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The Influence of Immobilization on Osteocyte Morphology

Osteocyte Differential Count and Electron Microscopical Studies

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Summary. Differential counts and electron microscopical studies of osteocytes were performed on rats immobilized by spinal cord severing, plaster cast and ischiatic nerive dissection. In undecalcified ground sections of tibia and femur (100 micron) stained wtth basic fuchsin, osteocytes were differentiated into small (metabolically inactive) osteocy es enlarged (metabolically activated) osteocytes and empty lacunae. In rats (immobilizedfor' three weeks) with functioning parathyroid glands, but not after parathyroidectomy, the number of activated cells is markedly increased, whereas the number of small osteocytes is reduced. In animals with spinal cord severing the number of empty lacunae is also increased.

Electron microscopical studies of undecalcified tibiae taken from rats immobilized for ten days showed a periosteocytic osteolysis with destruction of the lacunar wall, fragmentation of collagen fibres and loss of mineral crystals. The cytoplasmic seams of osteocytes were broadened, mitochondria were enlarged, and the cytoplasma showed vacuoles containing amorphous material which could be found in the pericellular space. Deep invaginations of the cytoplasma and an increase of the cell processes were typical findings.

The results of the investigation point to an activation of osteocyte metabolism by immobilization. The osteocytes thus play an important part at the onset of immobilization osteoporosis. Periosteocytic osteolysis can be inhibited by parathyroidectomy. Therefore, the response of osteocytes to endogenous parathyroid hormone must be altered under conditions of immobilization.

Osteocytes serve a dual function. They contribute to the homeostatic control of serum calcium and to the maintenance of skeletal homeostasis. On the one hand the efflux of calcium from the skeleton into the extracellular fluid is mediated by the action of osteocytes which are responsive to hormones (parathyroid hormone, calcitonin) and metabolic mediators (calcium, phosphorus, cyclic AMP). On the other hand osteocytes are thought to function as transducers which sense potentials resulting from elastic deformation of bone with its characteristic piezoelectric properties (Johnson, 1964; Bassett, 1968). Osteocytes, when stimulated, activate cells on the endosteal surface by signals of unknown nature and thus induce bone modelling and remodelling.

While activation of osteocytes in response to metabolic or hormonal stimuli has been investigated repeatedly (for literature see Rasmussen and Bordier, 1974), the reaction of osteocytes to the presence or absence of mechanical bone deformation activity has not been studied to any great extent. The model of immobilization has often been used to study changes in osteoclast and osteoblast morphology and to evaluate the contribution which osteoclasts and osteo-

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blasts make to the pathogenesis of immobilization osteoporosis (Albright *et al.*, 1941; Heaney, 1962; Frost, 1963; Uehlinger, 1963; Landry and Fleisch, 1964; Burkhart and Jowsey, 1967; Eichler, 1970; Kreinsen, and Krempien, 1974).

The behaviour of osteocytes under these conditions, however, has not as yet been thoroughly investigated.

The immobilization of bones provides an excellent model to study the response of osteocytes to the loss of mechanical stress as well as the response of osteocytes to hormonal stimuli in the absence of mechanical stimuli, from which insights into the interaction of mechanical and hormonal stimuli can be gained. Apart from its inherent theoretical interest, such an investigation is of considerable practical importance. The response of osteocytes to the withdrawal of mechanical stress is relevant to the pathogenesis of immobilization osteoporosis and thus has obvious implication for the long-term rehabilitation of patients with long-standing immobilization or for the response of the organism to weightlessness. Observations in hibernating animals and in human beings with immobilization due to fractures suggest that not osteoclasia but osteolysis plays a major part in the onset of immobilization osteoporosis (Whalen *et al.*, 1972; Westlin, 1974).

In order to evaluate the influence of acute immobilization on osteocyte morphology and the responsiveness of osteocytes to hormonal stimuli, ultrastructural studies and osteocyte differential counts according to the criteria given by Baud and Auil (1971) were performed. Both methods provide insight into the functional and metabolic behaviour of osteocytes. This investigation was designed to answer the following questions:

Does the distribution of osteocytes into morphologically defined classes of different metabolic activity change under the conditions of immobilization?

Is the response of osteocytes to skeletal hormones (e.g. endogenous parathyroid hormone) altered under conditions of immobilization?

Does immobilization result in changes of the ultrastructure of osteocytes and/or the osseous matrix surrounding the osteocytes?

Material and Methods

A. Osteocyte Differential Counts

Female Wistar rats weighing 150 grams were partially immobilized by (a) spinal paraplegia (severing of the spinal cord of the lumbar vertebral column 2 cm above the pelvis) or by (b) application of a pelvic plaster cast to the lower extremities. Some of the animals of group a were parathyroidectomized before the immobilization procedure was performed, according to a method outlined by Bommer (1975). Shame operations were performed on several rats to serve as a control. Animals' food and water intake were unrestricted. They were kept in individual cages in acclimatized rooms with 12 hours of day and night illumination. Because of bladder paralysis the urinary bladders of the paraplegmized animals were artificially emptied by imposing pressure upon the abdomen. Upon conclusion of the experiment renal function was determined in paraplegmized animals by measuring serum urea (blood drawn from the plexus orbitalis: enzymatic method, Boehringer & Co., Mannheim). Only animals with normal values were included in the evaluation of osteocyte morphology. Each animal's weight was recorded at the beginning and again at the experiment's conclusion.

The rats were killed after three weeks of immobilization under ether anesthesia, since at this time a steady state in bone formation and bone resorption had been reached (Landry

and Fleisch, 1964; Eichler, 1970). Tibiae and femora were removed, freed of soft tissues, and fixed in a solution of 70% alcohol. Undecalcified portions of the diaphyses (tibia at the point where the fibula inserts, femur at the middle of the diaphysis) were embedded in methylmetacrylate. Cross sections of bone were ground on Carborundum paper to a final thickness of 100 microns. The specimens were stained according to a method described by Baud and Auil (1971) in a 5% fuchsine base solution (each imersed in increasing concentrations of alcohol for dehydration for one minute subsequent to the removal of the methylmetacrylate) and placed in 100% alcohol for 10 seconds to remove the excess staining material.

Microscopical examination ensued following inclusion in Cedax at a magnification of 1:1,000 in oil immersion. For the tabulation of an osteocyte histogram, 500 osteocytes from 6 equal areas of a thin bone section taken from each animal were classified according to the criteria presented by Baud and Auil (1971) into the following types: small osteocytes (Fig. 1a, flattened ellipsoid lacunae with a smooth and regular outline, the short axis not exceeding 4 micron, dense and homogeneously stained nucleus, little cytoplasma), enlarged osteocytes (Fig. 1b, irregular oval lacunae, small axis exceeding 4 micron, swollen nucleus with coarse granula of chromatin, enclosed by broad cytoplasmic rims), and empty lacunae (Fig. 1c). Details are given by Baud and Auil (1971), Krempien *et al.* (1973), and by Manegold (1975). Statistical evaluation of the results followed by means of the Wilcoxon test, whereby individual statistics of 10 animals per group were compared.

B. Electron Microscopical Studies[1]

In order to overcome the difficulty of artificially emptying the animals' bladders and to avoid the skin and bladder infection often arising in the course of experiments involving immobilization, a new immobilization technique was developed for the electron microscopical studies: The skin from the right rear extremity of female Wistar rats weighing 150 grams was removed, the paw amputated, the ischiatic nerve severed at the midpoint of the femur, and the extremity sewn into the abdominal wall. Muscles and circulation remained fully intact. In previous experiments we had clarified the fact that no ischaemia of the bone or bone marrow occurred. Ten days following the onset of immobilization the animals were anesthesized in ether and perfunded through the abdominal aorta with 2% cacodylate buffer.

Small bone specimens of the tibiae were then further fixed in cacodylate buffer through immersion and post-fixed for two hours in osmiumtetroxide. Similar bone specimens from the corresponding nonimmobilized tibia taken from each animal served as a control. After embedding in Epon 812, ultra thin sections of undecalcified bone were prepared with a diamond knife, stained with uranyl acetate and lead citrate and examined with an electron microscope (EM 9, Zeiss). A detailed account of all methods used has been outlined elsewhere (Manegold, 1975).

Results

A. Microscopical Studies

a) Differential Counts of Osteocytes Performed on the Cortical Tibial Bone of Rats Immobilized by Severing of the Spinal Cord or Plaster Cast Application (Table 1, Fig. 2a).

In the cortical bone of nonimmobilized animals the majority of osteocytes appeared to be small and were located in a flattened ellipsoid lacuna with a smooth and regular outline. Conversely, the cortical bone of the paraplegmized animals and of those immobilized by plaster cast showed a great increase in the number of enlarged osteocytes which were located in an irregular oval lacuna. Differential counts of osteocytes confirmed these findings. Furthermore, the histogram not only showed an increase in the number of activated osteocytes

1 We thank Ms. I. Schütz for technical help.

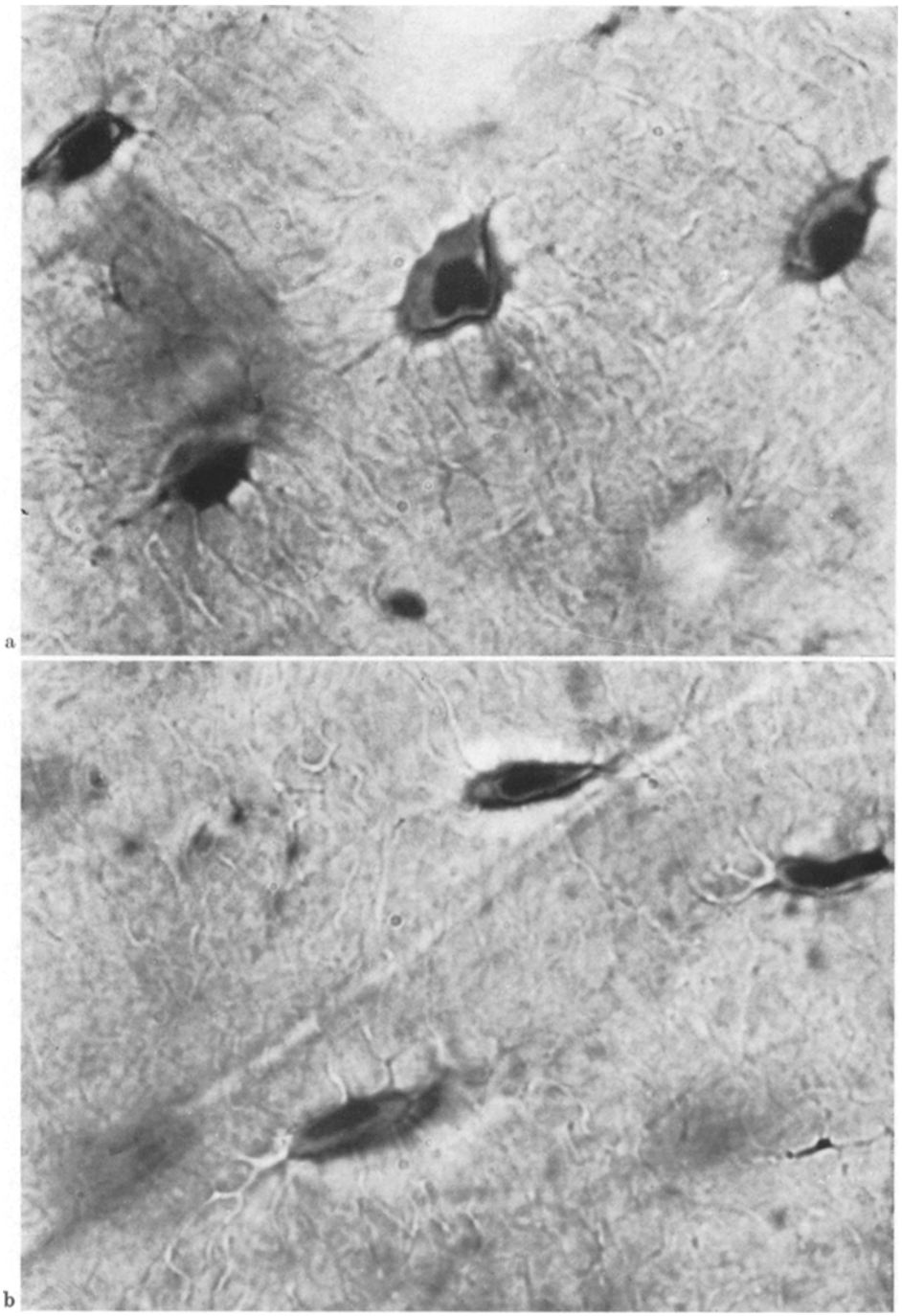
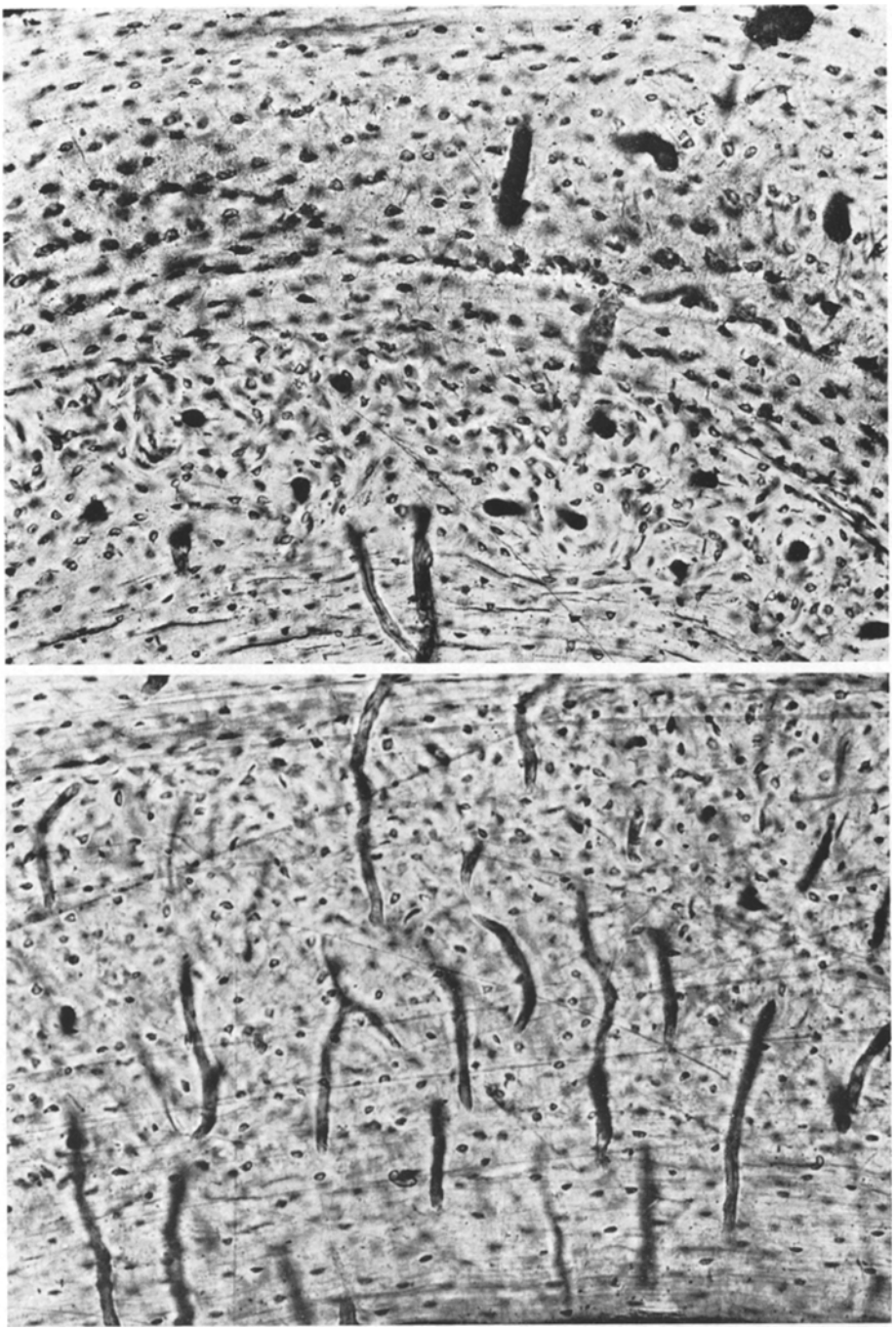


Fig. 1a–d. Undecalcified ground sections, basic fuchsin stain. Microphotographs. (a) Group of enlarged osteocytes with oval formed lacunae and large nuclei. (b) Group of small osteocytes lying in spindle-shaped lacunae. Enl. 1:1,000, oil immersion. (c) and (d) Part of a



cross section of the tibia, showing the total thickness of cortical bone. Enl. 1:240. (c) Immobilized rat without parathyroidectomy: The majority of osteocytes is remarkably enlarged. (d) Immobilized rat with parathyroidectomy: The majority of cells is represented by small osteocytes

Table 1. Osteocyte differential counts in immobilized and nonimmobilized tibiae of rats

	Control (%)	Spinal cord (%)	Plaster cast (%)	Wilcoxon test	
Small osteocytes	51.5 ± 3.0	30.5 ± 3.6	36.1 ± 5.5	$p < 0.01$	$p < 0.01$
Enlarged osteocytes	34.0 ± 2.8	46.6 ± 2.0	48.6 ± 4.0	$p < 0.01$	$p < 0.01$
Empty lacunae	14.5 ± 3.5	22.8 ± 2.0	15.0 ± 3.2	$p < 0.01$	n.s.

Table 2. Osteocyte differential counts in immobilized tibiae of rats with (PTX) and without (PTO) parathyroidectomy

	PTX (%)	PTO (%)	Wilcoxon test
Small osteocytes	47.1 ± 8.20	36.7 ± 10.7	$p < 0.01$
Enlarged osteocytes	44.5 ± 5.16	55.0 ± 10.0	$p < 0.02$
Empty lacunae	8.46 ± 4.48	7.6 ± 3.6	n.s.

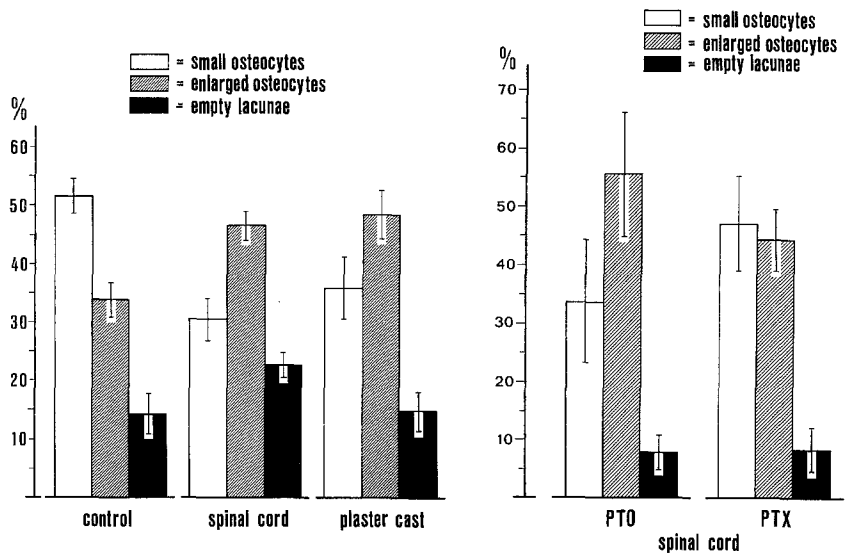
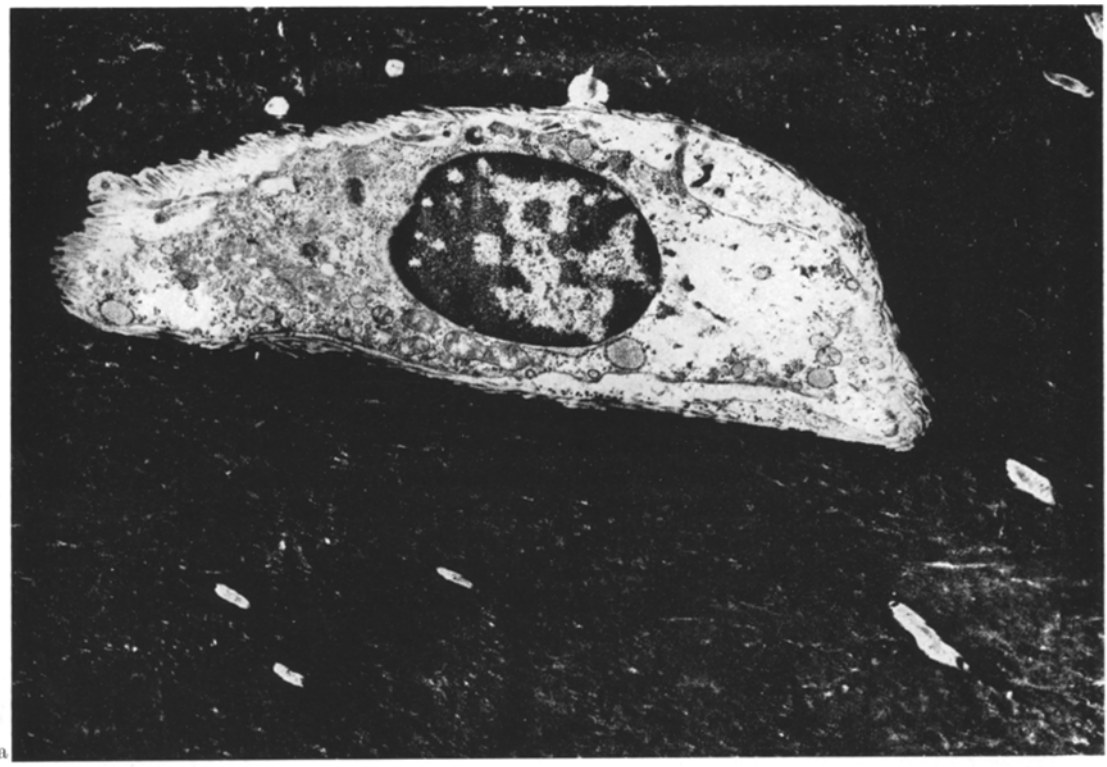


Fig. 2a and b. Histogram showing the percentage of small osteocytes, enlarged osteocytes and of empty lacunae in cortical bone of immobilized rats (a) and of immobilized rats with (*PTX*) and without (*PTO*) parathyroidectomy (b)

Fig. 3a–e. Transmission electron micrographs of osteocytes in cortical bone of immobilized rat tibiae. (a) Enlarged, resorptive osteocyte: Broad cytoplasmic seam, irregular outline of the lacunar wall with denuded collagen fibres and widened canaliculi. Enl. 1:10,200. (b) Section of a lacunar wall: Numerous collagen fibres, partly fragmented and mixed with a flocculent, confluent material and loosened mineral crystals. Destroyed, irregular borderline of the lacuna. Enl. 1:54,000. (c) Enlarged osteocyte: Numerous cellular processes, strikingly large vacuole in the cytoplasma containing amorphous flocculent material. Enl. 1:18,600. (d) Part of an osteocyte with deep invaginations in the cytoplasm, enlarged mitochondria, and distinct vacuoles. Enl. 1:27,900. (e) Conspicuously deformed osteocyte with numerous sections through the cytoplasm and the cellular processes. Enlarged canaliculi and denuded collagen fibres at the lacunar borderline Enl. 1:10,200



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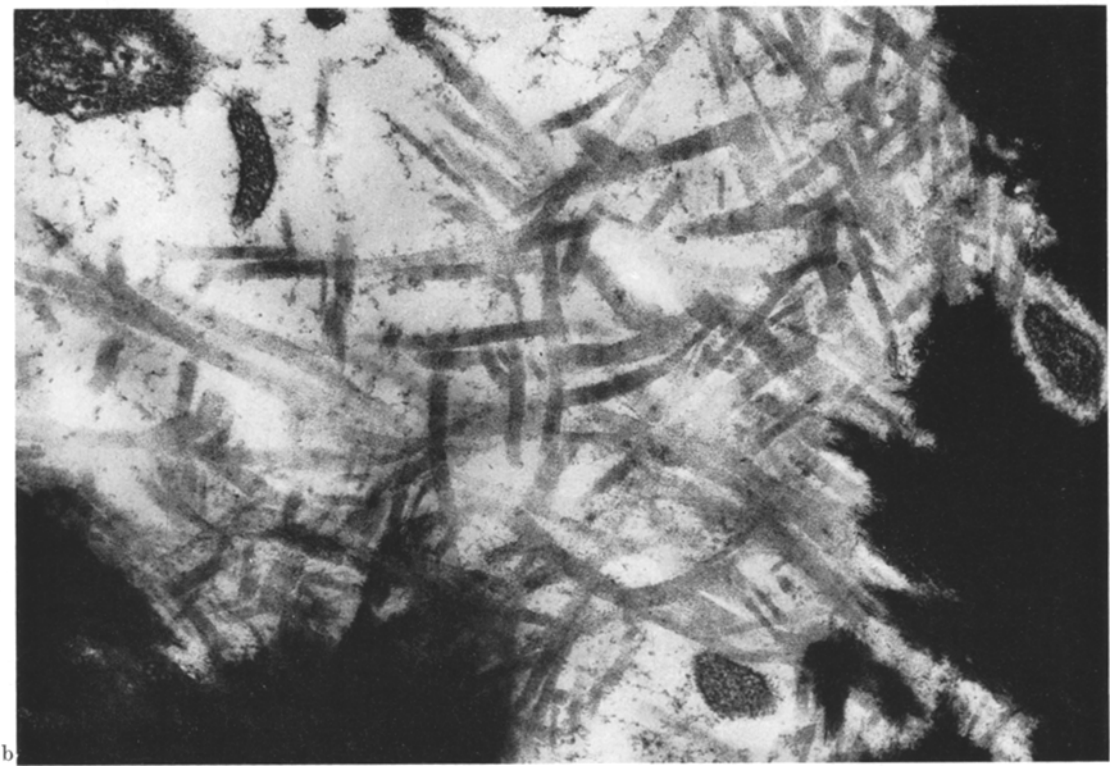

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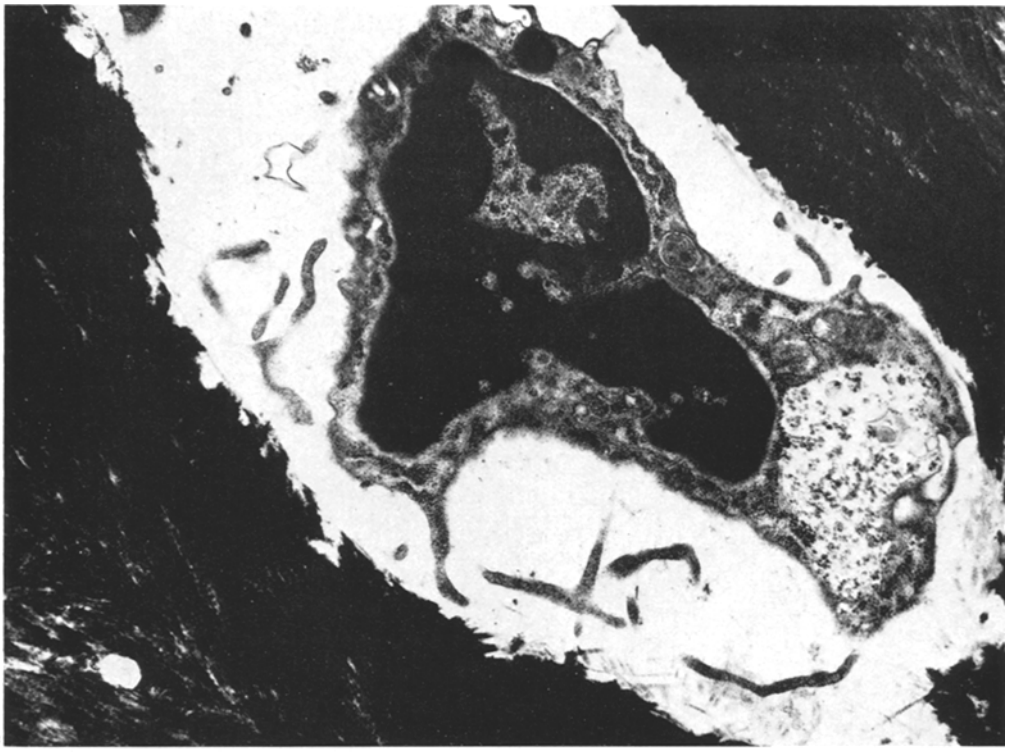

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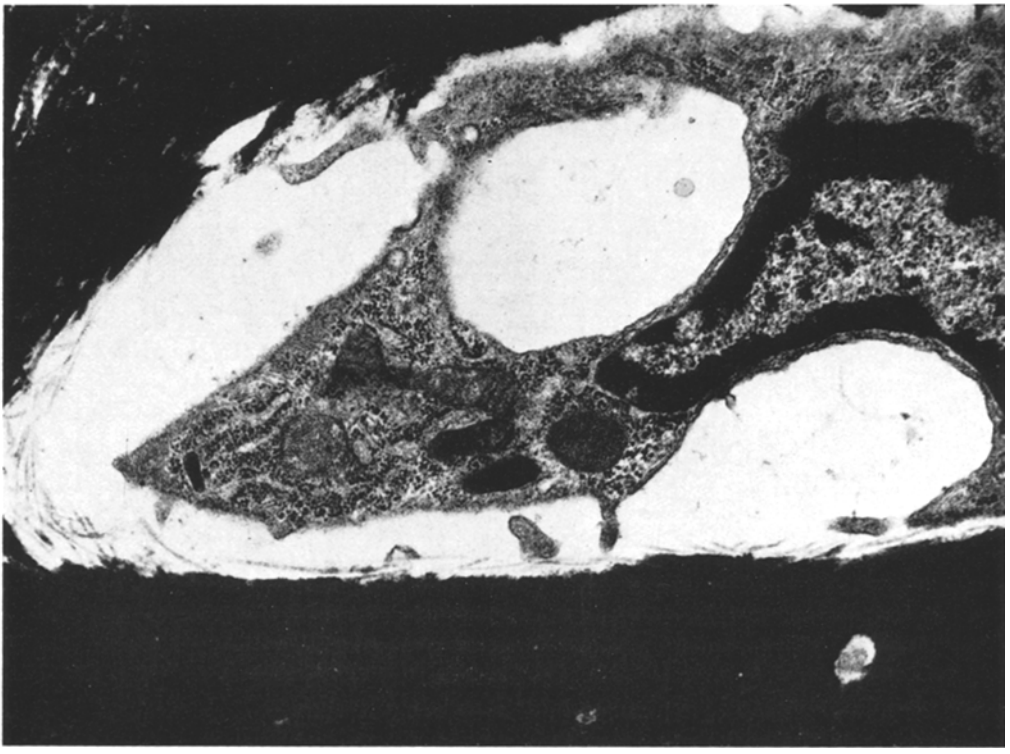

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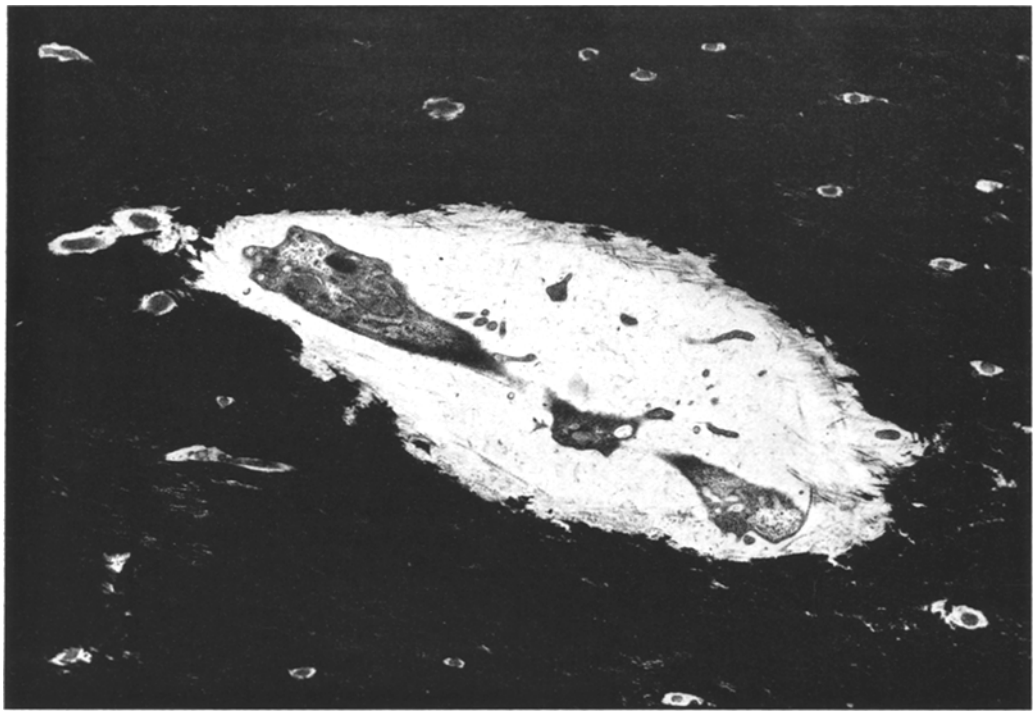

3e

in the paraplegmized animals, but also a significantly increased number of empty lacunae, that is, of dead osteocytes.

b) Differential Counts of Osteocytes Performed on the Cortical Femural of Rats Parathyroidectomized and Immobilized by Severing of the Spinal Cord (Table 2, Fig. 1d and e, 2b).

The histogram showed that the number of activated, enlarged osteocytes in the nonparathyroidectomized animals was significantly greater than the number of small osteocytes. At the same time the parathyroidectomized animals showed no increase in the number of enlarged osteocytes.

B. Electron Microscopical Studies

Under electron microscopical examination differences between the large activated osteocytes on the nonimmobilized as compared to the immobilized limbs could be seen in the periosteocytic bone matrix, the lacunal wall, the size and form of lacunae and canaliculi and in the osteocyte configuration, including cell organells. Especially noticeable was the fact that in the cortical bone specimens from immobilized limbs the majority of the enlarged, activated osteocytes lay in a significantly expanded, irregularly formed lacuna with a wall made inhomogeneous by expanded canaliculi (Fig. 3a and e). The pericellular matrix was no longer recognizable. Instead, the well developed pericellular space was filled with collagen fibrillae, partially fragmented which occasionally were mixed with

a flocculent material inclining to confluence and with loosened mineral crystals (Fig. 3b). The osteocytes themselves presented a bizarre, polymorphic appearance (Fig. 3c, d, and e). As a rule the cytoplasmic seam was broad. The mitochondria were enlarged. The Golgi apparatus was well developed and the number of lysosomes was large. A distinctive vacuolization of nuclear and cytoplasmic structures was noted, whereby the vacuoli were filled with fine, granulated substances which were very similar to the flocculent material of the pericellular space (Fig. 3c). Also easily discernible was the formation of invaginations which cut deeply into the cytoplasm (Fig. 3d). In contrast to this the majority of the osteocytes in the cortical bones from nonimmobilized specimens differed considerably from these cell forms. Intracellular vacuoles were rare and the mitochondria were not enlarged. The cytoplasmic membrane showed no invaginations. The pericellular space was narrower and for the most part free of detritus, the lacunar wall was more rarely visibly interrupted by canaliculi. The number of osteocyte processes and canaliculi was noticeably smaller, the cytoplasmic seam of the cells in general narrow.

C. Body Weight of the Animals at the End of the Experiments

Control 221.85 ± 12.64 g, plaster cast 172.3 ± 14.60, spinal cord severing 190.14 ± 16.33.

PTO 190.0 ± 15.61, PTX 203.5 ± 13.24 g.

Discussion

Osteocytes are not a homogenous population of cells. Rather, the population of osteocytes consist of different groups of cells which differ by their respective metabolic activities. These groups can be distinguished both by their characteristic cell morphology and by the appearance of the osseous matrix surrounding the osteocytes (v. Recklinghausen, 1910; Baud, 1958; Vittali, 1968; Remagen, 1970; Jande and Belanger, 1971). The three categories developed by Baud and Auil (1971) to distinguish these characteristic cell groups were as follows: Large cells with active metabolism, small metabolically inactive cells and finally empty lacunae resulting from osteocyte necrosis. They were thus able to define a characteristic histogram of osteocytes in normal human bone. Hormonal stimulation by parathyroid hormone was shown to result in a marked alteration of the histogram of osteocytes, leading to an increase in the number of large, active osteocytes and of empty lacunae, at the expense of small, inactive cells (Krempien *et al.*, 1973). Similarly, the above study showed that acute immobilization resulted in an increase in the ratio of large osteocytes and of empty lacunae at the expense of small osteocytes, irrespective of the method of immobilization. Activation of osteocytes by immobilization could be prevented by prior parathyroidectomy. Immobilization is known to cause hypercalcaemia and hypercalciuria quickly followed by a reduction of bone mass (Albright *et al.*, 1941; Heaney, 1962; Landry and Fleisch, 1964; Eichler, 1970; Denham, 1973; Lockwood *et al.*, 1973; Whalen *et al.*, 1973; Westlin, 1974; Whedon *et al.*, 1975). The activation of osteocytes subsequent to acute immobilization resembles what has

previously been described as "Onkose" or "periosteocytic osteolysis" in hyperparathyroid bone disease or following the administration of parathyroid hormone (v. Recklinghausen, 1910; Belanger and Rasmussen, 1968; Jande, 1972; Bordier *et al.*, 1973). The activation of osteocytes found in this study suggests that periosteocytic osteolysis is also a major factor for the increased mobilization of calcium originating from the skeleton under the influence of immobilization. While the appearance of enlarged osteocytes suggests increased metabolic activity, degeneration cannot firmly be excluded as the cause of osteocyte onkosis. Enlargement of osteocytes and their lacunae are not only morphological equivalents for increased metabolic activity, but can also signify degenerative changes. These findings were confirmed by v. Recklinghausen (1910), Majno and Roullier (1951), Rutishauser *et al.* (1960), Remagen, (1970) and Jande (1972), who found evidence of both increased osteocytic metabolism and osteocytic degeneration in metabolic bone disease of human beings and experimental animals. This agrees with our own previous studies in uremic individuals which suggested that increased metabolic activity of osteocytes is associated with a shortened osteocyte survival time (Krempien *et al.*, 1973). In our experiments of chronic immobilization we found an increase of empty lacunae in animals immobilized by spinal cord severing for three weeks. This finding indicates that a chronic stimulation of osteocytes due to immobilization will also lead to degenerative changes.

In our study concerning the influence of acute immobilization on osteocyte morphology, activation (as opposed to degeneration) of osteocytes could be clearly demonstrated by electron microscopy. In contrast to the osteocytes of the nonimmobilized tibia of the contralateral side, osteocytes in the cortical bone of the immobilized tibia exhibited swelling of cytoplasma and increase of ergastoplasmic reticulum. In addition, striking invaginations and protrusions of the osteocytic cytoplasma were regularly found, suggesting amoeboid motility with pseudopod formation. The cell surface is strikingly increased when compared to control osteocytes due to an increase of cellular processes. Perhaps osteocytes have contractile elements in their cytoplasma, a feature postulated previously for osteoblasts (Weinger and Holtrop, 1974). Changes in the electric potentials alter the electrical potentials across the cell membrane and alter thereby the ion fluxes within the cell. Membrane depolarization and/or ionic changes of the cytosol, caused by altered piezoelectric currents in the course of immobilization could, therefore, trigger cell contraction. According to the studies of Mears (1971) depolarization of the cell membrane through electropositivity leads to an increase in the activity of osteoclasts. Parathyroid hormone also causes a depolarization of the surface membrane of osteoclasts with consequent activation of osteoclasts' metabolism. In this connection it is interesting to note that under the influence of large doses of parathyroid hormone conspicuous changes in the form of osteoblasts and of their cell membranes can be observed (Krempien *et al.*, 1974, 1975).

While altered cell surface morphology might well result from contraction of subcellular elements, a hydrolic origin cannot be excluded. Using radioactively labelled serum proteins, the rapid translocation of fluid through bone, particularly along intraosseous spaces like canaliculi, could be demonstrated by Owen

et al. (1973). Frost (1963) suggested that the osteocyte might function as a water pump. To this process Ramussen and Bordier (1974) coined the term "minicirculation of bone". After mechanical stress, a long-lasting increase of 99 mTc in the knee joint was found by Boerbooms and Buys (1975). In addition, muscular contractions have been shown by Geiser and Trueta (1958) to profoundly affect bone metabolism. It is therefore conceivable that withdrawal of mechanical deformation after immobilization results in changes of transosseous fluid transport which could secondarily affect osteocyte surface morphology. These invaginations and protrusions of osteocytes seem to be to some degree specific for immobilization, since analogous changes have not been described as the result of the action of bone hormones. The lacunar wall around the osteocytes of acutely immobilized animals pointed to the presence of intensive resorptive processes. These were indicated by denuded and partially fragmented collagen fibres and loosened mineral crystals. Analogous findings have been found after the administration of parathyroid hormone (Jande, 1972). However, studies in immobilized human beings (Sevastik and Mattson, 1971; Arnstein *et al.*, 1973; Heath *et al.*, 1973) and experimental animals (Burkhart and Jowsey, 1967; Jowsey and Raisz, 1968; Conaway *et al.*, 1973) did not show any evidence of increased parathyroid hormone activity. Rather, parathyroid hormone secretion should be suppressed as a result of hypercalcemia. Activated osteocytes were found exclusively in immobilized bones and not generalized throughout the skeleton.

This finding also argues against systemic hormonal stimulation. While increased parathyroid activity in this model is therefore rather unlikely, the changes of osteocytic morphology could be suppressed by prior parathyroidectomy. Similar findings were obtained by Burkhart and Jowsey (1967). These results point to the permissive rather than causative role of the parathyroid hormone in the development of osteocyte activation in immobilization. Under these circumstances the effect of the parathyroid hormone—perhaps in the course of altered membrane polarization as outlined above—would be mediated by changes of cytosol calcium concentration; after parathyroidectomy lower cytosol calcium concentration could conceivably raise the activation threshold of osteocytes.

The above results underline the dual role of osteocytes in the maintenance of bone mineral homeostasis and of the supportive function of the skeleton. The morphological study of osteocytes in immobilized bone suggests that hormonal stimuli and mechanical stimuli use the final common pathway of osteocyte activation. A further conclusion stemming from this investigation is that osteocytes play an important part at the onset of immobilization osteoporosis.

References

Albright, F., Burnett, Ch. H., Copp, O., Parson, W.: Acute atrophy of bone (Osteoporosis) Simulating Hyperparathyroidism. J. clin. Endocr. **1**, 711–716 (1941)

Arnstein, A. R., McCann, D. S., Blumenthal, F. S., Prunty, J.: Serum immunoreactive parathyroid hormone in patients with paralysis due to spinal cord trauma. In: Clinical aspects of metabolic bone disease (B. Frame, A. M. Parfitt, H. Duncan eds.), p. 253–256. Amsterdam: Excerpta Medica 1973

Bassett, C. A.: Biologic significance of piezoelectricity. Calcif. Tiss. Res. **1**, 252–272 (1968)

Baud, C. A.: Submicroscopic Structure and Functional Aspects of the Osteocyte. Clin. Orthop. **56**, 227–236 (1968)

Baud, C. A., Auil, E.: Osteocyte differential count in normal human alveolar bone. Acta anat. (Basel) **78**, 321–328 (1971)

Bélanger, L. F., Rasmussen, H.: Inhibition of osteocytic osteolysis by thyrocalcitonin and some antigrowth factors. In: Parathyroid hormone and thyrocalcitonin (Calcitonin). Edited by R. V. Talmage and L. F. Bélanger. Excerpta Medica Int. Congr. Ser. **159**, 156–168 (1968)

Bélanger, L. F.: Osteocytic Osteolysis. Calcif. Tiss. Res. **4**, 1–12 (1969)

Boerbooms, A., Buys, W.: Rapid assessment of 99 mTc-Pertechnetate uptake in the knee joint as parameter of inflammatory activity. Scand. J. Rheumatol., Suppl. 8 (1975)

Bohr, H.: On calcium metabolism during immobilization. In: Calcium metabolism bone and metabolic bone disease. (Kuhlencordt and H. P. Kruse, eds.), p. 39–42. Berlin-Heidelberg-New York: Springer 1975

Bommer, J.: Der Einfluß von Thyroxin auf den Phosphatstoffwechsel und das Knochenwachstum. Habil. Schrift, Heidelberg, 1975

Bordier, P., Arnaud, C., Hawker, C., Tun-Chot, S., Hioco, D.: Relationship between Serum I. PTH, osteoclastic and osteocytic Bone resorptions and serum calcium in Primary hyperparathyroidism and osteomalacia. In: Clinical aspects of metabolic bone diseases. (B. Frame, A. M. Parfitt, and H. Duncan eds.), p. 222–228. Amsterdam: Excerpta Medica Foundation 1973

Burkhart, J. M., Jowsey, J.: Parathyroid and thyroid hormones in the development of immobilization osteoporosis. Endocrinology **81**, 1053–1082 (1967)

Conaway, H. H., Waite, C. C., Kenny, A. D.: Immobilization and bone mass in rats. Effects of parathyroidectomy and acetazolamide. Calcif. Tiss. Res. **11**, 323–330 (1973)

Denham, M. J.: Progressive osteoporosis in hemiplegia. Geront clin. (Basel) **15**, 361–365 (1973)

Eichler, J.: Inaktivitätsosteoporose. Akt. Orthop. Heft 3, 1970

Frost, H. M.: The osteocyte as a water pump. Bone remodelling, skeletal modelling errors. Orthop. Lect. **4**, 119 (1963)

Geiser, M., Trueta, J.: Muscle action, bone rarefication and bone formation. An experimental study. J. Bone, Jt Surg. **40** B, 282–311 (1958)

Heaney, R. P.: Radiocalcium metabolism in disuse osteoporosis in man. Amer. J. Med. **33**, 188–200 (1962)

Heath, H., Schaaf, M., Wray, H. L., Monchik, J. M., Earll, J. M.: Parathyroid activity and Immobilization-induced changes in calcium metabolism. In: Clinical aspects of metabolic bone disease. (B. Frame, A. M. Parfitt, H. Duncan, eds.), p. 257–260. Amsterdam: Excerpta Medica 1973

Jowsey, J., Raisz, L. G.: Experimental osteoporosis and parathyroid activity. Endocrinology **82**, 348–396 (1968)

Kreinsen, U., Krempien, B.: Aktivität und Richtung der Umbau- und Wachstumsvorgänge an den Oberflächen der Knochen unter experimenteller Immobilisation. Verh. dtsch. Ges. Path. **58**, 411–414 (1974)

Krempien, B., Geiger, G., Ritz, E., Büttner, S.: Osteocytes in chronic uremia. Differential count of osteocytes in human femoral bone. Virchows Arch. Abt. A **360**, 1–9 (1973)

Krempien, B., Geiger, G., Ritz, E.: Alteration of bone tissue structure in secondary hyperparathyroidism. A scanning electron microscopical study. In: Vitamin D and problems related to uremic bone disease. (A. W. Normann, K. Schaefer, H. G. Grigoleit, D. v. Herrath, E. Ritz, eds.), p. 156–165. Berlin-New York: W. DeGruyter 1975

Krempien, B., Geiger, G., Ritz, E.: Effects of acute and chronic Parathyroid hormone stimulation on osteoblasts and the underlying bone matrix. Experimental studies in rats with scanning and transmission electron-microscopy. XIth Europ. Symp. Calcif. Tiss. 1975, in the press

Landry, M., Fleisch, H.: The influence of immobilisation on bone formation as evaluated by osseous incorporation of tetracycline. J. bone, Jt Surg. **46** B, 764–771 (1964)

Lindgren, U.: Disuse osteoporosis and parathyroid function. XIth Europ. Symp. Calcif. Tiss. 1975 (in press)

Lockwood, D. R., Lammert, J. E., Vogel, M. J., Hulley, S. B.: Bone mineral loss during bedrest. In: Clinical aspects of metabolic bone disease. (B. Frame, A. M. Parfitt, H. Duncan, eds.), p. 261–265. Amsterdam: Excerpta Medica 1973

Majno, G., Rouiller, C.: Die alkalische Phosphatase in der Biologie des Knochengewebes. Virchows Arch. Path. Anat. **321**, 1–61 (1951)

Manegold, Ch.: Morphologische Untersuchungen an Osteocyten bei experimenteller Immobilisation. Inaugural Dissertation, Heidelberg, 1975

Mears, D. C.: Relationship between membrane potential and metabolic activity of osteoclasts. Exp. Cell Res. **58**, 427–429 (1969)

Owen, M., Triffit, J. T., Melick, R. A.: Albumin in bone. In: Hard tissue, growth, repair and remineralization. Ciba Foundation Symposium 11 (new series), p. 263–287. Amsterdam-London-New York: Elsevier, Excerpta Medica, North Holland 1973

Rasmussen, H., Bordier, Ph.: The physiological and cellular basis of metabolic bone disease. Baltimore: Williams and Wilkins Comp. 1974

Recklinghausen, F. v.: Untersuchungen über Rachitis und Osteomalacie. Jena: Fischer 1910

Remagen, W.: Calciumkinetik und Knochenmorphologie. Stuttgart: G. Thieme 1970

Rutishauser, E., Rohner, A., Held, D.: Experimentelle Untersuchungen über die Wirkung der Ischämie auf den Knochen und das Mark. Virchows Arch. Path. Anat. **323**, 101–118 (1960)

Sevastik, J. A., Mattsson, S.: Osteoporosis and Parathyroid Glands. Israel J. med. Sci. **7**, 346–349 (1971)

Uehlinger, E.: Die Kinetik des Calciumstoffwechsels. Verh. dtsch. Ges. Path. **47**, 69–91 (1963)

Vittali, P.: Osteocyte activity Clin. Orthop. **56**, 213–226 1968)

Weininger, J. M., Holtrop, M. E.: An ultrastructural study of bone cells: The occurrence of microtubules, microfilaments and tight junctions. Calcif. Tiss. Res. **14**, 15–29 (1974)

Westlin, N.: Loss of bone mineral after Colles' fracture. Clin. Orthop. **102**, 194–199 (1974)

Whalen, J. P., Krook, L., Nunez, E. A.: A radiographic and histologic study of bone in the active and hibernating bat (Myotis Lucifugus). Anat. Rec. **172**, 97–108

Whedon, G. D., Lutwak, L., Rambaut, P., Whittle, M., Leach, C., Reid, J., Smith, M.: Effect of weightlessness on mineral metabolism, metabolic studies on skylab orbital space flights. XIth Europ. Symp. Calcif. Tiss. 1975 (in press)

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