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Bone Modelling Processes at the Endosteal Surface of Human Femora

Scanning Electron Microscopical Studies in Normal Bone and in Renal Osteodystrophy

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Summary. In femoral bone of 10 adult patients without bone disease and of 15 patients with secondary hyperparathyroidism, the endosteal surface of the diaphysis was studied by scanning electron microscopy after non-mineralised organic material had been removed from the endosteal surface by sodium hypochlorite. This technique permits one to analyse the effects of past osteoblastic and osteoclastic activities. In *normal bone,* the endosteal envelope shows a highly ordered texture: The main part of the inner surface is represented by fully mineralised smooth surfaces without evidence of apposition or resorption (so called neutral surfaces). In apposition areas, collagen fibers are still incompletely mineralised. Ordered mineral deposits are observed, consisting of spindleshaped calcospherites of uniform size. The resorption areas are sharply delimited. The resorption layer shows a small difference of level with respect to the surrounding neutral surface. Resorption areas consist of numerous lacunae with a smooth bottom. Individual lacunae are encircled by shallow ridges which run almost perpendicularly to the main direction of collagen fibers that have been exposed by resorption. These findings suggest that in normal bone osteoclasts act as a coordinated group of cells. The direction of advance of the resorption area is to some extent influenced by the collagen pattern of bone.

In patients with *secondary hyperparathyroidism,* domain formation of the endosteal surface can no longer be recognized. The size and shape of calcospherites are extremely heterogeneous, a finding interpreted as evidence of formation of woven bone. Resorption areas are irregularly determined and often resemble worm-eaten wood. The planes of resorption vary in direction and depth and in general resorption cavities penetrate deeper than in normal bone. These findings point to loss of coordinated cell action under the influence of hyperparathyroidism.

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The observations suggest that in hyperparathyroidism endosteal cells do not respond to local factors which influence endosteal cell activities in modelling processes of normal bone. Such local factors consist of the pattern of collagen and lamellar organisation on one hand and mechanical forces presumably via pizo-electrical potentials, on the other. In hyperparathyroidism the interdependence between bone matrix texture and spatial orientation of bone surface lining cell activities is lost.

Key words: Bone modelling – Normal bone – Renal osteodystrophy – Scanning electron microscopy.

Introduction

Bone structure and bone mass are maintained by the concerted action of systemic hormones and local mechanisms (Rasmussen and Bordier, 1974). The latter are presumably the direct or indirect consequences of mechanical factors (Pauwels, 1965; Basset, 1968). The constancy of bone geometry and bone volume points to the action of homeostatic regulatory mechanisms. The form and structure of bone are the result of both skeletal renewal by remodelling and cellular activities which change the shape of bone by modelling processes (Frost, 1963; Frost, 1966). These mechanisms respond to metabolic stimuli as well as to mechanical strain.

Skeletal homeostasis, i.e. the maintenance of form and structure of bone, is disturbed in metabolic bone disease. In patients with secondary hyperparathyroidism, the derangement of internal remodelling has been studied to a considerable extent in spongy bone, using histological (Recklinghausen, 1947; Hamperl and Wallis, 1933; Berner, 1944; Gilmour, 1947; Uehlinger, 1956; Ball, 1960; Ammann, 1963) and micromorphometric techniques (Duursma et al., 1972; Krempien et al., 1972; Ritz et al., 1973; Binswanger et al., 1974; Delling, 1975).

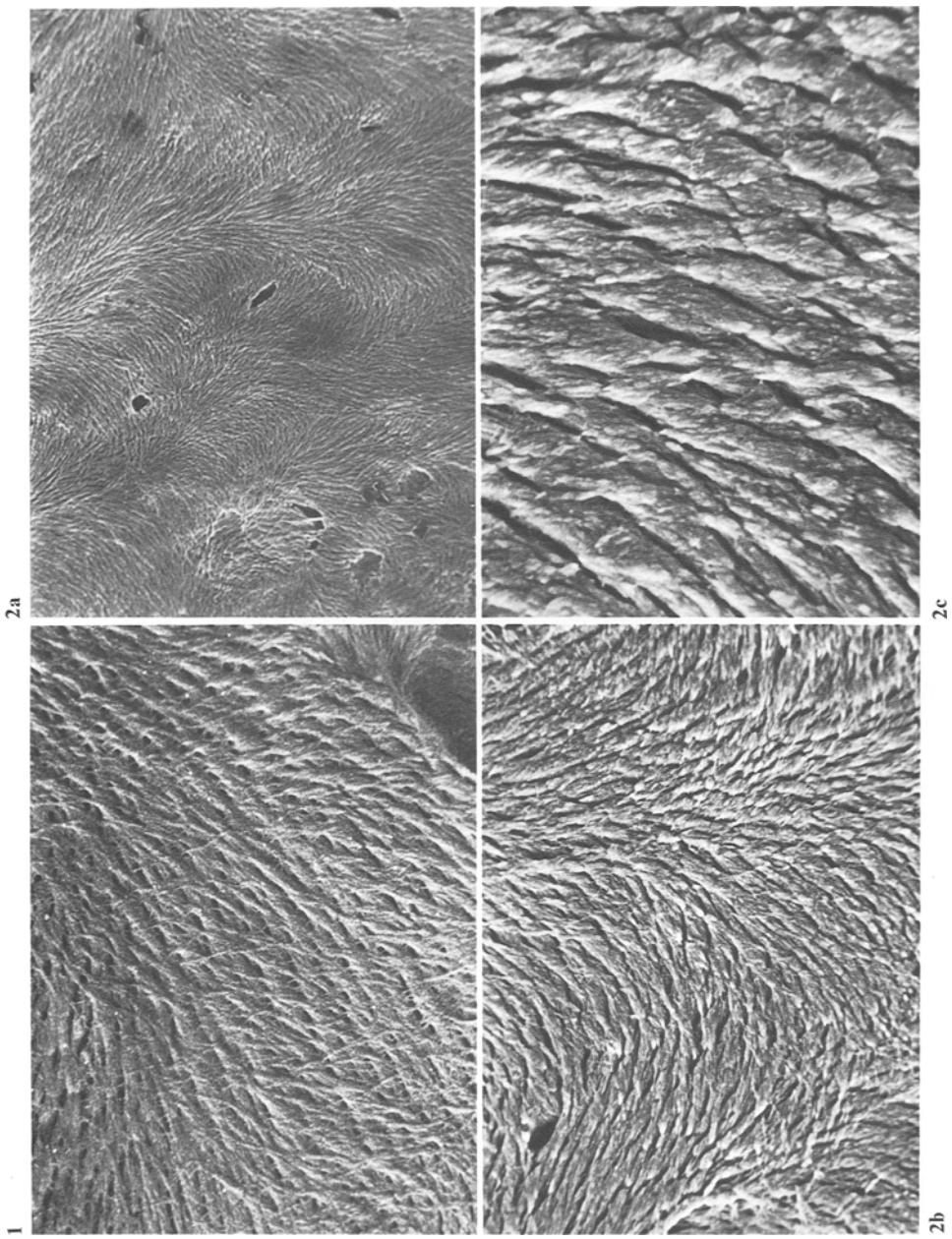
However, the influence of hyperparathyroid bone disease on modelling processes has received little attention.

The structure of bone is the result of the activities of cells lining the bone surfaces. It has been suggested that bone surface cell activities and bone surface structure are closely interdependent (Krempien et al., 1976). A disturbance of bone cell function may result in a disturbance of bone surface structures, as

Figs. 1–6. Scanning electron micrographs of the endosteal surface of diaphyses of human femora. Treatment with sodium hypochlorite

Fig. 1. Neutral surface of a control patient: smooth endosteal plane, consisting of densely packed collagen fiber bundles, which are fully mineralized and do not exhibit calcospherites. A layer of strong fiber bundles with uniform direction is covered by a layer of smaller bundles with a different parallel alignment. (Enl. 1:1,000)

Fig. 2a–c. Apposition surfaces of a control patient. **a** The endosteal surface consists of areas in which the direction of collagen fiber bundles follows one preferential direction ("domains"). The direction of collagen fiber bundles appears to alternate. Whereas the main direction of the domains runs from top to bottom of the figure, an exchange of fiber bundles between neighbouring



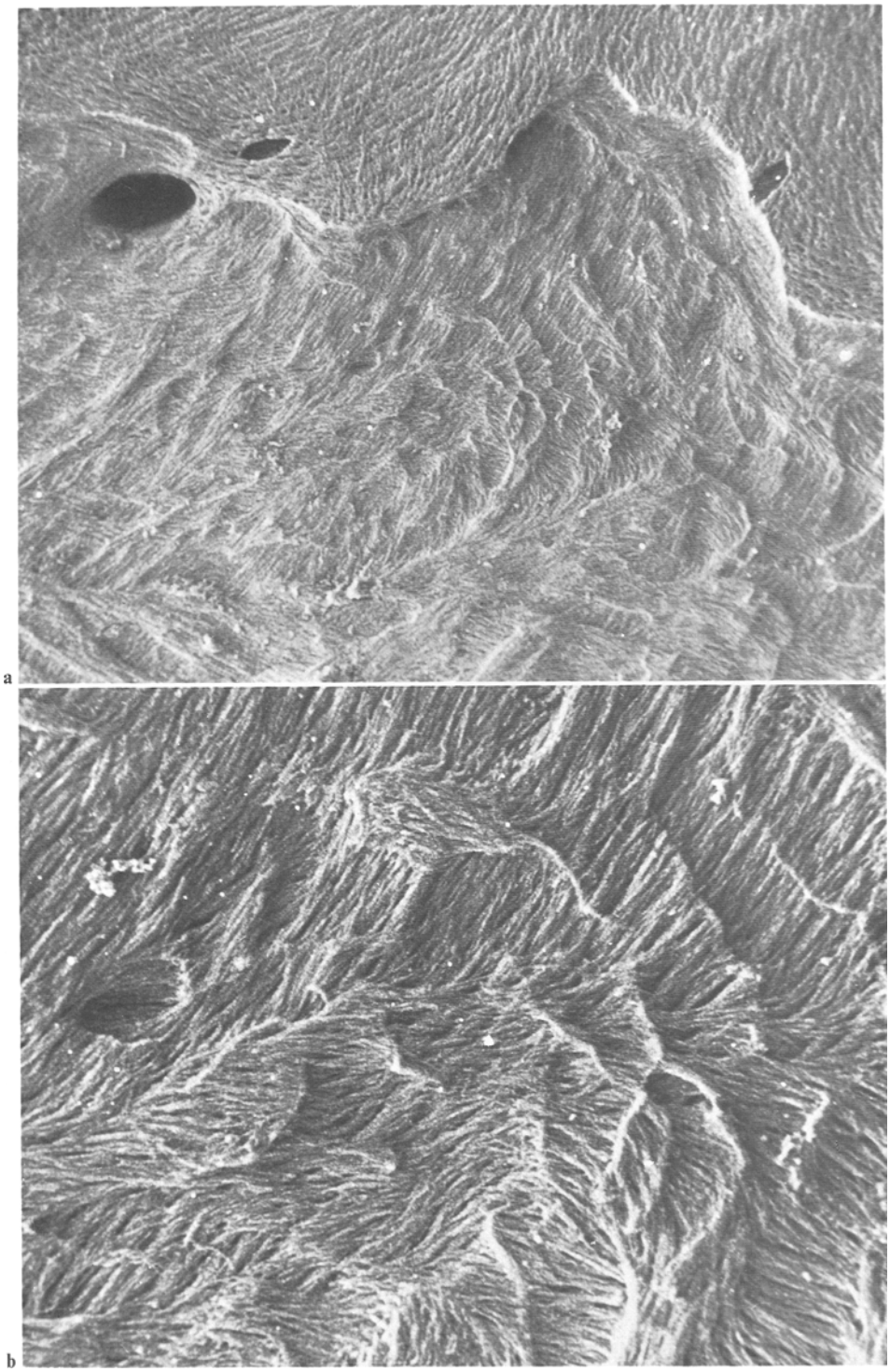
domains can be seen in the direction perpendicular to it. Few osteocytic lacunae can be recognized, which are not completely buried. (Enl. 1:300). **b** This higher magnification shows typical calcospherites. The apposition surface differs from the neutral surface (Fig. 1) because mineralization of collagen fiber bundles is not completed and interspaces between fiber bundles are still wide. Domains are clearly visible. (Enl. 1:1,000). **c** Detail of interspaces between collegen fiber bundles. Calcospherites appear as tightly packed spindle-shaped mineral deposits. Although they are composed of needle- or plate-like substructures, the surface of individual calcospherites is almost smooth. (Enl. 1:3,000)

described by Jones and Boyde (1976) and by Krempien et al. (1977). However, the question of whether the surface structure of bone can also influence the activities of bone surface lining cells has not been examined.

In the present study, the endosteal surface of femoral bone specimes of patients with and without bone disease was analysed by scanning electron microscopy. On this bone surface, endosteal cells are engaged exclusively in modelling processes. This investigation addresses the problem of whether apposition and resorption areas in hyperparathyroid patients are different from those in patients without bone disease and further, whether an interrelation between bone surface structure and bone surface activity can be demonstrated under normal and pathological conditions. Surface lining cells are rapidly destroyed by autolytic processes, thus a systematical scanning electron microscopical investigation of the endosteal cell population in human cortical bone is not possible. In order to circumvent this problem, we used a more indirect method, which allows to analyze traces of cellular activity. Surface structures were uncovered by treatment of bone specimens with sodium hyperchlorite, which dissolves the unmineralized material of bone. After this treatment, areas with different morphological characteristics can be described.

To define our terms, surface areas with completely mineralized collagen fiber bundles are called "neutral resting surfaces", because these areas are covered with resting lining cells as revealed by transmission electron microscopy. When incompletely mineralized surfaces are treated with sodium hyperchlorite, the non-mineralized part of the organic matrix is dissolved and characteristical mineral clusters, so called "calcospherites", are left behind. These surfaces are named "apposition areas", though no information is available whether these areas had been covered with active osteoblasts or not. In transmission electron microscopical studies, we confirmed that in normal bone only part of this area is engaged in active matrix synthesis. The other portion has to be considered as a "resting apposition area", where mineralization has ceased before being completed. Surfaces with traces of osteoclastic resorption are named "resorption areas". This term does not make assumptions as to whether resorption had been active or inactive (Krempien, 1979). Since the radius of curvature of the femoral cross-section is large, the small areas of the endosteal envelope that are studied by scanning electron microscopy appear nearly plane. Distortion may therefore be neglected.

Fig. 3 a and b. Resorption surfaces of control patients. **a** On top of the figure a neutral surface, underneath a resorption surface. There is an abrupt transition between both surfaces. The resorption area is flat, showing the same depth everywhere. The bottom of the resorption area is subdivided by numerous shallow ridges. To the left more pronounced funnel-shaped resorption, which opens a vascular channel. (Enl. 1:300). **b** Detail of a resorption area which shows the shallow ridges more clearly. In this higher magnification their preferential direction that runs perpendicular to the main direction of the collagen fiber bundles is evident. Within the areas, subdivided by the ridges, collagen fiber bundles show a parallel alignment. Both figures produce the impression that the osteoclasts have been moving upon the endosteal surface from the left-hand side of the figure to the right side. (Enl. 1:1,000)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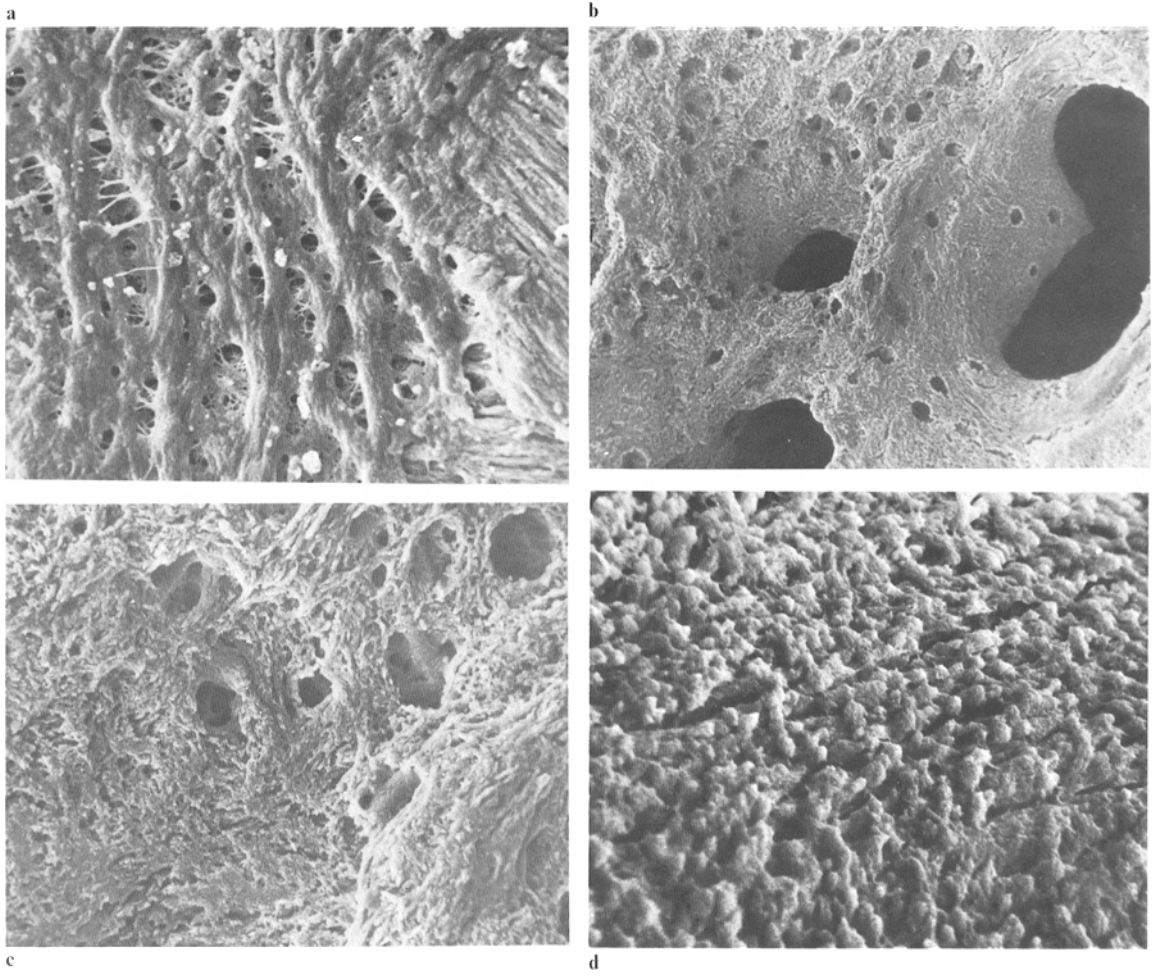


Fig. 4a–d. Neutral surfaces of uraemic patients.
a In contrast to the normal neutral surface (Fig. 1), coarse collagen fiber bundles of varying diameter run crisscross and mingle with finer collagen bundles in between. Wide gaps in the surface correspond to canaliculi. The collagen fiber bundles are completely mineralized. (Enl. 1:1,000).
b Endosteal surface of a uraemic patient with enlarged vascular channels surrounded by woven bone. Numerous osteolytic lacunae which are closely packed, irregularly distributed and incompletely buried into bone. (Enl. 1:300).
c A higher magnification shows coarse and wide gaps of canaliculi at the bottom of the osteolytic lacunae. The surrounding mineralized bone matrix looks like crusted snow. No individual calcospherites or fiber bundles are visible. (Enl. 1:3,000).
d Endosteal area with a higher degree of disorganisation. The surface consists of partly cemented, coarse and globiform knob-like mineral deposits. Mineral deposits of this kind are never found in control patients. (Enl. 1:1,000)

Material and Methods

Bone specimens were obtained during autopsy from the anterior quadrant of the right femoral mid-diaphyses of 15 patients with chronic renal failure (without dialysis) and of 10 age-matched control patients without skeletal disease that had died in traffic accidents. The mean age of both groups was 35 years with a range from 25–47 years.

The tissue was stored in 70% ethanol. Bone marrow and unmineralized bone matrix were subsequently removed with 7% sodium hypochlorite.

The specimens were then washed with distilled water, dehydrated in ethanol-acetone, defatted and air dried. The endosteal surface of specimens measuring 1×1 cm was coated with gold and examined by scanning electron microscopy (Joel JS 1).

Results

Determination of the different types of surface area planimetrically was not possible, for especially in severe cases of secondary hyperparathyroid bone disease, it is not possible to delimite resting areas from apposition and resorption areas with certainty.

a) Normal Individuals

The endosteal surface of femoral diaphyses of individuals without skeletal disease exhibits fully mineralised smooth *neutral surfaces* when studied by scanning electron microscopy (Fig. 1). The texture of collagen fiber bundles can easily be recognized since the arrangement of bone mineral crystals follows that of collagen fiber bundles. The bundles are quite uniform in thickness and are deposited side by side, exhibiting a highly ordered pattern.

Apposition areas are considerably less frequent (Fig. 2a–c). In such areas of bone formation, regular "domains" can be seen (Fig. 2a). These domains are the result of the concerted action of a group of osteoblasts, which deposit collagen fiber bundles in one prefered direction. Since collagen fiber bundles are incompletely mineralised, their unmineralised part is dissolved by sodium hypochlorite. The other part, that had already been enveloped by mineral crystals, is left behind. These mineral deposits, which are called "calcospherites", are regularly spindleshaped macrocrystals (Fig. 2b). The ordered alignment of calcospherites represents the underlying order of collagen fiber bundles. At a higher magnification the calcospherites show a substructure of densely packed plates and needles (Fig. 2c).

Within the endosteal surface, resorption areas are regularly outlined and sharply delimited (Fig. 3a). Individual resorption areas show a uniform depth. This leads to the conclusion that osteoclasts penetrate in a coordinated manner, removing one lamella after the other. Consequently, the bottom of the resorption area is smooth. The bottom is subdevided by shallow ridges, which encircle the areas of Howship's lacunae (Fig. 3b). Within the lacunae, the regular fibrillar texture of collagen is exposed and can be easily identified. There is a suggestion that the direction of the ridges mentioned above is determined by the direction of the collagen fiber bundles, running perpendicular to their preferential direction.

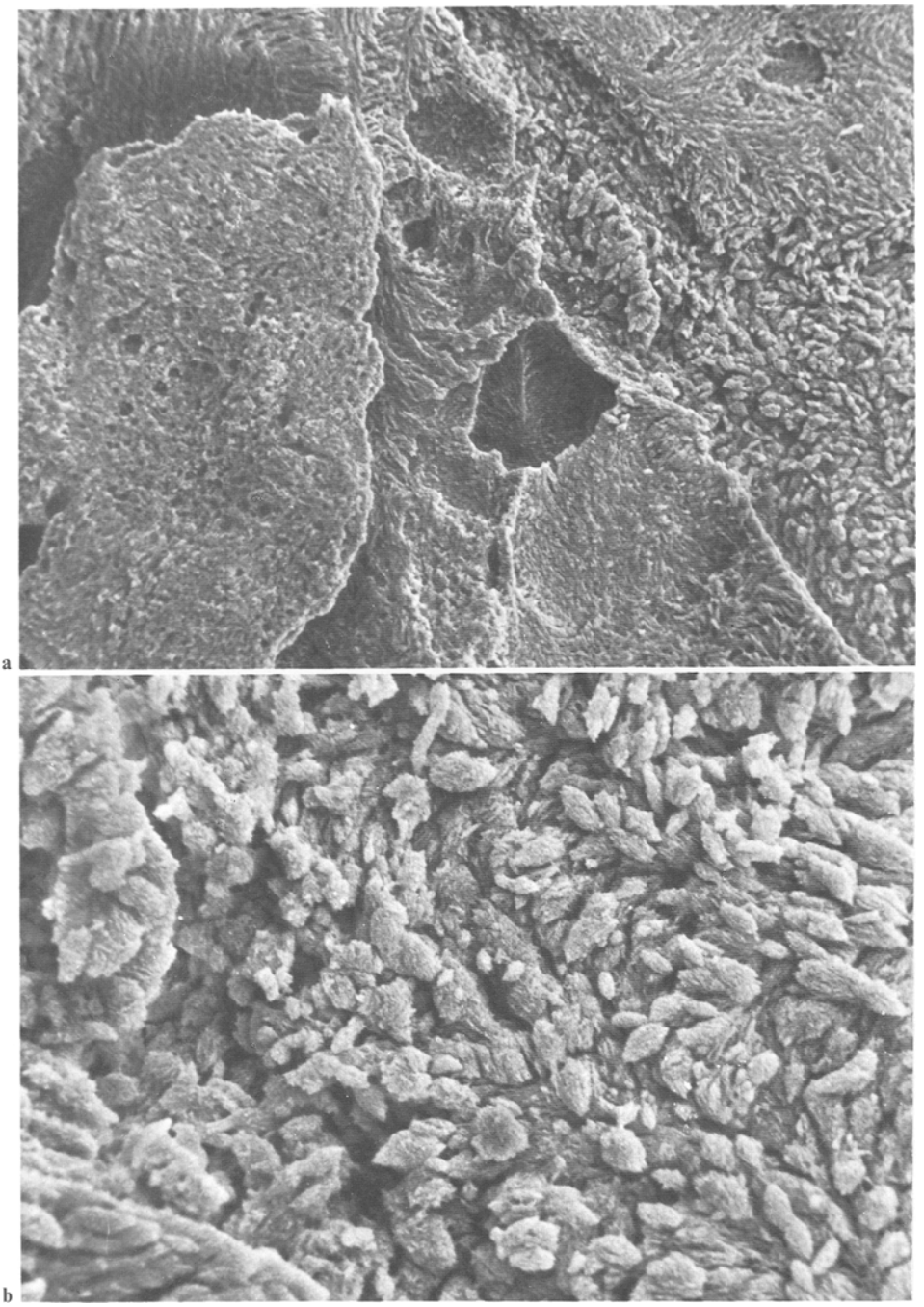
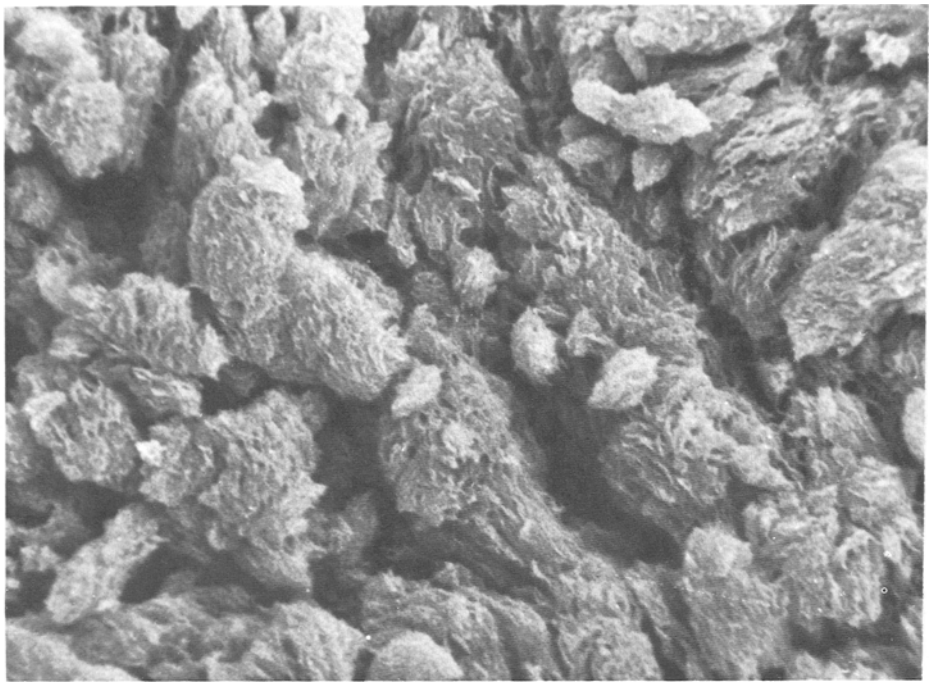


Fig. 5a–c. Apposition surfaces of uraemic patients. **a** To the right an apposition area with completely irregular pebble-like deposits. To the left there is an elevated plateau, consisting of bone with woven collagen texture and rising above the level of the surrounding bone surface. In the vicinity of the plateau are traces of resorption. In the middle the opening of a vascular channel is seen. (Enl. 1:300). **b** Detail of the apposition area of Fig. 5a: Numerous pebble-like deposists of varying size and shape without domain formation. Note clustering of the deposits (left top) and loose packing of the deposits (underneath), leaving wide empty spaces in between.(Enl. 1:3,000). **c** Detail of Fig. 5b: The mineral deposits are highly irregular in size, their surface is frayed, exhibiting disordered bunches of poorly defined needles. This observation demonstrates that not only the macrostructure but also the microstructure of the mineral is completely disturbed in secondary hyperparathyroidism. (Enl. 1:6,000)



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b) Individuals with Secondary Hyperparathyroidism

In patients with secondary hyperparathyroidism the neutral surface of the endosteal envelope is composed of normal bone with a normal ordered texture in some areas and newly formed woven bone in others (Figs. 4a and b, 6a–d). In contrast to normal bone, the surface is not smooth, but interrupted by incompletely "buried" osteocytic lacunae of variable size, which form densely packed clusters (Fig. 4a and b). As far as mineralised collagen fiber bundles can be identified, they look coarse and frayed (Fig. 4c). In woven bone a disordered, partly homogeneous, partly disrupted mineralised bone matrix takes the place of well defined mineralised collagen fiber bundles (Fig. 4b). As a striking feature in bone specimens of uraemic patients we found areas with globiform mineral deposits of variable size, partly cemented together (Fig. 4d). This finding is interpreted as a gradual transition between apposition and neutral surfaces.

A further typical finding in bone specimens of uraemic patients is a confusion of resting areas and of areas of apposition and resorption (Fig. 5a). Compared with normal bone, the part of the endosteal surface which can be characterized as the *apposition area,* is increased. Such apposition areas no longer show the typical "domain" formation. Calcospherites are extremely variable in size and shape. In contrast to normal bone they show no ordered alignment and are distributed in dense clusters with wide gaps in between (Fig. 5b). The substruc-

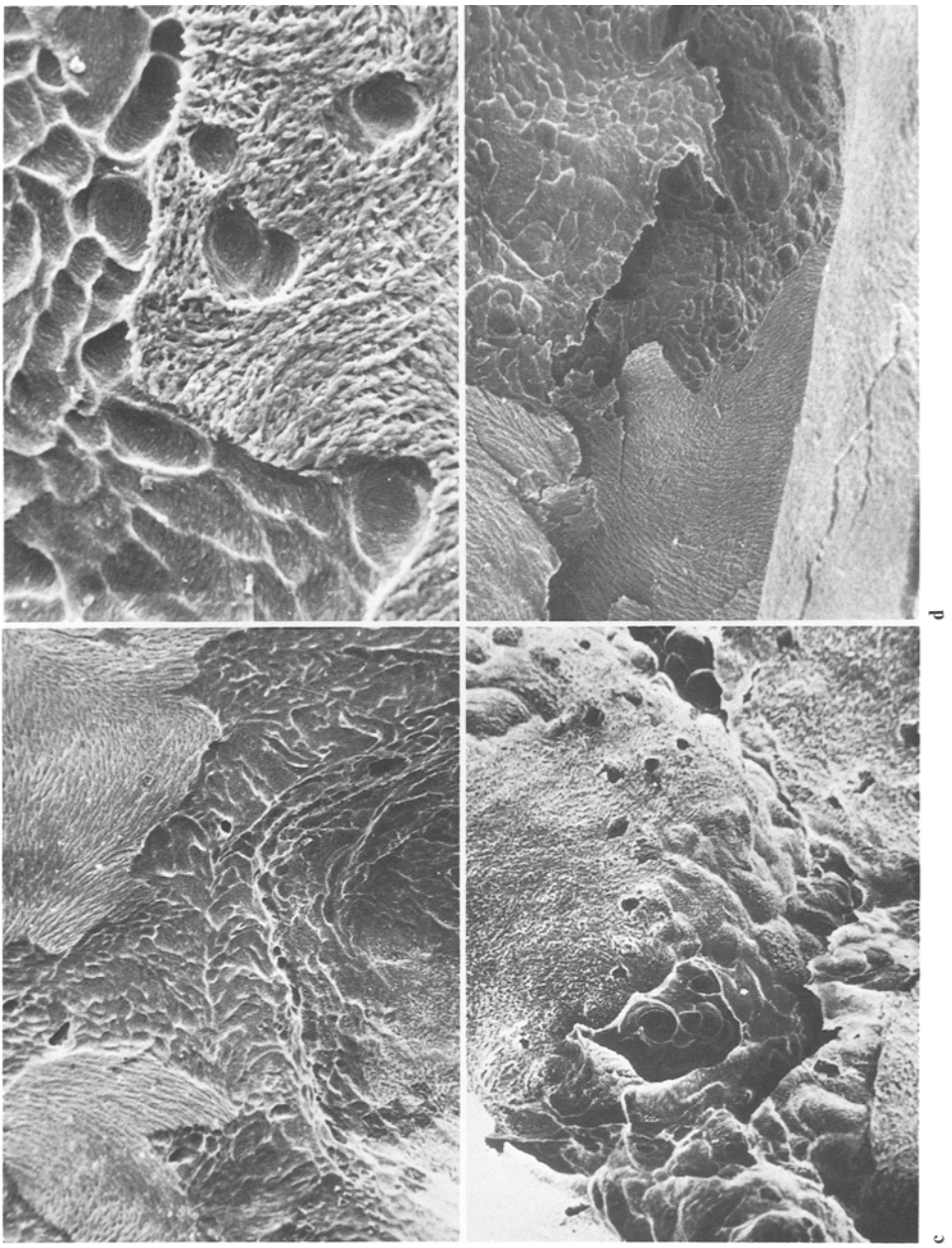

Fig. 6a–d. Resorption areas of uraemic patients. **a** Neutral surface at the top, resorption area at the bottom of the figure, The resorption area is not plain, but descends in several irregular steps (in this particular view from the top to the bottom). The resorption cavities, separated by ridges on the bottom of the resorption area are highly irregular in size and outline. (Enl. 1:300). **b** Transition between resorption area (on top) and neutral resting surface (on the bottom of the figure). Note the resorption areas within the resting surface which are not connected to the large resorption area on the top of the figure. Individual resorption cavities penetrate more deeply into bone than in normal controls. (Enl. 1:1,000). **c** Irregular resorption area with deeply penetrating, funnel-shaped cutting cones, which consist of numerous individual resorption cavities. (Enl. 1:300). **d** Regular lamellar normal bone on the left. At the top right a resorption area is seen. Note the flint-blade-like remnant of cortical bone which protudes over another area of resorption. This apperance results from undermining osteoclastic resorption of cortical bone, leading to a highly irregular endosteal surface and to spongiosation of cortical bone. (Enl. 1:100)

ture of the calcospherites consists of loosely packed filamentous crystals, which are irregularly situated in contrast to normal bone (Fig. 2c) (Fig. 5c).

There is a marked increase in *resorption areas* at the expense of neutral endosteal surfaces. Resorption areas are irregularly delimited and have serrated fuzzy borders (Fig. 6a). Occasionally, distinct resorption areas are no longer visible, their dissappearance leaving a maze of furrows and tunnels (Fig. 6c). As a consequence, the endosteal surface of cortical bone resembles worm-eaten wood in places (Fig. 6b). Resorption areas tend to penetrate far more deeply than they do in the bone of non-hyperparathyroid patients (Fig. 6b). The bottom of the resorption areas is no longer regularly subdivided by shallow ridges, but deep troughs are visible, which may reflect an exaggerated resorptive activity of osteoclasts (Fig. 6a–d). At places, undermining osteoclastic resorption results in overhanging cortical plates (Fig. 6d). This finding suggests that osteoclasts no longer resorb lamellae sequentially. This constributes to "spongiosation" of cortical bone in hyperparathyroid bone disease.

Discussion

The endosteal envelope of cortical bone is covered by osteoblasts and osteoclasts which are engaged in surface modelling processes throughout life. With scanning electron microscopy, one is able to visualize the traces left from past action of bone surface cells. Since, in contrast to the tetracycline double labelling technique, no time marker is available in SEM-studies, no conclusions can be drawn on rates of cellular activities using this methodology.

Cellular activity at the endosteal surface of cortical bone differs from that at the surface of cancellous bone in several respects. In cancellous bone, bone surface cells are primarily involved in bone *remodelling,* which is related to two different functions: the adaptation of skeletal structure to load and the homeostasis of plasma calcium. In contrast, at the endosteal surface of cortical bone, bone surface cells are particularly involved in bone *modelling,* a process which mediates changes of bone shape and bone mass. The activity of the cells at the endosteal surface of cortical bone is thought to be influenced mainly by mechanical factors. This becomes obvious under conditions of faulty loading (Pauwels, 1965; Frost, 1973). These observations lead to the conclusion that the endosteal cells in cortical bone are primarily under the control of local factors, which result from static and dynamic deformation of bone tissue.

The present study demonstrates a comprehensive disturbance of the endosteal modelling processes in femora of patients with secondary hyperparathyroid bone disease. This disturbance is shown at different structural levels of the endosteal envelope: firstly at the level of the entire endosteal surface which shows a disordered composition of neutral surfaces, apposition and resorption areas; secondly, at the level of these different surfaces, which show an altered pattern of collagen fiber bundles and calcospherites; and thirdly, at the submicroscopical level of individual fiber bundles and calcospherites.

The endosteal envelope of the femoral diaphyses in patients without bone disease consists mainly of fully mineralized neutral surfaces. However in uremic

patients a preponderance of apposition and resorption areas at the expense of neutral surfaces is generally observed. This preponderance is due to increased bone turnover induced by the oversecretion of parathyroid hormone, in turn related to a disturbed mineralization process. The different surface areas, which are clearly marked off in normal bone, cannot be specifically identified in uraemic patients. It is conceivable that in these cases remnants of highly ordered normal bone are found next to bone tissue of disorganized texture. The degree of the derangement of the endosteal surface depends on both the duration and the severity of renal insufficiency. In individuals with advanced secondary hyperparathyroidism even the fully mineralized surface areas differ from normal: they exhibit an irregular orientation of collagen fiber texture and show numerous enlarged osteocytic lacunae, which are incompletely buried in bone. Both features are hallmarks of woven bone.

In *normal femoral bone,* areas of bone formation (whether active or inactive) can be recognized by collagen fiber bundles that are not completely mineralized. When the technique of sodium hypochlorite digestion is used the non-mineralized organic material is removed and the inorganic portion is left behind. The remaining mineral deposits, so-called "calcospherites" follow the main axis of collagen fiber bundles. The substructure of the spindle-shaped macrocrystals is composed of needles and plates, which are packed close together. Numerous investigators have shown that during the process of mineralization a close interaction exists between collagen fibers on the one hand and mineral deposits on the other (Zipkin, 1973; Höhling et al., 1974). In *apposition surfaces,* collagen fiber bundles are deposited in parallel, forming a lamellar pattern. The calcospherites are deposited between these collagen fiber bundles and more or less closely reflect their pattern. Bone surface areas with uniform orientation of calcospherites and presumably also of collagen fiber bundles have been described as "domains" (Jones and Boyde, 1976).

In *hyperparathyroid bone disease,* domain formation can no longer be recognised. The alignment of calcospherites is highly disordered, and their size and shape varies greatly. Furthermore, a severe disturbance of their substructure can be recognized. Similar alterations of the substructure of calcospherites were obtained in experimental chronic uremia, rickets and after a prolonged administration of parathyroid hormone (Krempien et al., 1977). Whereas the loss of the regular alignment of calcospherites might be caused by a disturbance of regular collagen fiber arrangement, the disarray of the substructure of calcospherites leads to the assumption that the mineralization process is itself affected.

The factors which determine the regular orientation of collagen fiber bundles in the matrix of lamellar bone are unknown. According to Jones and Boyde (1976) and Jones et al. (1977), formative cells must be able to move upon the bone matrix surface in order to achieve a parallel orientation of the collagen fiber bundles. Other authors have proposed that contraction of the cytoskeleton is involved in the spatial orientation of collagen fiber bundles (Weinger and Holtrop, 1974). A disorganized "woven" texture of the bone matrix is commonly encountered in the skeleton of individuals with hyperparathyroid bone disease (Krempien et al., 1975; 1978). This observation presumably indicates that in

hyperparathyroid bone disease the formative cells have lost their abiltity to deposit collagen in preferential directions by coordinated action of cohorts of cells.

In uraemic patients surfaces with globiform and conglomerated mineral deposits and poorly defined fibrillar texture were identified, in addition to the types of surfaces allready defined. We suppose that these areas represent transitional stages between the initial and final steps of mineralization and that they may be considered to be an expression of a prolonged mineralization process in connection with delayed matrix formation. Similar findings have been described by Lindenfelser et al. (1974) in cases of idiopathic necrosis of the femoral head. Whether these findings are caused by an increased formation of non-fibrous bone matrix – as proposed by Lindenfelser – cannot be decided using scanning electron microscopy.

Resorption areas at the endosteal envelope of *normal bone* are characterized by their uniform depth and their almost uniformly smooth bottom. We interpret this finding as evidence of cellular coordination of osteoclasts, which obviously determines the advance of the plane of resorption at the endosteal envelope. Since individual collagen fiber bundles can be traced clearly at the bottom of resorption areas, one must conclude that in normal bone osteoclasts resorb one lamella after the other. The resorptive activity of osteoclasts at the endosteal surface of femoral diaphyses is thought to be mainly directed by mechanical factors, which act on bone cells via pizo-electrical signals in the micro-environment. Such signals might be generated by the known ability of collagen to produce pizo-electrical potentials under the influence of deformation (Basset, 1968). At a higher magnification, shallow ridges can be recognized at the bottom of resorption surfaces. The exact significance of such ridges remains unknown. It is conceivable that they delimite the resorption areas of neighbouring osteoclasts or that they corresponde to successive resorption phases of individual osteoclasts. The ridges run almost perpendicularly to the main direction of collagen fiber bundles, which are exposed at the bottom of the resorption areas. It is therefore tempting to suppose that the spatial movement of osteoclasts during normal bone resorption is determined, to some extent, by the fiber pattern of the underlying bone matrix.

In patients with *secondary hyperparathyroidism,* such evidence of intercellular coordination between osteoclasts is largely lost. In these patients, in whom cells are exposed to excessive levels of parathyroid hormone, analysis by scanning electron microscopy shows irregularly outlined areas of resorption that penetrate deeply into cortical bone. This finding must be interpreted as evidence of uncoordinated removal of bone tissue by individual osteoclasts or groups of osteoclasts. The resorption front apparently no longer proceedes in a direction perpendicular to the endosteal surface, but rather in all directions without coordination and without respect to bone matrix fiber pattern.

The findings in bone resorption and bone apposition areas lead to the conclusion that under the influence of high levels of parathyroid hormone osteoblasts and osteoclasts are no longer responsive to local signals of mechanical, pizo-electrical or chemical nature. We can only speculate on whether the cells loose

their ability to perceive such signals, or the ability to adjust their activity according to the signals they receive.

Lerchenthal (1974) has emphazised that in hard tissue the action of mechanical forces leads to an inhomogeneous stress in cell membranes and may thus produce local differences in dipole density and dipole orientation on the surface of such membranes. These differences may produce attractive or repulsive forces that are capable of orientating the agglomeration pattern of cells. There is some evidence that the agglomeration pattern of cells is able to influence the spatial orientation of newly formed fiber matrix.

The value of Lerchenthal's speculation in understanding the alteration of intercellular coordination in hyperparathyroidism is not yet clear. In this context it is interesting to note that in animal experiments the absence of mechanical factors due to immobilisation leads to an increased reactivity of osteoblasts to exogenous parathyroid hormone (Krempien et al., 1977). We may suppose that systemic hormonal factors and local factors are interdependent in their effect on bone cell morphology and bone cell function. Consequently, the relative activity of one of these factors alters the effectiveness of others.

Parathyroid hormone modulates cellular calcium homeostasis (Rasmussen and Bordier, 1974). Since calcium has been shown to play a critical role in the assembly and disassembly of microtubuli (Weisenberg et al., 1972), it is tempting to assume that the effect of parathyroid hormone on bone cell movement, contraction or coordination is related to its effect on the cytosol calcium and the cytoskeleton (Krempien and Ritz, 1978). A close interaction of formative cells on apposition surfaces is suggested by the observation of Jeansonne et al. (1972), that dye, when microinjected into one single osteoblast, rapidly spreads into neighbouring cells. This observation leads to the assumption that lowresistance nexus exist between osteoblasts (Weinger and Holtrop, 1974) and it is conceivable that parathyroid hormone affects the number or the properties of such intercellular channels. This assumption might explain why such cells are no longer able to interact. When large doses of parathyroid hormone are administered to rats for a prolonged period, one observes a loss of domain formation similar to that seen in patients with hyperparathyroid bone disease (Krempien et al., 1976).

In conclusion, analysis of the traces of cellular activity on the endosteal surface of cortical bone in the femoral diaphysis suggests that in hyperparathyroid bone disease the signals coordinating interaction between bone surface cells and between bone matrix and bone surface lining cells, are no longer effective.

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