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Action of 1,25-Dihydroxycholecalciferol on Cartilage Mineralization and on Endosteal Lining Cells of Bone

Experimental in vitro Studies*

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Summary. The aim of our study was to answer the following questions: 1) Does 1,25-dihydroxycholecalciferol influence the mineralization process directly? 2) How does 1,25-D_3 act on the endosteal cell envelope of bone, which is thought to be engaged in the moment to moment regulation of calcium homeostasis? We studied the endosteal surface of calvaria and the epiphyseal cartilage of rachitic rats in a tissue culture system in order to avoid the secondary effects of endogenous hormones or mineral sources. Investigations were carried out using transmission and scanning electron microscopy. Compared with untreated rachitic control specimens, 1,25-D_3 (4×10^{-8} M) did not enhance the mineralization process. We therefore conclude that the established in vivo effect of 1,25-D_3 on the mineralization process must be an indirect one. Within 2 h the flat-shaped surface lining cells of calvaria showed a rapid transformation into globiform cells and a loss of cell adhesion. Within two days a second slower transformation into spindle-shaped fibroblast-like proliferating cells occured. All changes of cell shape were reversible within several days in the absence of 1,25-D_3. Obviously, bone matrix fiber texture influences the spatial movement of proliferating cells. The endosteal cell envelope is considered to act as a functional membrane of bone. Starting from this consideration we conclude that the changes of cellular shape described are the morphological basis for the action of 1,25-D_3 in the regulation of calcium homeostasis in bone at the cellular level.

Key words: 1,25-dihydroxycholecalciferol – Mineralization – Endosteal cell envelope – Calcium homeostasis.

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*** To Prof. Doerr on the occasion of his 65th birthday

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Introduction

Since 1,25-dihydroxycholecalciferol (1,25 D_3) is the most potent metabolite of vitamin D available, it has attained clinical importance for the therapy of vitamin D deficiency and of vitamin D resistance. The two known target organs of 1,25 D_3 are intestine and bone. Whereas the action of 1,25 D_3 on intestinal calcium absorption has been widely clarified (DeLuca 1979; Haussler and Mc Cain 1979), its action on the skeleton is only incompletely investigated. It has been clearly demonstrated that 1,25 D_3 heals osteitis fibrosa and osteomalacia (Coburn and Massry 1980), but the question is open, whether this improvement is caused by a direct action of 1,25 D_3 on the mineralization process or by an indirect effect resulting from increased calcium absorption through the gut. Nevertheless, 1,25 D_3 directly activates bone resorption (Raisz et al. 1972). However, the action of 1,25 D_3 on bone cells which are not involved in bone resorption, for instance endosteal lining cells, is unknown. These bone surface lining cells have been postulated to be engaged in the moment to moment regulation of calcium homeostasis (Neuman and Ramp 1971; Davis et al. 1975). We therefore tried to answer the following questions:

1) Is there a direct effect of 1,25 D_3 on the mineralization process?

2) How does 1,25 D_3 act on the endosteal cell envelope of bone?

The experiments were done in rachitic rats. In order to avoid systemic effects of other hormones like parathyroid hormone and in order to eliminate hormonal effects on other mineral sources like the intestine or mineralized bone, we used a tissue culture system with epiphyseal cartilage and calvaria.

Material and Methods

Pregnant Wistar rats were kept in darkness and fed a vitamin D deficient, low phosphorus diet. The newborn offspring of these rats were kept in the same conditions and showed symptoms of severe rickets at the age of four weeks. Calvaria were excised under anesthesia and immersed immediately into culture medium (37° C). Then the pachymeninges were carfully removed. The epiphyseal cartilage of the proximal tibia was excised without adjacent bone, using a razor blade. All preparation procedures were done under sterile conditions. The following experiments were performed at 37° C under an H_2O supersaturated atmosphere of 95% air and 5% CO_2 using Hanks BSS with 10% heat inactivated fetal calf serum:

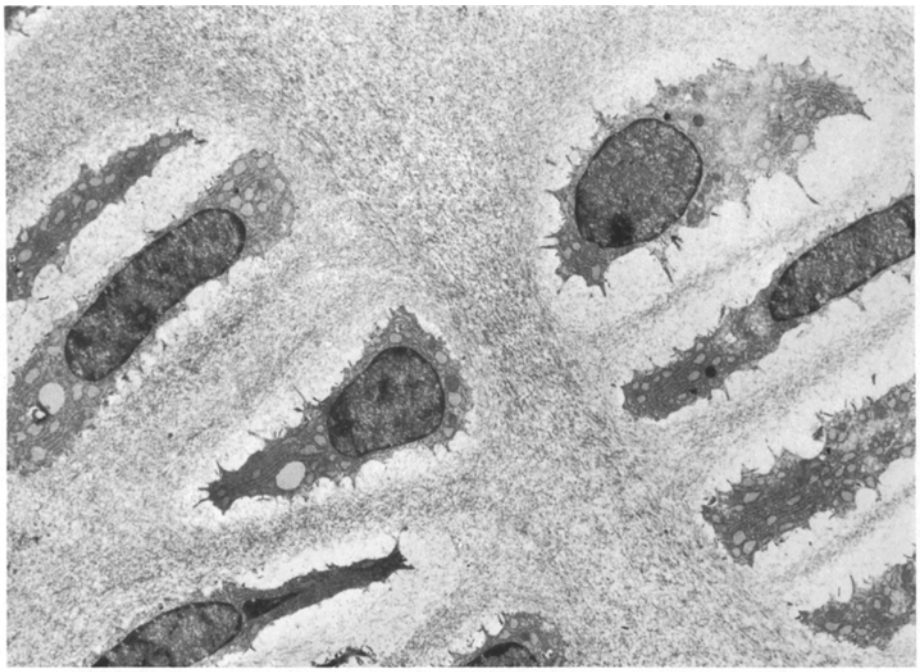
1) Tissue specimens (calvaria or cartilage) were in cubated for an incubation period of 1–7 days with or without 1,25 D_3 (4×10^{-8} M)[1].

2) In a recovery experiment calvaria were incubated with 1,25 D_3 (4×10^{-8} M) for 1 day, then transferred into a medium without 1,25 D_3 and cultivated for a second period of 1–7 days.

3) Changes of the endosteal cell envelope, which were induced by 1,25 D_3 (4×10^{-8} M), were studied at hourly intervals from 1–24 h.

The culture medium (with or without 1,25 D_3) was renewed at day 3 of the incubation period. Cartilage and bone specimens were fixed by immersion in 2% glutaraldehyde and cacodylate buffer at pH 7.4 and 37° C. Cartilage specimens were postfixed in osmium 1% for 2 h and embedded in Spurrs medium. Ultrathin sections were prepared using a diamond knife and stained with uranyl-acetate and lead according to Reynolds. Rat calvaria were dried by the critical point drying method and covered with gold using a sputter coater. TEM studies were performed with a transmission electron microscope ZEISS EM 9, SEM studies with a scanning electron microscope JEOL JS 1.

[1] 1,25 D_3 was a gift of E. Hoffmann-La Roche Co.



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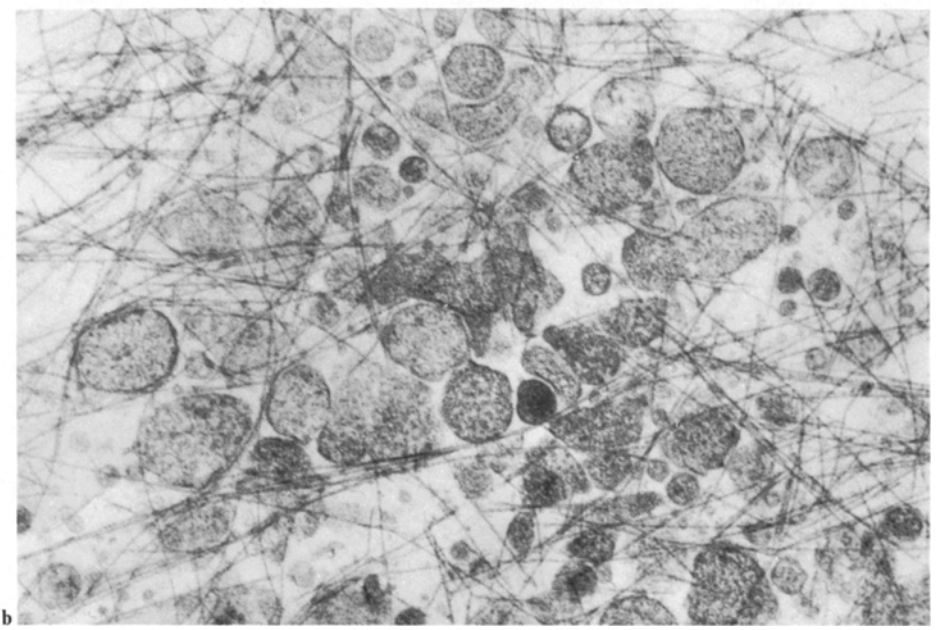

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Fig. 1a and b. Cartilage of a rachitic rat incubated for one day without 1,25 D_3. **a** broad and unmineralized longitudinal septum between two columns of chondrocytes. TEM, enl. 1:5,100. **b** longitudinal septum of **a** in detail with numerous type I and type II vesicles. No mineral is visible. TEM, enl. 1:60,000

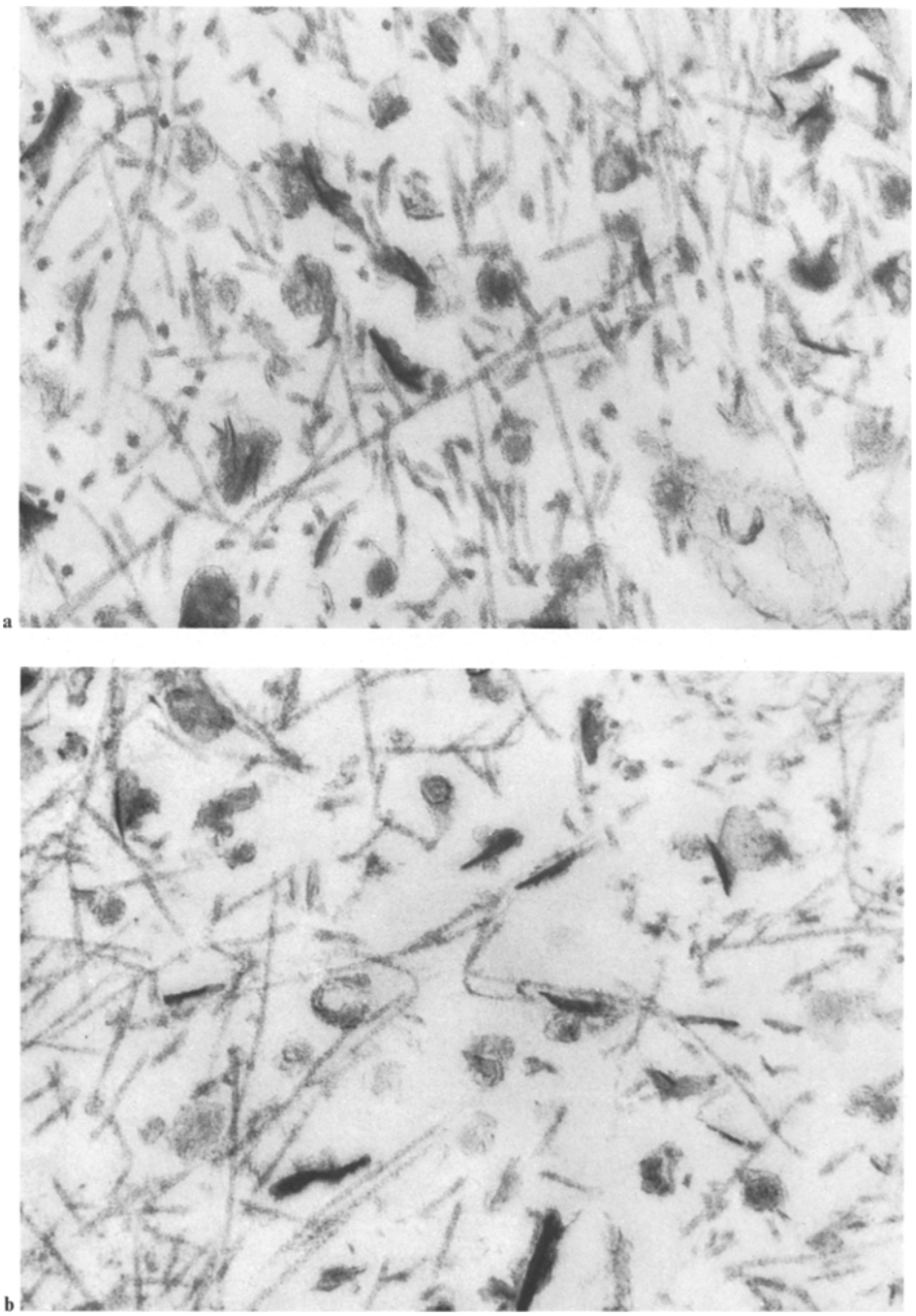


Fig. 2a and b. Second day of the incubation period. Longitudinal septa of rachitic cartilage. Needle shaped mineral crystals within the matrix vesicles and outside of them. There is no obvious difference in the degree of mineralization between cartilage, which has been incubated (**a**) without and (**b**) with 1,25 D_3. TEM, enl. 1:124,000

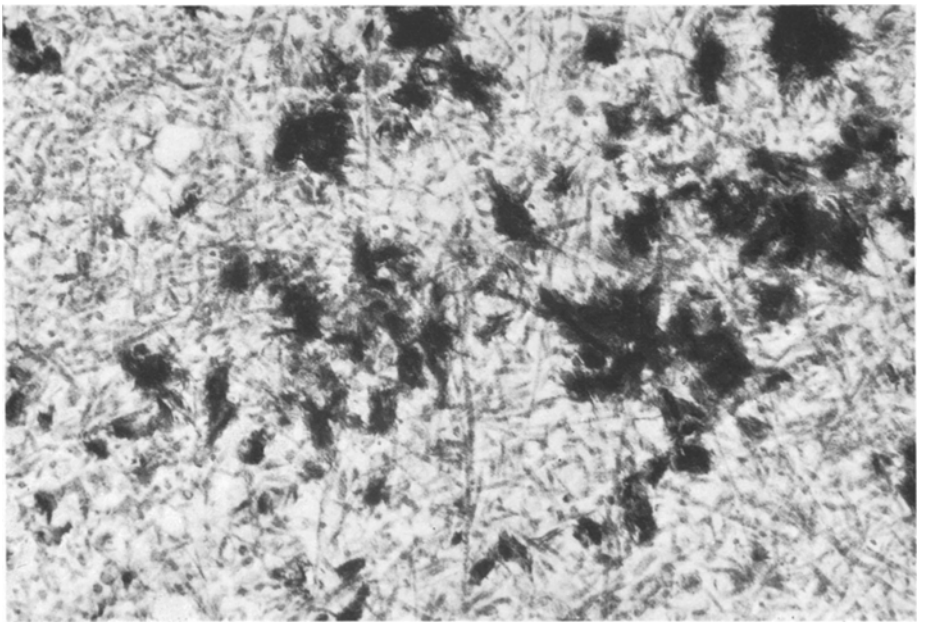


Fig. 3. Third day of incubation without 1,25 D_3. Longitudinal septum of epiphyseal cartilage with dense mineral clusters of needle shaped crystals. No matrix vesicles visible. TEM, enl. 1:45,000

Results

Using TEM we were not able to detect any significant influence of 1,25 D_3 on the mineralization process of rachitic cartilage in vitro. At the beginning of our experiment rachitic cartilage contained many type I and II vesicles, which were completely unmineralized (Fig. 1 a and b). After an incubation period of 1 day (with and without 1,25 D_3) the first mineral crystals appeared within or outside type II vesicles (Fig. 2a and b). The formation of mineral clusters started at day 3 (Fig. 3). At day 6 (with and without 1,25 D_3) longitudinal septa showed a nearly complete mineralization (Fig. 4a and b).

Calvaria of rachitic rats (incubated without 1,25 D_3 and studied by SEM) were covered by a dense layer of flat surface lining cells (Fig. 5a). In contrast to normal rats of the same age, these lining cells did not pocess surface structures like microvilli, which are typical for active osteoblasts. These findings were obtained during the incubation period of 7 days (Fig. 5b). When 1,25 D_3 was added, dramatic changes of surface lining cells appeared: all cells were rounded off and transformed into globiform cells. They lost their contact to the underlying bone matrix and to neighbouring cells (Fig. 6a). These changes were often accompanied by a rupture of the cell membrane. The transformation started earlier than two hours after incubation. In the following days of incubation with 1,25 D_3 an increasing number of spindle shaped cells appeared and the globiform cells disappeared (Fig. 6b). At day 3 streams of proliferating fibroblast-like

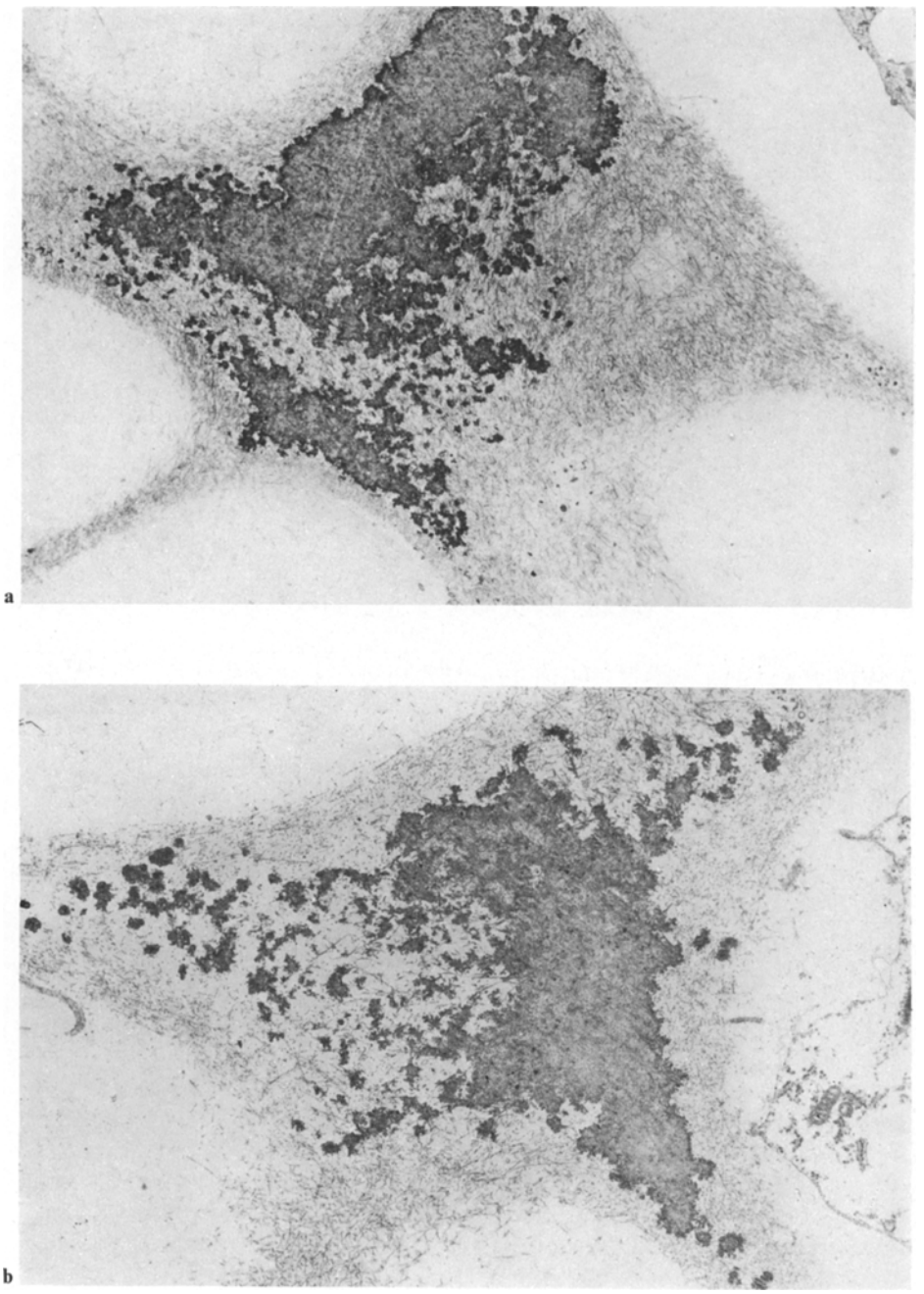
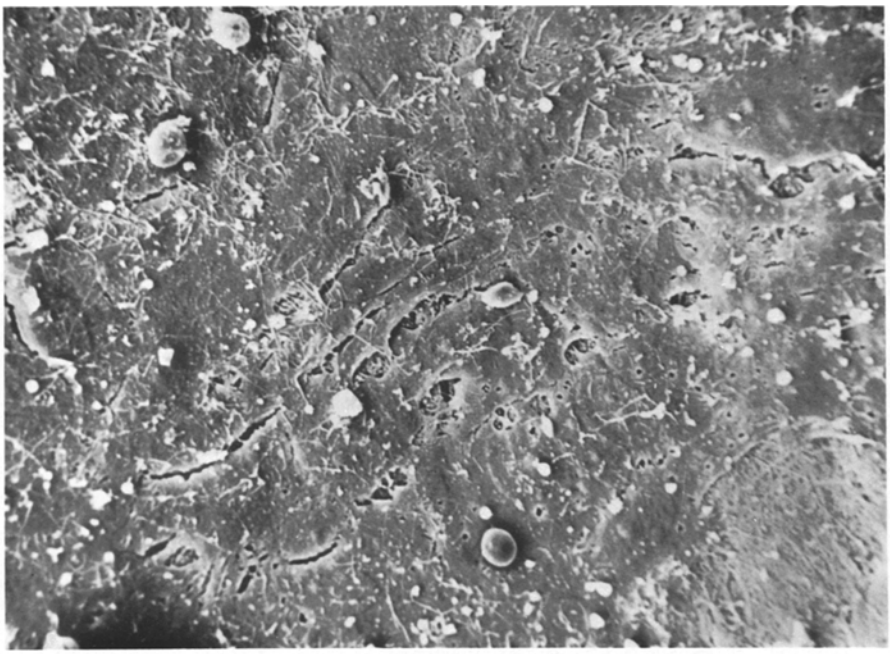


Fig. 4a and b. Horizontal section through longitudinal septa of rachitic cartilage at day 6 of the incubation period. The specimen of (**a**) has been incubated without 1,25 D_3, the specimen of (**b**) with 1,25 D_3. Both septa are completely mineralized. TEM, enl. 1:3,400



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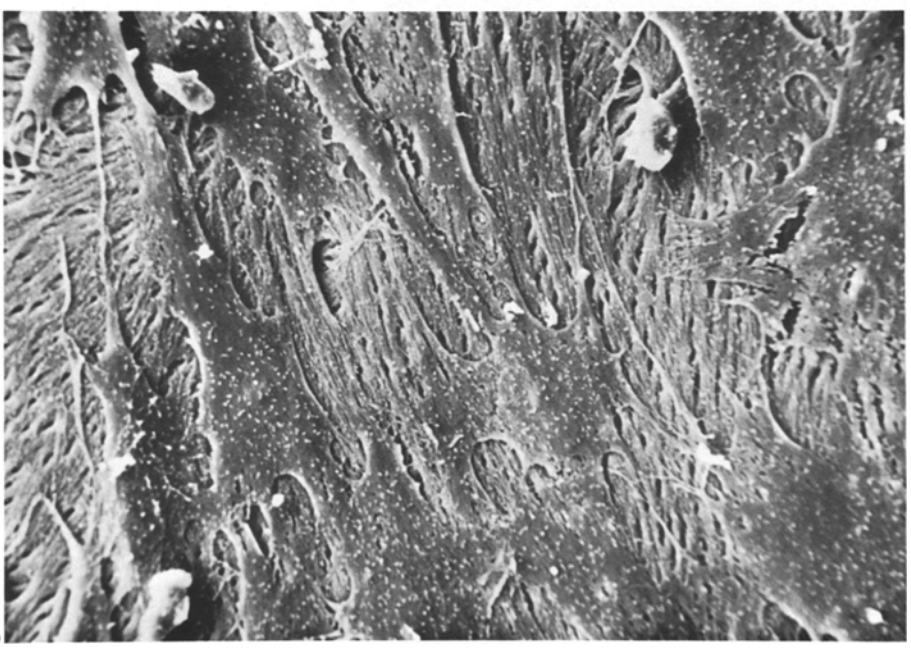


b

Fig. 5. a Calvarium of a rachitic rat, which has been incubated for 1 day without 1,25 D_3. The endosteal surface is covered by a monolayer of extremely flat and thin surface lining cells. The cell membranes are nearly smooth exhibiting only few microvilli. SEM, enl. 1:300. **b** endosteal cell envelope of rat calvaria at day 6 of the incubation period without 1,25 D_3. The shape of the surface lining cells has not changed during the incubation period. The endosteal surface of bone is partly visible. SEM, enl. 1:1,000

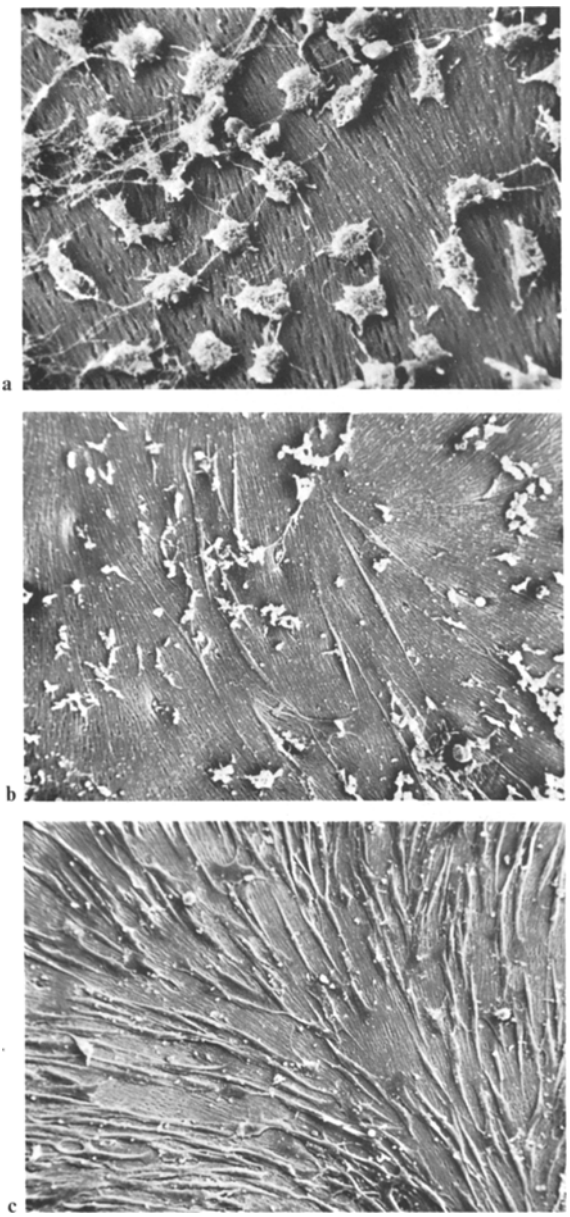
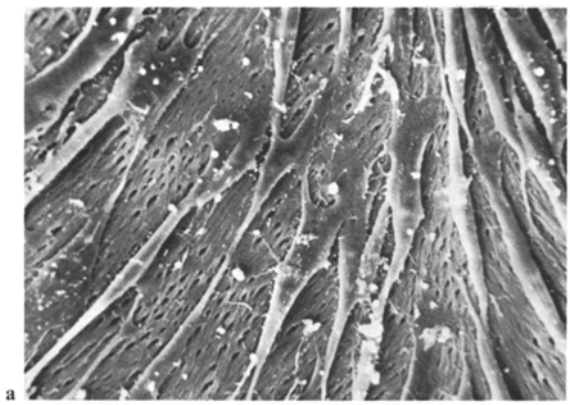
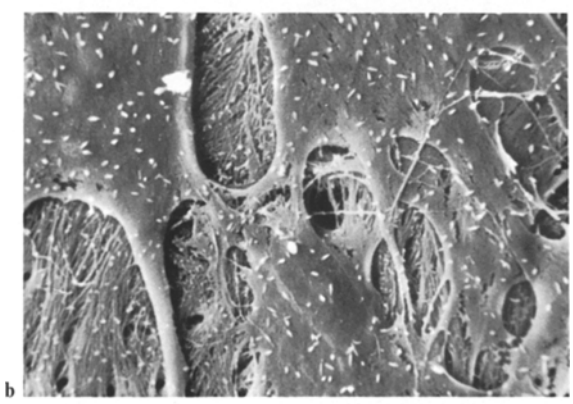


Fig. 6a–c. Calvaria of rachitic rats, which have been incubated with 1,25 D_3. **a** calvarium at day one of the incubation period. In contrast to untreated control specimens (see Fig. 5a) all bone surface lining cells are transformed into globiform cells. They have lost their adhesion to the underlying bone matrix and to neighbouring cells. The cell membranes often show disruptions. SEM, enl. 1:1,000. **b** calvarium at the third day of incubation. The number of cells is markedly reduced and the endosteal bone surface is almost completely denuded of surface lining cells. Only few elongated spindle shaped cells are visible. SEM, enl. 1:300. **c** calvarium at day 5 of incubation. The endosteal surface of bone is covered by numerous elongated spindle shaped cells. The longitudinal axis of these cells obviously follows the pattern of the collagen fiber bundles of the endosteum. SEM, enl. 1:100





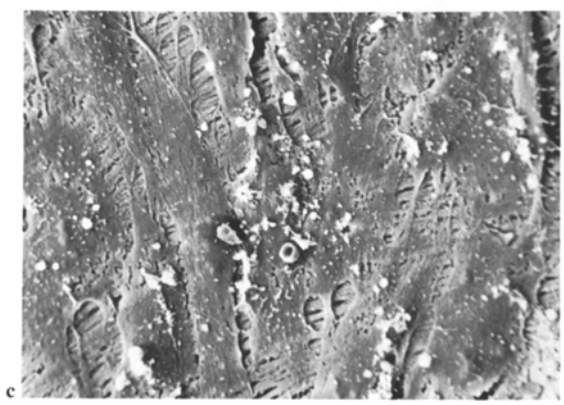


Fig. 7a–c. Calvaria of rachitic rats, incubated for one day with 1,25 D_3, recovery period of incubation without 1,25 D_3. **a** Third day of the recovery period without 1,25 D_3: the surface cells of bone are still elongated and spindle shaped. No flat surface cells are visible. SEM, enl. 1:1,000. **b** Day 6 of the recovery period: The endosteal surface is incompletely covered by flat surface cells. A few elongated cell processes are remnants of the spindle shaped cells. SEM, enl. 1:3,000. **c** Day 7 of the recovery period: the 1,25 D_3-induced changes of cell morphology are completely reversed. The flat surface cells are undiscernable from the endosteal cells of untreated control specimens (s. Fig. 5a). SEM, enl. 1:1,000

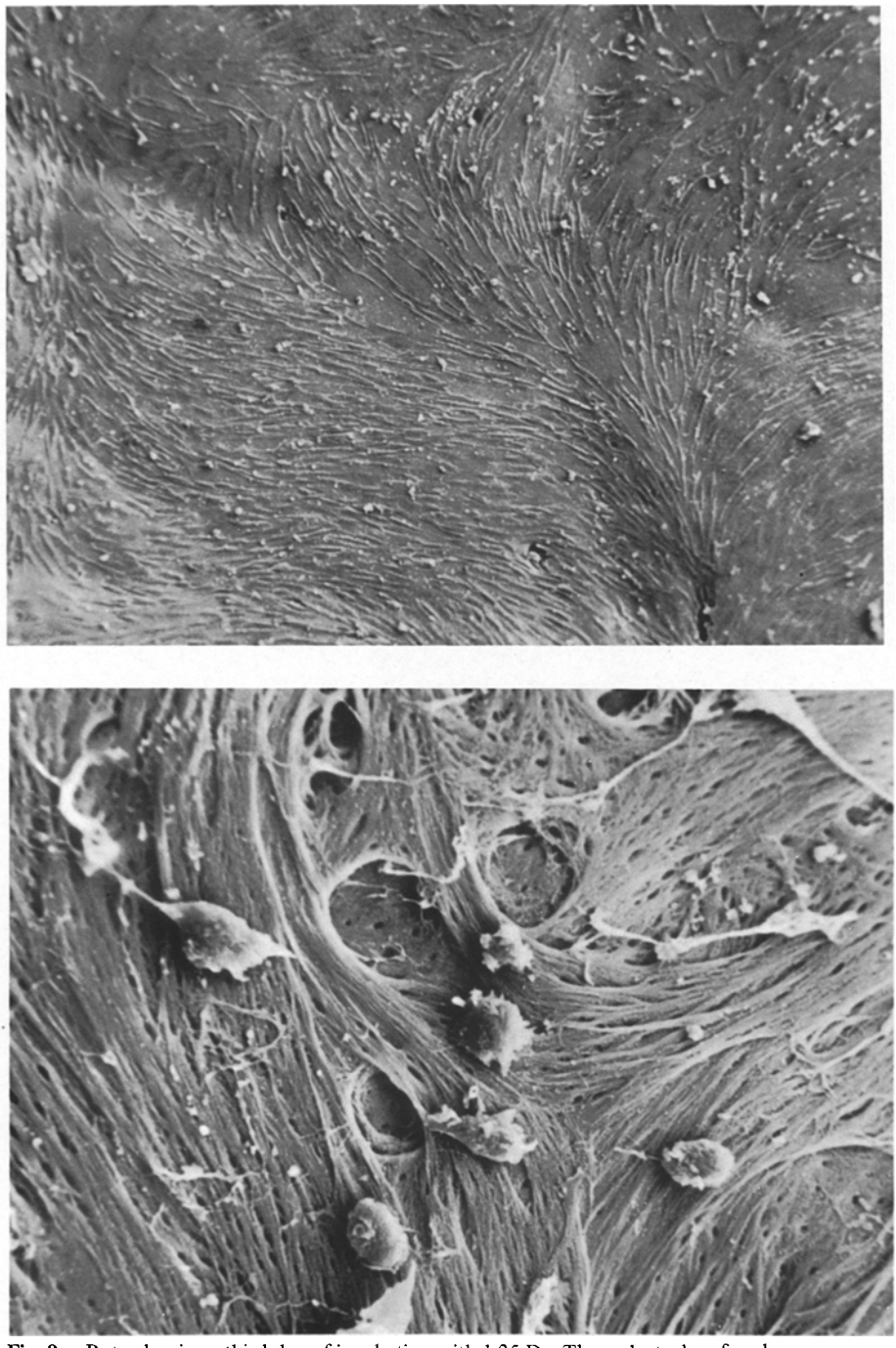


Fig. 8. a Rat calvarium, third day of incubation with 1,25 D_3. The endosteal surface bears numerous elongated cells, which are arranged in streams. Neighbouring cells lie in parallel, the main direction of the longitudinal axis of cells changes from area to area. SEM, enl. 1:100. **b** Endosteal surface of rat calvaria. Note bundles of collagen fibers running in curves. When compared with a, the spatial arrangement of surface cells is a copy of the matrix fiber texture of bone. SEM, enl. 1:1,000

cells covered the endosteal surface of the calvaria (Fig. 6c). Even at the end of the incubation period with 1,25 D_3 no flat surface cells could be detected by SEM.

In a 7 days recovery experiment the 1,25 D_3 induced changes of cellular morphology were slowly reversible. At the end of this experiment numerous flat surface lining cells had reappeared (Fig. 7a–c).

There was a striking conformity of bone matrix fiber texture and the long axis of proliferating cells. This finding points to an influence of bone matrix fiber texture on the spatial movement of proliferating surface cells of bone. During the whole incubation period with 1,25 D_3 no bone resorbing cells could be detected at the endosteal surface of rat calvaria (Fig. 6b and c; Fig. 8a and b).

Discussion

The epiphyseal cartilage of rachitic rats is known to mineralize in vitro, if calcium and phosphorus are available (Anderson et al. 1975). This observation is not surprising, since rachitic cartilage contains numerous type I and type II vesicles which are involved in the mineralization process (Bonucci 1971; Anderson et al. 1975; Thyberg and Friberg 1978).

Whereas in our in vivo experiment the mineralization process in rachitic rats is immediately enhanced after the injection of 1,25 D_3 (Krempien et al. 1977), our in vitro experiment shows no obvious difference in the mineralization process of cartilage specimens incubated with or without 1,25 D_3. We therefore conclude that 1,25 D_3 in supraphysiological doses fails to promote cartilage mineralization and that the in vivo effect of 1,25 D_3 on mineralization must be an indirect one. In contrast, 1,25 D_3 has a direct stimulating effect on the synthesis of proteoglycans by chondrocytes (Corvol et al. 1978).

The endosteal envelope of bone is formed by a monolayer of cells, which act as osteoblasts or resting surface lining cells. These cells have been considered to be involved in the moment to moment regulation of calcium homeostasis (Neuman and Ramp 1971; Davis et al. 1975). The morphology of these cells is modulated by hormones which are engaged in the regulation of the calcium homeostasis, like parathyroid hormone, calcitonin and 1,25 D_3 (Krempien et al. 1976; Davis et al. 1976; Jones and Boyde 1977). When calvaria of rachitic rats are studied by scanning electron microscopy, the endosteal surface is covered by extremely flattened and thin surface lining cells. A reduction of cytoplasm and a loss of microvilli reveal that these cells are not active matrix synthesizing cells (Krempien et al. 1977). We therefore prefer to call them surface lining cells rather osteoblasts. Surface lining cells of the calvaria of rachitic rats and normal osteoblasts in healthy control animals are both rapidly transformed by 1,25 D_3 into globiform cells, loosing their attachment to the bone matrix and their adhesion to neighbouring cells. This rapid change of cell shape, which occured within the first 2 h after exposure to 1,25 D_3, is followed by a second slower transformation into proliferating spindle shaped cells which resemble fibroblasts. These changes only take place when 1,25 D_3

is present. They are completely reversible within several days in a recovery period without 1,25 D_3. The rapid transformation of the flat surface lining cells into globiform cells was also observed in our in vivo experiments (Krempien et al. 1977). This rapid transformation often leads to rupture of the cellular membrane. In previous experiments with parathyroid hormone we found comparable changes of the morphology of osteoblasts and osteocytes (Krempien et al. 1976; Krempien and Ritz 1978). These changes of shape and surface morphology of bone cells were accompanied by an alteration of microtubuli and microfilaments. The cytoskeleton might therefore be involved in the cellular response to parathyroid hormone as well as to 1,25 D_3. We suppose that both hormones alter the cytosol calcium, thus affecting the cytoskeleton with changes of cell shape and loss of cellular junctions. Herrmann-Erlee and Gaillard (1978) have obtained comparable changes in cell morphology under the influence of 1,25 D_3 and PTH. Using embryonic bone in a tissue culture system, they found cellular changes under the influence of 1,25 D_3 which are typically known from experiments with high doses of PTH.

According to Neuman and Ramp (1971) the extracellular fluid of bone and the extracellular fluid outside the skeleton differ in their ionic composition and are actively separated by a functional membrane. If the assumption that the endosteal cell envelope of bone acts as such a membrane is true the 1,25 D_3-induced changes in the morphology of surface lining cells in return must influence the movement of ions through this membrane. It therefore seems reasonable to conclude that the changes of cell morphology of the endosteal cell envelope of bone which are induced by PTH as well as by 1,25 D_3, are the morphological basis of the hormonal regulation of calcium homeostasis at the cellular level.

Levene and Lawson (1977) have described a similar loss of cell adhesion in endothelial cells of the pig aorta under the influence of 1,25 D_3. They suppose that this phenomenon indicates a general effect of vitamin D differing from the action of 1,25 D_3 on the skeleton. According to our results, however, we believe that the 1,25 D_3-induced changes of bone cell morphology and of endothelial cells are comparable and represent the main effect of 1,25 D_3. Both types of cells, endothelial cells and the cells of the endosteal envelope of bone are enabled to act as functional membranes, which separate and control different fluid compartments.

Perris (1971) has shown that parathyroid hormone induces mitotic activity in different mammalian tissues. Our in vitro experiment leads to the assumption that 1,25 D_3 has a similar effect, since we found an increasing number of spindle shaped cells during the culture period of 7 days. We are sure that these spindle shaped cells derive from the endosteal surface lining cells. The reasons for this conclusion are as follows: we never found spindle shaped cells in the calvaria of controls; spindle shaped cells were numerous in the beginning of our recovery experiment but were nearly absent at the end of this period; we never saw cells at the cut edges of the calvaria climbing up from their outer surface.

Finally, we observed a striking conformity between the fiber texture of bone and the spatial pattern of sprouting surface cells. This observation suggests

that the alignment of the surface lining cells with respect to the bone surface depends on the matrix texture of the bone surface. A comparable influence of bone matrix texture on cell movement along the bone surface has been evaluated in a previous study on the spatial resorption activity of human osteoclasts (Krempien 1979). The interaction between bone cell movement along the endosteal surface and the matrix texture of bone might therefore influence modelling and remodelling processes of the skeleton.

References

Anderson HCl, Russel C, Sajdera StW (1975) Calcification of rachitic rat cartilage in vitro by extracellular matrix vesicles. Am J Pathol 79:237–254

Bonucci E (1971) The locus of initial calcification of cartilage and bone. Clin Orthop 78:108–139

Coburn JW, Massry SG (ed) (1980) Uses and action of 1,25-dihydroxyvitamin D_3 in uremia. Contributions to Nephrol, vol 18, Karger, Basel

Corvol MT, Dumontier MF, Garabedian M, Rappaport R (1978) Vitamin D and cartilage. II. Biological activity of 25-hydroxycholecalciferol and 24,25 and 1,25-dihydroxycholecalciferols on cultured growth plate chondrocytes. Endocrinology 102:1269–1274

Davis WL, Matthews JL, Martin JH, Kennedy III JW, Talmage RV (1975) The endosteum as a functional membrane. In: Talmage RV, Owen M, Parsons JA (eds) Calcium-regulating Hormones. Proc. 5th Parathyroid Conf. Oxford 1974. Excerpta Medica Amsterdam, p 275

De Luca HF (1979) Vitamin D metabolism and function. Springer, Berlin Heidelberg New York

Haussler MR, Mc Cain TA (1977) Basis and clinical concepts related to vitamin D metabolism and action. N Engl J Med 297:1041–1050

Herrmann-Erlee Gaillard PJ (1978) The effects of 1,25-dihydroxycholecalciferol on embryonic bone in vitro: a biochemical and histological study. Calcif Tissue Res 25:111–118

Krempien B, Geiger G, Ritz E (1976) Effects of acute and chronic PTH stimulation on osteoblasts and the underlying bone matrix. Calcif Tissue Res [Suppl] 21:260–266

Krempien B, Friedrich G, Geiger G, Ritz E (1977) Ultrastructural studies of bone: influence of vitamin D, PTH and uremia on bone cell ultrastructure and bone cell/bone matrix interaction. In: Norman AW, Schaefer K, Coburn JW, DeLuca HF, Fraser D, Grigoleit HG, v Herrath D (eds) Vitamin D. Biochemical, chemical and clinical aspects related to calcium metabolism. De Gruyter, Berlin New York, pp 381–390

Krempien B, Ritz E (1978) Effects of parathyroid hormone on osteocytes. Ultrastructural evidence of anisotropic osteolysis and involvement of the cytoskeleton. Metabol Bone Dis Rel Res 1:55–65

Krempien B (1979) Bone modelling processes at the endosteal surface of human femora. Scanning electron microscopical studies in normal bone and in renal osteodystrophy. Virchows Arch [Pathol Anat] 382:73–88

Levene CI, Lawson DEM (1977) A possible effect of vitamin D metabolites on cell adhesion. Cell Biol Int Rep 1:93–97

Neuman WF, Ramp WK (1971) The concept of a bone membrane: some implications. In: Nichols G jr, Wasserman RH (eds) Cellular mechanisms for calcium transfer and homeostasis. Academic Press, New York London, pp 197–206

Perris AD (1971) The calcium homeostatic system as a physiological regulator of cell proliferation in mammalian tissues. In: Cellular mechanisms (s. Neuman and Ramp), pp 101–126

Raisz LG, Trummel CL, Holick MF, DeLuca HF (1972) 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. Science 175:768–769

Thyberg J, Friberg U (1978) The lysosomal system in enchondral growth. Fischer, Stuttgart New York

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