normally calcified bone matrix (above) and osteoid tissue (below). Three osteocyte-like cells are recognizable between uncalcified collagen fibrils. Uranyl acetate and lead citrate, × 2,500

ultrathin sections, these crystals showed intrinsic electron density and measured about 35 Å in thickness. In some cases, they were placed within and/or on the surface of roundish bodies surrounded by a membrane and consisting of amorphous, homogeneous matrix.

Where the calcification process was more advanced, but still largely incomplete, the calcified areas were coalescing. In this case, most of the crystals had the same orientation as the axis of the fibrils. Where the calcification process was still more advanced, but not yet complete, the inorganic substance showed a close relationship with the collagen fibrils and appeared to be deposited according to the collagen periodic banding.

The incompletely calcified areas often contained roundish osteocytes whose lacunae appeared enlarged under the light microscope. The electron microscope showed that these lacunae were of practically normal size and that the enlarged appearance was due to the presence of either uncalcified or incompletely calcified matrix around the osteocytes.

The Process of Bone Resorption

All of the patients showed an increment of the bone resorption rate, sometimes to such an extent as to be predominating in respect to the osteomalacic picture.

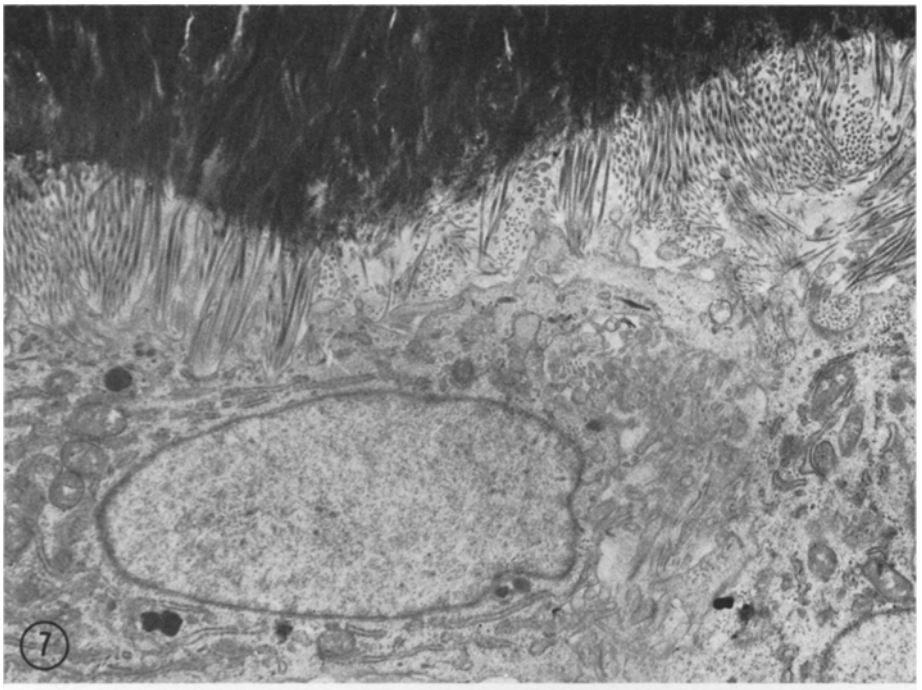
Under the optical microscope, isolated osteoclasts and osteoclasts collected in clusters were frequently observed along the border of the trabeculae (Figs. 9, 10). In some cases, they were found also within the trabeculae and consequently their activity had a dissecting character. Moreover, where osteoclasts were not present, Howship's lacunae showed that they had been there previously.

Under the electron microscope, the osteoclasts showed the same morphology as that of osteoclasts of normal subjects (Fig. 11). Usually, they were characterized by a developed ruffled border which was in contact with disrupted and disaggregated bone matrix. Isolated crystals were present between the infoldings of the ruffled border and within cytoplasmic vacuoles. From 3 to 6, and exceptionally more than 6 nuclei were visible in each osteoclast. Mitochondria were numerous and were usually collected in a cytoplasmic area opposite to that of the ruffled border. Vesicles of Golgi apparatus were recognizable near the nuclei.

In some cases, osteoclasts were found near, but not in contact with the bone matrix. In these cases, the ruffled border was lacking and mitochondria were present throughout the cytoplasm.

In other cases, osteoblasts were placed side by side with osteoblasts on the same edge of the same trabecula, so that thick osteoid borders and Howship's lacunae were at the same time recognizable along the border of the calcified matrix. Usually, the osteoid tissue was not reabsorbed by the osteoclasts, so that sometimes it remained as the only limit between large osteoclastic lacunae and bone marrow spaces (Fig. 10). However, in other cases the osteoclasts were in very close contact with the osteoid tissue and electron microscopy showed the presence of collagen fibrils within the channels of their ruffled borders as if fibril resorption was in progress (Fig. 12).

Independently on the presence of osteoclasts, most of the osteocytes showed enlarged and irregular lacunae, and the lacunae not rarely seemed to coalesce.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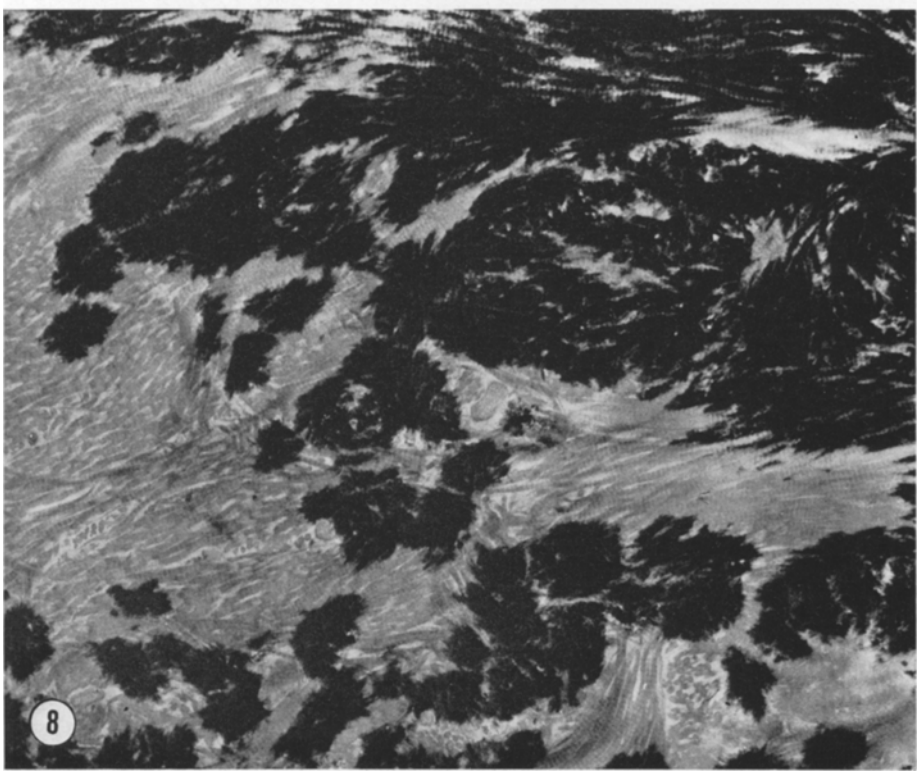
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The osteocyte canaliculi, too, were enlarged and coalescing. These pictures were controlled in seriated semithin sections which were particularly useful for establishing the true shape of the lacunae.

Under the electron microscope, most of the osteocytes appeared to be contained within enlarged and irregular lacunae (Fig. 13). The osteocytes themselves were irregular, mainly due to many cytoplasmic processes and arched outline of the cell membrane. Moreover, a pericellular space was often present. It contained irregularly distributed filamentous and flocculent material and small fragments of collagen fibrils. The mitochondria of the osteocytes whose lacunae were enlarged often contained clusters of thin, intrinsically electron-dense crystals similar to those of the calcified bone matrix (Fig. 13).

Not all of the osteocyte lacunae were enlarged and irregular. In this case, their border was not smooth due to the presence of many crystals protruding from the calcified matrix towards the osteocytic membrane. These crystals either were regularly arranged and resembled the hairs of a brush, or were disarranged, irregularly oriented and detached from the calcified bone matrix.

The bone marrow spaces of the patients with the highest degrees of bone resorption were occupied to a variable extent by fibrous tissue (Fig. 9). In some cases, this tissue was represented only by a few fibroblasts and collagen fibrils forming thin bundles which were oriented almost parallel to the osteoid border. In the most severe cases, the fibrous tissue was present in almost all of the bone marrow spaces, which were completely obliterated by thick and irregular bundles of collagen fibrils. A distinction between the fibrous tissue and the uncalcified osteoid matrix was not always possible. Elongated fibroblasts, mast cells, osteoclast-like giant cells and a few residual hemopoietic elements were present between the collagen fibrils in these cases.

Discussion

In agreement with previous investigations the present results show that in uremic, hemodialized patients skeletal changes occur which are similar to those found in bone biopsies of patients with chronic renal failure treated conservatively. This is true for both the histological and the ultrastructural changes (cfr. Bonucci et al., 1975; Maschio et al., 1974). Bone loss and "osteoporotic-like" modifications (Bishop et al., 1972; Parfitt et al., 1972; Woods et al., 1972) have not been observed. This could be due either to the fact that quantitative measurements of bone volume have not been carried out, so that a slight reduction of the bone mass may have been overlooked, or to the fact that because of different

Fig. 7. Part of Howship's lacuna after the disappearance of the osteoclast and the beginning of ossification; small bundles of collagen fibrils protrude from the calcified matrix (above) and are almost perpendicular to the membrane of an osteoblast. Uranyl acetate and lead citrate, $\times 8{,}000$

Fig. 8. Detail of incompletely calcified bone matrix; small calcified areas, partly coalescing at the upper right corner, are scattered through the matrix. Many collagen fibrils are uncalcified. Uranyl acetate and lead citrate. $\times 12{,}000$

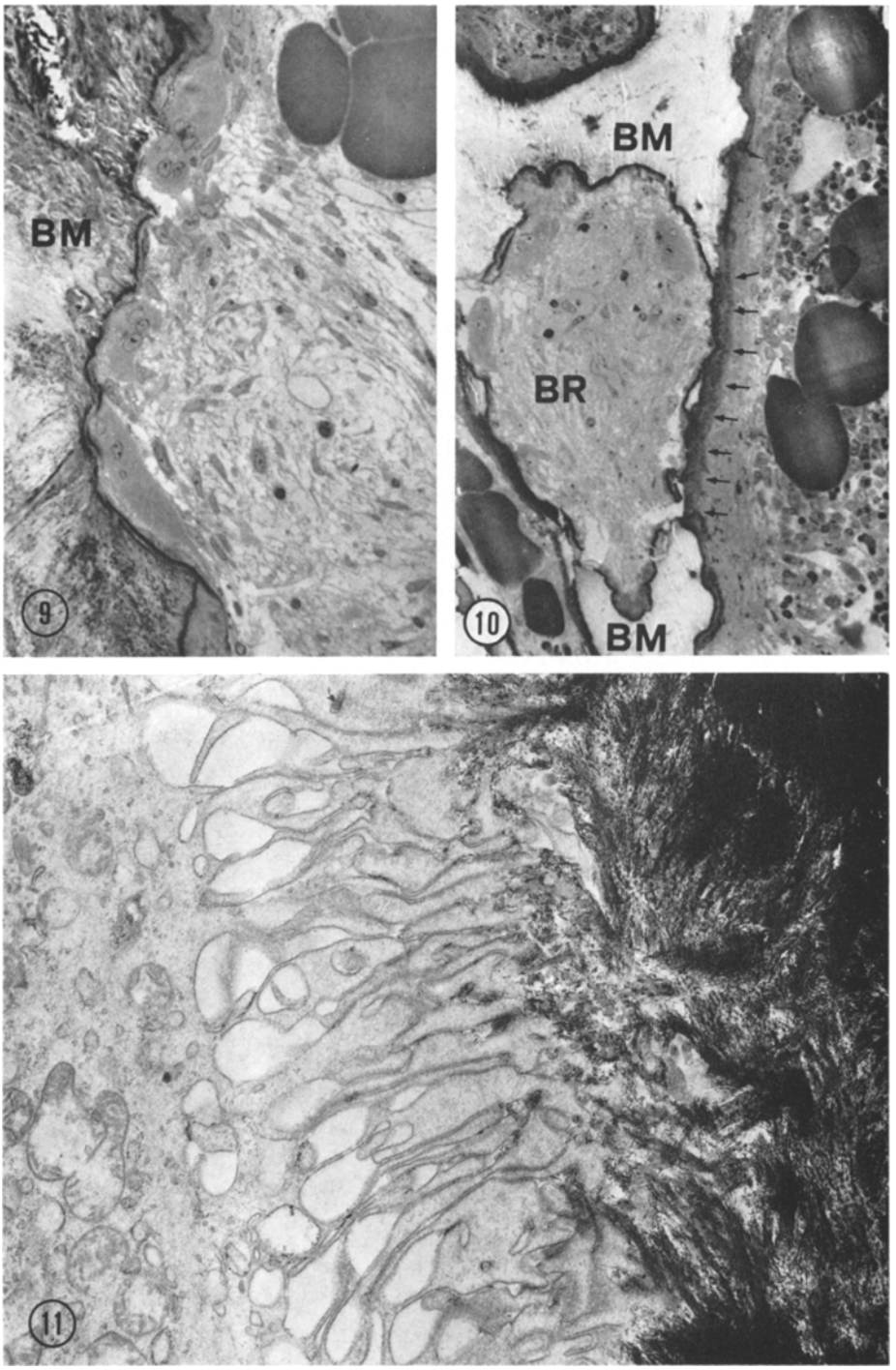


Fig. 9. Area of bone resorption; note the presence of osteoclasts in close contact with calcified bone matrix (*BM*) and the fibrosis of the marrow space. Azure II-Methylene blue, ×350

diets and therapeutic regimens osteodystrophic changes could follow different courses in different groups of patients during hemodialysis (Fiaschi et al,, 1972; Parfitt et al., 1972; Rubini et al., 1969; Stanbury, 1972).

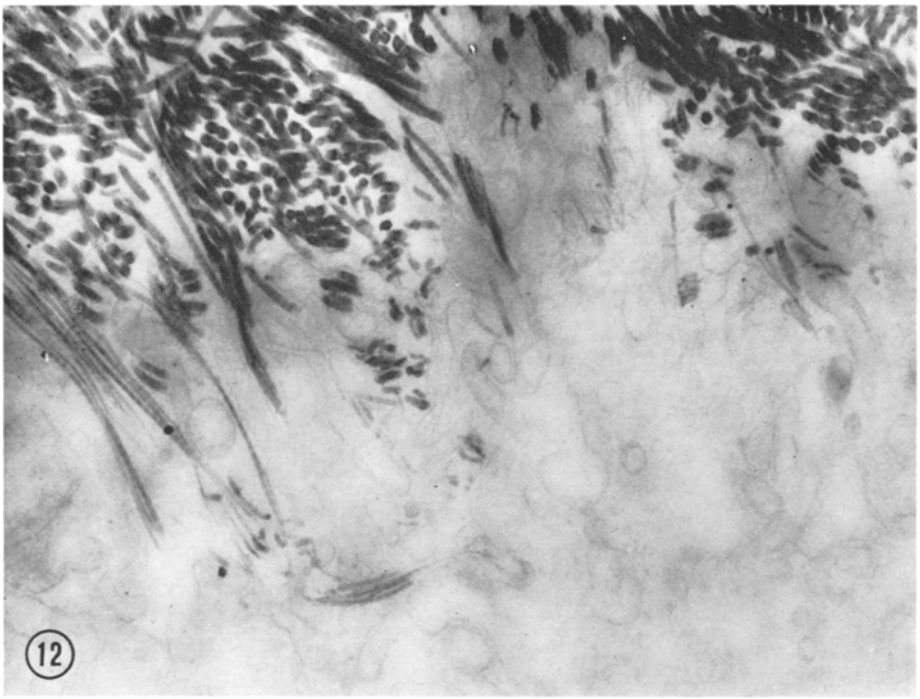
As far as the cells are concerned, two types of osteoblasts have been found, one having large cytoplasm with abundant ergastoplasmic cysternae, the second characterized by elongated shape and reduced number of cytoplasmic organelles. While the first type undoubtedly corresponds to active synthesizing osteoblasts, it is possible that the second type represents the so-called "resting" osteoblast (Dudley and Spiro, 1961). It is also possible that these cells are undifferentiated elements which could evolve along different cellular lines.

A few cells, classifiable as osteoblasts because of their cytoplasmic characteristics (chiefly their highly developed rough ergastoplasmic reticulum) showed small intracytoplasmic clusters of inorganic crystals, probably contained within mitochondria. This observation could be tentatively explained in different ways. It could be suggested that these osteoblasts had acquired osteoclastic properties in consequence of the severe secondary hyperparathyroidism (cfr. McLean, 1956) which was present in all of the patients (Mioni et al., 1976); that they had directly derived from differentiated osteoclasts (cfr. Rasmussen and Bordier, 1974), with persistence in their cytoplasm of the inorganic clusters already present in the cytoplasm of the osteoclasts; or that they were normal osteoblasts in which intramitochondrial accumulation of inorganic material had occurred in consequence of increased intracellular concentration due to hyperparathyroidism. At present, this latter hypothesis offers the most probable explanation. In fact, it is known that mitochondria are very active in controlling and regulating the intracellular concentration and movement of calcium ions and that they can accumulate both amorphous and crystalline inorganic material (Rasmussen, 1966; Lehninger, 1970).

It has been shown that in vitamin D deficiency there is increase of the extent of lysine hydroxylation (Barnes et al., 1973; Toole et al., 1972) and that the degree of crosslinks which stabilize the collagen fibrils can be reduced (Mechanic et al., 1972). The osteoid tissue observed in the present series of patients consisted of loosely arranged collagen fibrils whose fine structure was similar to that of fibris of normal subjects. The abnormal amount of hydroxylysine, if present in renal osteodystrophy as in conditions of experimental vitamin D deprivation, does not seem to cause changes of fibril ultrastructure recognizable under the electron microscope. However, as previously reported in nutritional osteomalacia (Bonucci et al., 1969), the collagen fibrils of the osteoid tissue were not so closely packed and laterally aggregated as they are in normal bone matrix. Only in areas of advanced calcification side-by-side aggregation of collagen fibrils with their

Fig. 10. Area of bone resorption (*BR*) containing osteoclasts and loose fibrous tissue; it is surrounded by calcified bone matrix (*BM*) and by osteoid tissue (arrows) which seems to have resisted the action of the osteoclasts. Azure II–Methylene blue, ×190

Fig. 11. Detail of the ruffled border of an osteoclast and of the bone matrix undergoing resorption. Uranyl acetate and lead citrate, ×15,000



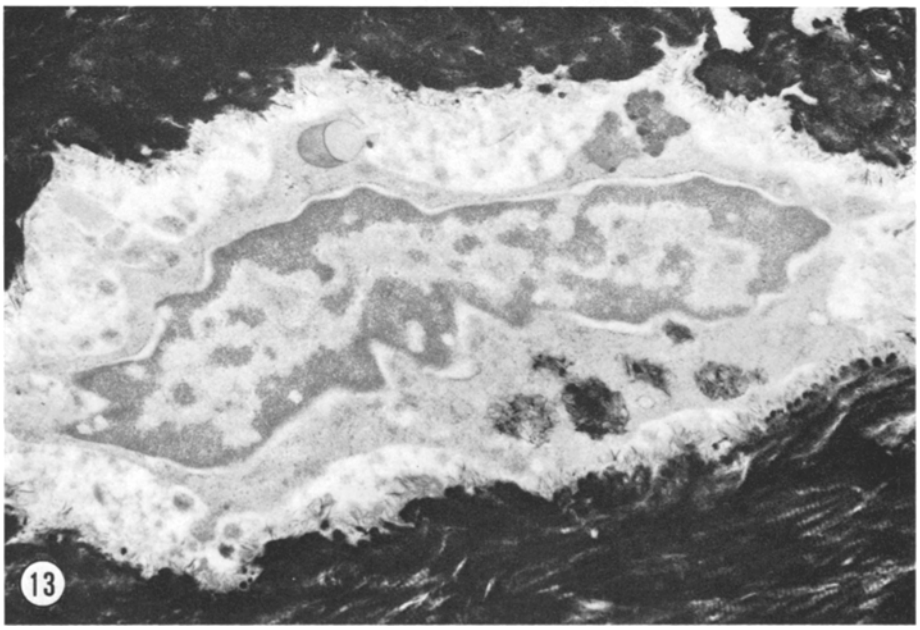


Fig. 12. Detail of an osteoclast in contact with osteoid tissue; uncalcified collagen fibrils are present between the infolding of the ruffled border. Uranyl acetate and lead citrate, $\times 24{,}000$

Fig. 13. Electron micrograph of an osteocyte with enlarged and irregular lacuna. Note crystals protruding from the calcified matrix into the lacunar space, granular and flocculent material in this space, and intracytoplasmic clusters of inorganic material. Urany acetate andlead citrate, $\times 12{,}000$

periodic banding in register was evident. It seems that the possibility of lateral aggregation of the collagen fibrils in bone is closely related to and dependent on the process of matrix calcification.

Roundish bodies surrounded by a membrane and consisting of homogeneous, amorphous, osmiophilic matrix were present between the collagen fibrils of the osteoid tissue. A few of these bodies contained clusters of crystals, showing that, as previously reported (Bonucci and Dearden, 1976), they have a calcium affinity and calcium-binding potentiality greater than those of the other components of the matrix and that they can be active in initiating the calcification process even in cases of severe osteomalacia. Most of them, however, were uncalcified, a finding similar to that observed in experimental rickets (Anderson et al., 1975; Simon et al., 1973).

The morphology of the osteoclasts found in the present series of patients was similar to that of the osteoclasts previously described in normal subjects and in animals. However, they were often small and contained few nuclei. They usually had a developed ruffled border which was in contact with disaggregated bone matrix. In some cases, they were also in contact with the osteoid tissue which, however, was not reabsorbed, confirming the previously reported observation that osteoid resists resorption much longer than the calcified matrix (Irving and Handelman, 1963; Weinmann and Schour, 1945a, b). However, in a few cases uncalcified collagen fibrils were contained within the channels of the ruffled borders as if they were reabsorbed by the osteoclasts. This finding seems to show that in cases of severe hyperparathyroidism associated to osteomalacia the bone matrix can be reabsorbed even before its calcification, as previously shown by Carnes (1958) in rachitic rats treated with parathormone.

In the present series of patients, as well as in the osteodystrophy of uremic patients treated conservatively (Bonucci et al., 1975; Krempien et al., 1973; Ritz et al., 1973a, b) and in other pathological conditions involving the skeleton (cfr. Bélanger, 1971), bone resorption was due not only to osteoclastic activity but also, and in some cases predominantly, to osteocytic osteolysis. This process is chiefly characterized by enlargement and irregularity of the osteocyte lacunae. However, it is not always recognizable on the basis of these characteristics alone under the optical microscope, because in cases associated to osteomalacia an apparent enlargement of the osteocytic lacunae can be due to osteomalacic defective calcification of the pericellular matrix rather than to periosteocytic bone resorption (Sissons, 1969; Steendijk et al., 1966). Electron microscopy has made it possible to differentiate between true and apparent enlargement of the osteocytic lacunae. As has been previously reported in other physio-pathological conditions of the skeleton (Baud, 1962; Jande, 1971, 1972; Jande and Bélanger, 1973; Remagen et al., 1969), so also in hemodialyzed patients osteocytic osteolysis was characterized by enlargement of the lacunae, irregularity of their border, and presence of a periosteocytic space which contained flocculent and granular material and fragments of collagen fibrils. Moreover, most of the osteocytes engaged in osteolysis showed intramitochondrial accumulation of inorganic material, probably due to an increased intracellular concentration of calcium ions. Conversly the pericellular space was not visible when the lacunae were enlarged in consequence of defective calcification, the osteocytes were in direct contact with uncalcified collagen fibrils and no evidence of bone matrix resorption could be found.

A few or the osteocytes showed lacunae of normal shape. Under the electron microscope, protruding crystals were frequently present along the border of these lacunae. They were either regularly arranged like the hairs of a brush, or were disarranged and detached from the perilacunar bone matrix. These crystals could have a very important role in calcium and phosphate homeostasis. They are in close contact with the osteocytic membrane and are loosely arranged, so that they could be rapidly reabsorbed by the osteocytes. For this reason, they could represent a disposable, easily utilizable reservoir of mineral ions and their removal could be the first step of the process of osteocytic osteolysis. Further studies are in progress to clarify this point.

In the present group of patients the most evident bone changes consisted of increased amounts of uncalcified osteoid tissue (osteomalacic changes) and of an enhancement of bone resorption processes (comparable to those which occur in the most severe cases of primary hyperparathyroidism). These changes were easily recognizable by using the light microscope alone, so that electron microscopy cannot be considered indispensable for diagnostic purpose. However, the comparative examination of the same biopsy by light and electron microscopy furnished information which otherwise could have been lost, particularly with regard to the structure of the osteocytes and the process of osteocytic osteolysis. Moreover, even without using the electron microscope, it is evident that cutting 1 μ thick sections from undecalcified bone biopsies embedded in epoxy resins is itself extremely advantageous: it furnishes a great improvement in the resolution of histological detail; it allows the use of undecalcified sections in which discrimination between calcified matrix and osteoid tissue is very easy; and it makes it possible to obtain an almost unlimited number of seriated sections, even of single cells.

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