Electronmicroscopic study of mineralization in induced heterotopic bone formation in guinea pigs

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Abstract – Mineralization of heterotopic bone was studied in a bone induction model using allogenic demineralized dentin implanted in the abdominal wall of guinea pigs. There was a high yield of newly formed osteoid and bone as well as some cartilage together with areas of resorption of the dentin, and fibroblast proliferation. The osteoid contained many matrix vesicles II and less of lysosome-like type I vesicles. Early cartilage formation had more type I vesicles. The implanted dentin contained no matrix vesicles. The first signs of mineralization occurred mainly as irregular clusters of mineral crystals in the matrix close to the surface of collagen fibrils. Crystal-like figures were also found inside some type II matrix vesicles, although most of these vesicles in the mineralization zone had no crystals. The type I vesicles of both bone and cartilage exhibited often crystals near the outer membrane. The mineralizing bone showed a reduction in the size and number of proteoglycan particles. Remineralization of the implanted dentin was also often found and the mineralization pattern resembled the mineralization of bone except for the absence of matrix vesicles. Electron diffraction of selected areas showed that the crystals in the new bone and the mineralized dentin were hydroxyapatite.

Key words: bone; collagen; dentin; electronmicroscopy; mineralization.

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The mechanism of mineralization of hard tissue such as bone, dentin and cartilage has been studied extensively, and several theories have been proposed during recent decades.

Until about 1970 the effect and importance of some macromolecular species were emphasized, and mineralization was regarded as an extracellular process (7, 11). A relationship between collagen and crystals was first described by ROBINSON & WATSON (35). Crystal-like particles have been shown to be sequentially orientated in collagen fibrils (25). GLIMCHER & KRANE (22) suggested also that the collagen fibrils were the substrate for nucleation and that the initial stages showed crystals in "holes" in the collagen fibrils. Other investigators have proposed that the initiation of mineralization also involves the effect of proteoglycans (10, 19, 41), lipids (24, 51), and alkaline phosphatases (27, 36, 39).

During the past decade the role of cells involved in mineralization has been emphasized (20, 49). ANDERSON (2) and BONUCCI (15) demonstrated membrane-bounded vesicles in

the matrix of hard tissues having a diameter of about 100 nm. They proposed that the initial crystals appeared inside these vesicles. Later two subtypes of these matrix vesicles have been described, type I having a dark, lysosome-like content and type II having a content resembling intracellular matrix (31, 44). Several investigators have proposed that matrix vesicles constitute the initial site of the mineralization in cartilage (3, 5, 16) as well as early mineralization of dentin (13, 26) and bone (4, 14, 17). THYBERG (42), however, failed to demonstrate initial crystals in matrix vesicles of mineralized cartilage.

Using an experimental bone induction model (8, 32, 48) we are able to study bone formation that occurs de novo in postfetal life. Previous light microscopic studies of induced heterotopic bone formation in guinea pigs showed increasing amount of osteoid and bone between 16 and 28 d (9). Some cartilage formation was also found. The bone induction was performed by implanting freeze-dried allogenic dentin in the abdominal muscles of guinea pigs (9).

A previous study of the cellular reactions in this model (31) showed many type II matrix vesicles in the osteoid. Type I matrix were also observed but less frequently in osteoid than in the early cartilage formation. The aim of the present study was to investigate the ultrastructure of the matrix and the cellular components in the initial phase of mineralization of newly formed heterotopic hard tissues. Special emphasis was laid on correlating this process with the remineralization of the implanted dentin.

Material and methods

Fifteen randomly bred guinea pigs were used. They were fed a standard mixed diet (Norwegian standard for guinea pigs and rabbits, Statens Institutt for folkehelse) supplemented with swedes and hay and given water ad libitum. Under general anesthesia (Nembutal ®) demineralized and freeze-dried allogenic teeth were implanted intramuscularly as described previously (8). Four implants were used in each animal. The dentin implants were obtained from incisors and molars of adult male guinea pigs. The apical soft part and the pulp were removed. The demineralization was performed in 0.6 M hydrochloride acid (HCl) at room temperature (20° C).

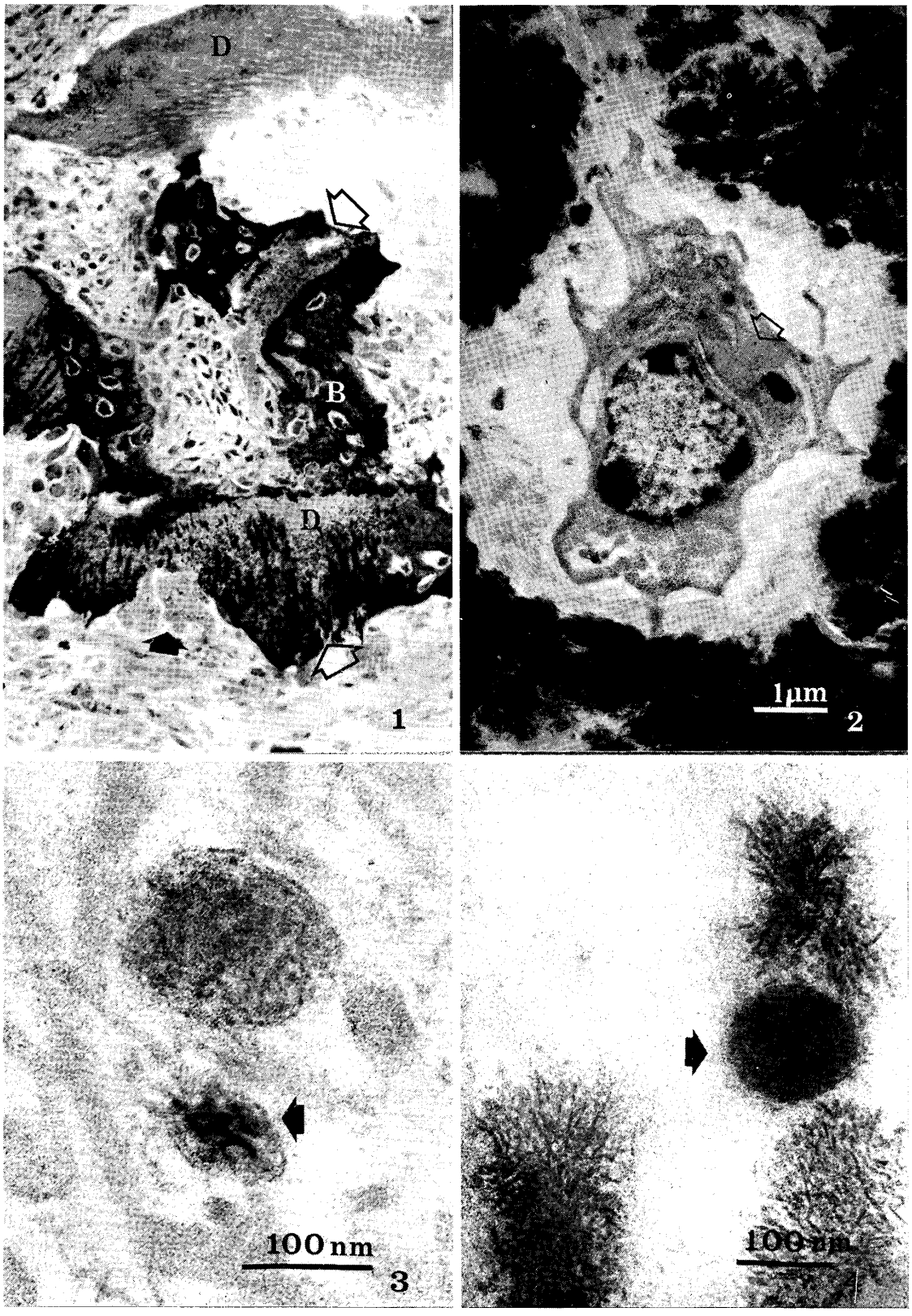
After 16, 21 or 28 d the implanted teeth with surrounding tissue were taken out under general anesthesia, immediately divided into small pieces (1 mm), and placed in ice-cold 2% glutaraldehyde buffered with 0.1 M cacodylate buffer with 7.5% sucrose. The specimens were postfixed in 2% OsO_4 in the same buffer, dehydrated and embedded in Epon 812®. Sections 1-µm-thick were cut with glass knives and stained with toluidine blue (1%). Areas of osteoid, bone cartilage and remineralized dentin were prepared and sectioned at 70 nm on a Reichert OM U3 ultramicrotome using a diamond knife. Both uncoated grids and Formvare® coated grids were used, and the floating time was as brief as possible. The sections were stained with uranyl acetate and lead citrate. Some sections were also examined unstained. All the sections were examined in a Philips 300 electron microscope at 80 kV accelerating voltage. Selected area electron diffraction was performed in the electron microscope on areas approximately 0.9 µm in diameter. The diffraction pattern was compared with unstained ultrathin sections from synthetic hydroxyapatite embedded in Araldite® (14% hydroxyapatite).

Results

Three types of cellular reactions were observed: 1) resorption, 2) fibroblastic proliferation and 3) zones of new matrix production. New matrix production was found consistently close to the implanted dentin (Fig. 1).

At 16 d the osteoid was dominating while at 21 d there was more mineralized bone. After 28 d there was abundant mineralized bone and remineralized dentin. Cartilage formation was observed less frequently than bone and mostly at 16 and 21 d.

The cells of the outer part of the osteoid resembled osteoblasts with polarized cytoplasm, having a prominent endoplasmic reticulum and Golgi apparatus. The osteocytes in the deeper parts of bone (Fig. 2) became more irregular, with diminished size, less prominent Golgi apparatus, smaller endoplasmic reticula and more free ribosomes, and their nuclei had more indentations and were more hyperchromatic. Electron dense granules were found regularly in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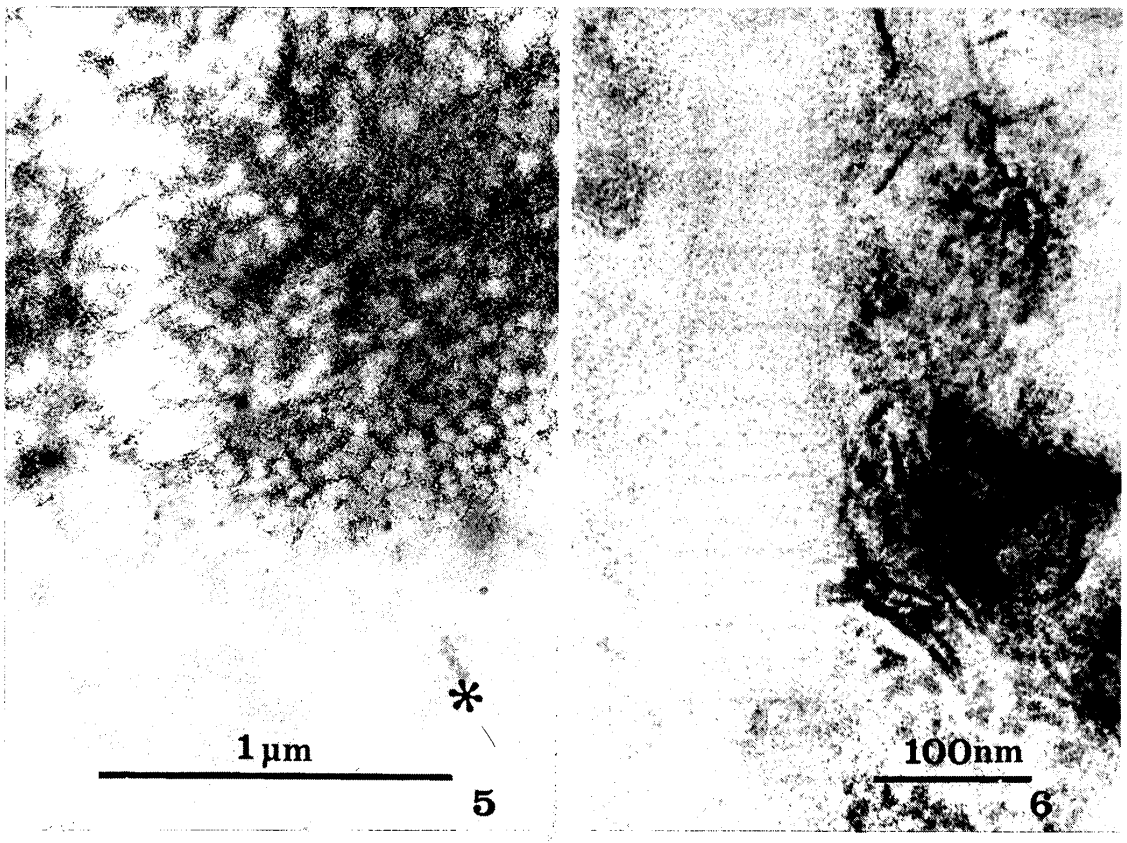

Fig. 5. Mineralization of the collagen matrix of osteoid with initial crystals surrounding cross-sectioned collagen fibrils. Note amorphous area in unmineralized matrix (*). Uranyl acetate and lead citrate. × 43 100. *Fig. 6.* Crystal located close to, but not inside, a collagen fibril from remineralizing dentin (see Fig. 1). Uranyl acetate and lead citrate. × 200 000.

the mitochondria of (Fig. 2) matrix-producing cells. Extrusion of mitochondria or these granules was not seen. There was always a distinct crystal-free zone between the cell membrane of the osteocyte and the mineralization front. This feature was also obvious along most cytoplasmic extensions, although the unmineralized zone here was narrower.

The collagen in the dentin and the osteoid both showed a periodic banding of 50–60 nm. The collagen in the dentin was about twice as thick as that of the bone. Between the collagen fibrils in bone and cartilage small granules (about 5–10 nm) were found. Early cartilage had more of these granules and less collagen in the matrix. The granules decreased in size and number during the mineralization.

Type II matrix vesicles were frequently found in the hard tissue forming area. They had a trilaminar unit membrane (Fig. 3) and had a

Fig. 1. Light microscopic picture of implanted dentin (D) with remineralized areas (open arrow). Outside dentin there is bone formation (B) and resorption of hard tissue with giant cells (arrow). Toluidine blue. × 225. *Fig. 2.* Osteocyte in induced heterotopic bone with fusiform appearance, and many free ribosomes. Mitochondria granule (open arrow). Uranyl acetate and lead citrate. × 9100. *Fig. 3.* Type II matrix vesicles with trilaminar membrane. One of the vesicles (arrow) contains crystal-like particles. Uranyl acetate and lead citrate. × 19 800. *Fig. 4.* Type I matrix vesicle (arrow) with a trilaminar membrane and electron dense content appearing close to some clusters of crystals. Uranyl acetate and lead citrate. × 130 000.

diameter from 40 nm to 150 nm. Type I matrix vesicles (Fig. 4), with a more osmiophilic content than type II, were also found, but less frequently in osteoid and bone than in cartilage.

Crystals were regularly found close to the membrane of type I vesicles (Fig. 4), while type II matrix vesicles were surrounded by a crystal-free zone. Crystal-like structures were seen inside some of these vesicles (Fig. 2) while others contained electrondense granules. Most of the matrix vesicles, however, had a content like the cytoplasm of the cells.

In areas of the initial mineralization of the osteoid radially orientated crystals were found near the collagen in small clusters. In other areas they seemed to increase in size and coalesce. The new crystals were mostly found surrounding the collagen fibrils, forming a filigree pattern on cross-section (Fig. 5). Intrafibrillar crystals were not found in areas of initial mineralization.

Areas with an amorphous appearance were frequently observed close to areas of initial mineralization (Fig. 5), but without any sharp delineation to the more distinct collagen matrix. The electron density homogeneous areas seemed to be equal to that of collagen.

Remineralization of the implanted dentin occurred regularly, mostly close to the mineralizing osteoid. Cellular components resembling matrix vesicles were not observed in the dentin. The initial crystals of the dentin appeared in small clusters close to the surface of the collagen fibrils, but without any further specific orientation (Fig. 6).

The initial crystals were not found inside fibrils. The size and shape of the crystals resembled those of bone.

Selected area electron diffraction of the initial clusters of crystals in bone and remineralized dentin showed diffraction patterns identical to that of synthetic hydroxyapatite. The diffraction rings were continuous and diffusely outlined.

Discussion

The present study supports the previous findings with regard (8, 31) to type of cellular reaction, including the amount of osteoid, bone and cartilage formation. More mineralized bone and remineralized dentin were observed, however, than in the previous electron microscope study (31) due to the longer experimental period in the present experiment.

The osteocyte decreased in size and appeared structurally simplified as the amount of bone increased, supporting earlier observations with this model (31) and previous reports of aging of bone cells (46, 47), showing that the osteocytes represent the terminal stages of the matrix-producing cell.

Electron-dense granules was found in the mitochondriae in osteoblasts and osteocytes which is in keeping with other reports (1, 21). GAY & SCHAER (21) and ALI *et al.* (1) have proposed that these granules are involved in calcium accumulation and that the mitochondriae were involved in concentration of calcium and phosphorus and the export of these elements to the matrix. No type of extrusion of such granules was observed in the present study.

The predominance of type II matrix vesicles over type I in mineralizing bone was in keeping with earlier findings (31). In contrast to the findings in osteoid, early cartilage formation exhibited more type I matrix vesicles, confirming another study of metaphyseal cartilage (45).

THYBERG & FRIBERG (43, 44) and MEIKLE (30) showed that some of the type I vesicles in cartilage demonstrated acid phosphatase activity. Other investigators, however, failed to demonstrate acid phosphatase in matrix vesicles. In a recent light microscopic study of the present model, acid phosphatase activity was demonstrated to be higher in young cartilage, both intracellularly and extracellularly (unpublished data) than in osteoid (32). SILBERMAN & FROMMER (40) described also a possible contribution of acid phosphatase to mineralization of cartilage. These enzyme histochemical results together with the ultrastructural picture may indicate that type I matrix vesicles are lysosome-like and have a function in changing the matrix to a "calcifiable" stage, according to the view of URIST (49).

The granules between the collagen fibers presumably contain proteoglycans. They resemble those described in bone (45) and showed no specific morphologic relationship to crystals. The reduction of the amount and size of the proteoglycans in the areas of initial mineralization may, however, corroborate previous observations suggesting that this is an important initiating factor of mineralization (10, 29, 42). Cathepsin D, which is a lysosomal protease, has been proposed to reduce the proteoglycans in mineralizing tissue (33). Greater amounts of acid phosphatase were found in mineralizing cartilage than in osteoid (32), and could be explained by higher amounts of type I matrix vesicles reflecting the need of enzymes for degradation of the proteoglycans.

Some type II matrix vesicles contained crystal-like material in the area of initial mineralization although several other vesicles showed electron dense material. Other investigators have found that the initial crystals appeared inside the matrix vesicles in the early mineralization of cartilage (2, 3, 16), dentin (26) and bone (12, 17).

BERNARD (12) and BONUCCI & GHERARDI (17) have proposed that mineralization of the interfibrillar material occurred after the mineralization of matrix vesicles had started. HÖHLING *et al.* (24) proposed that the first crystals of apatite were found in matrix vesicles and that the general mineralization of collagen matrix was a secondary event. THYBERG (42), however, found that intravesicular crystals were only found in heavily mineralized cartilage. The present study shows that the initial crystals appeared extravesicularly but also to a certain extent inside the type II vesicles.

The dominating matrix vesicles in osteoid and bone were type II, which indicates possible budding-off of the cytoplasmic process (31, 34). The membrane of these vesicles exhibits alkaline phosphatase including ATPase (28, 44). These enzymes are also believed to take part in mineralization of hard tissue (27, 36, 39), ANDERSON & REYNOLDS (4) have suggested that matrix vesicles concentrate calcium phosphate by an enzymatic process and in that way initiate mineralization. Matrix vesicles from cartilage have been shown by means of electronprobe analysis (1) to be rich in calcium. ALI *et al.* (1) suggested that the mineral present in the matrix vesicles is crystallike in nature. The present study, however, showed only few intravesicular crystals.

The amorphous intravesicular material observed in a part of matrix vesicles type II may represent amorphous calcium phosphate, which is proposed to precede the crystal formation in matrix vesicles. It seems therefore likely that calcium and phosphorus may accumulate in osteoblasts and osteocytes and matrix vesicles. The present study, however, shows that the first crystals seem to appear mostly outside the matrix vesicles.

Early mineral deposits inside the fibrils were not found, in contrast to previous reports on bone (22, 25) and dental cementum (37, 38). Except for the crystals found in some type II matrix vesicles, initial crystals were mostly found in small clusters in the ground substance in between and close to collagen fibrils. This is in accordance with the findings of ASCENZI & BENEDETTI (6). Other specific patterns could not be observed although a periodic banding of the mineralized matrix was observed in some areas of more mineralized matrix using low magnification. This banding, however, may be an artefact (18).

The size of the crystals and their random orientation in small clusters between the collagen fibrils of the remineralized dentin resembled the crystal formation between the collagen fibrils of mineralizing bone. Matrix vesicles or other cellular material, however, were not observed in the dentin. Remineralization of the dentin seemed mostly to occur after mineralization of the corresponding osteoid had started, in keeping with the results of FIRSCHEIN & URIST (20). A direct cellular contribution to the remineralization of the dentin, however, was not observed. Remineralization of the implanted dentin may thus be explained by the fact that this matrix was altered by having been minera-

lized previously and thus had the criteria of a "calcifiable" matrix, according to URIST (49).

The present study and discussion show that the cells and the cell derived matrix vesicles seem to be important in the control of the initial mineralization of heterotopic bone. The cells and matrix vesicles may contribute to this initial mineralization by accumulating calcium, and by producing enzymes which could alter the matrix prior to the initial mineral deposition.

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References

1. ALI, S. Y., GRAY, J. C., WISBY, A. & PHILIPS, M.: Preparation of thin cryosections for electron probe analysis of calcifying cartilage. *J. Microsc.* 1977: **111**: 65–76.
2. ANDERSON, H. C.: Electronmicroscopic studies of induced cartilage development and calcification. *J. Cell Biol.* 1967: **35**: 81–101.
3. ANDERSON, H. C.: Vesicles association with calcification in the matrix of epiphyseal cartilage. *J. Cell Biol.* 1969: **41**: 59–72.
4. ANDERSON, H. C. & REYNOLDS, J. J.: Pyrophosphate stimulation of calcium uptake into cultured embryonic bones. Fine structure of matrix vesicles and their role in calcification. *Dev. Biol.* 1973: **34**: 211–227.
5. ANDERSON, H. C. & SAJDERE, S. W.: Calcification of rachitic cartilage to study matrix vesicles function. *Fed. Proc.* 1976: **35**: 148–153.
6. ASCENZI, A. & BENEDETTI, E. L.: An electron microscopic study of the foetal membranous ossification. *Acta Anat.* 1969: **37**: 370–385.
7. BACHRA, B. N.: Calcification of connective tissue. *Int. Rev. Connect. Tissue Res.* 1970: **5**: 165–208.
8. BANG, G.: Induction of heterotopic bone formation by demineralized dentine in rats and guinea pigs. *Scand. J. Dent. Res.* 1973: **81**: 230–239.
9. BANG, G.: Induction of heterotopic bone formation by demineralized dentine in guinea pigs: relationship to time. *Acta Pathol. Microbiol. Scand.* Sect. A. 1973: **236**: 60–70: Suppl.
10. BAYLINK, D., WERGEDAL, J. & THOMPSON, E.: Loss of proteinpolysaccharides at sites where bone mineralization is initiated. *J. Histochem. Cytochem.* 1972: **20**: 279–292.
11. BECKER, G. L.: Calcification mechanism: roles for cells and minerals. *J. Oral Pathol.* 1977: **6**: 307–315.
12. BERNARD, G. W.: The ultrastructural interface of bone crystals and organic matrix in woven bone and lamellar endochondral bone. *J. Dent. Res.* 1969: **48**: 781–788.
13. BERNARD, G. W.: Ultrastructural observation of calcification in dentine and enamel. *J. Ultrastruct. Res.* 1972: **41**: 1–12.
14. BERNARD, G. W. & PEARSE, D. C.: An electron microscopy study of intramembranous osteogenesis. *Am. J. Anat.* 1969: **125**: 271–290.
15. BONUCCI, E.: Fine structure of early cartilage calcification. *J. Ultrastruct. Res.* 1967: **20**: 33–50.
16. BONUCCI, E.: Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J. Cell Biol.* 1969: **41**: 59–72.
17. BONUCCI, E. & GHERARDI, G.: Histochemical and electron microscopic investigations on medullary bone. *Calcif. Tissue Res.* 1975: **163**: 81–97.
18. CAMERON, D. A.: The ultrastructure of bone. in BOURNE, B. G. (ed): *The biochemistry and physiology of bone.* Academic Press, London & New York 1972, vol. I, pp. 196–201.
19. ENGFELDT, B. & HJERPE, A.: Glycosaminoglycans and proteoglycans of human bone tissue at different stage of mineralization. *Acta Pathol. Microbiol. Scand.* Sect. A 1976: **84**: 95–106.
20. FIRSCHEIN, H. E. & URIST, M. R.: Enzyme induction, accumulation of collagen and calcification in implants of bone matrix. *Clin. Orthop.* 1972: **84**: 263–275.
21. GAY, C. & SCHAER, H.: Frozen thin section of rapidly forming bone: bone cell ultrastructure. *Calcif. Tissue Res.* 1975: **17**: 39–49.
22. GLIMCHER, M. J. & KRANE, S. M.: The organization and structure of bone and the mechanism of calcification. In GOULD, B. S. (ed.); *Treatise of collagen.* Vol 2. Academic Press, London and New York, 1969, pp. 239–241.
23. HÖHLING, H. J., STEFFENS, H., STAMM, G. & MAYS, U.: Transmission electron microscopy of freezedried unstaining epiphyseal cartilage of the guinea pigs. *Calcif. Tissue Res.* 1976: **167**: 243–263.
24. IRVING, J. T.: Interrelations of matrix lipids, vesicles and calcification. *Fed. Proc.* 1976: **35**: 109–111.

25. JACKSON, S. F.: The fine structure of developing bone in the embryonic fowl. *Proc. R. Soc. Biol.* 1957: **146**: 270–283.
26. LARSSON, A: Ultrastructural observation on early dentin formation with special reference to "dentinal globules" and alkaline phosphatase activity. *Z. Anat. Entwickl.-Gesch.* 1973: **142**: 103–105.
27. LINDE, A. & MAGNUSSON, B. C.: Inhibition studies of alkaline phosphatases in hard tissue forming cells. *J. Histochem. Cytochem.* 1975: **23**: 265–275.
28. MATSUZAWA, T. & ANDERSON, H. C.: Phosphatase of epiphyseal cartilage studied by electron microscopic cytochemical methods. *J. Histochem. Cytochem.* 1971: **19**: 801–808.
29. MATUKAS, V. J. & KRIKOS, G. A.: Evidence of changes in protein polysaccharide associated with the onset of calcification in cartilage. *J. Cell Biol.* 1968: **39**: 43–48.
30. MEIKLE, M. C.: The mineralization of condylar cartilage in the rat mandible, an electron microscopic enzyme histochemically study. *Arch. Oral Biol.* 1976: **21**: 33–43.
31. NILSEN, R.: Electronmicroscopy for induced heterotopic bone formation in guinea pigs. *Arch. Oral Biol.* 1977: **22**: 485–493.
32. NILSEN, R. & MAGNUSSON, B. C.: Enzyme histochemical studies on heterotopic bone formation in guinea pigs. *Arch. Oral Biol.* 1979: **24**: 833–841.
33. NYGREN, H., PERSLIDEN, B., HANSSON, H. A. & LINDE, A.: Cathepsin D: Ultra-immunohistochemical localization in relation to dentine formation. *Calcif. Tissue Int.* 1979: **29**: 251–256.
34. RABINOWITCH, A. L. & ANDERSON, H. C.: Biogenesis of matrix vesicles in cartilage growth plates. *Fed. Proc.* 1976: **35**: 112–116.
35. ROBINSON, R. A. & WATSON, M. L.: Crystal-collagen relationship in bone as seen in electromicroscope. III. Crystals and collagen morphology as function of age. *Ann. N.Y. Acad. Sci.* 1952: **60**: 396–410.
36. ROBIRSON, R. A.: The possible significance of hexosephosphoric esters in ossification. *Biochem. J.* 1923: **17**: 286–293.
37. SELVIG, K. A.: An ultrastructural study of cementum formation. *Acta Odontol. Scand.* 1963: **22**: 105–120.
38. SELVIG, K. A.: Nonbanded fibrils of collagenous nature in human periodontal connective tissue. *J. Periodontal Res.* 1968: **3**: 169–179.
39. SEVERSON, A. R., TONNA, E. A. & PAVLEC, M.: Histochemical demonstration of nucleoside triphosphate hydrolysis in the mouse dentition. *Acta Histochem. (Jena)* 1968: **40**: 86–97.
40. SILBERMAN, M. & FROMMER, J.: Ultrastructural localization of acid phosphatase in cartilage of young mandibular condyles. *Histochemie* 1973: **37**: 365–369.
41. SOBEL, A. E., BURGER, M. & NOBEL, S.: Mechanism of nuclei formation in mineralization tissue. *Clin. Orthop.* 1960: **17**: 103–123.
42. THYBERG, J.: Electron microscopy on the initial phases of calcification in guinea pig epiphyseal cartilage. *J. Ultrastruct. Res.* 1974: **46**: 206–218.
43. THYBERG, J. & FRIBERG, U.: Ultrastructure and acid phosphatase activity of matrix vesicles and cytoplasmic dense bodies in the epiphyseal plate. *J. Ultrastruct. Res.* 1970: **33**: 554–573.
44. THYBERG, J. & FRIBERG, U.: Electron microscopic enzyme histochemical studies on the cellular genesis of matrix vesicles in the epiphyseal plate. *J. Ultrastruct. Res.* 1972: **41**: 43–59.
45. THYBERG, J., NILSSON, S. & FRIBERG, U.: Electronmicroscopic and enzyme cytochemical studies on the guinea pig metaphysis with special references to the lysosomal system of different cell types. *Cell Tissue Res.* 1975: **156**: 273–299.
46. TONNA, E. A.: An electronmicroscopic study of skeletal cell aging. II. The osteocyte. *Exp. Gerontol.* 1973: **8**: 9–16.
47. TONNA, E. A.: Factors (aging) affecting bone and cementum. *J. Periodontol.* 1976: **47**: 267–280.
48. URIST, M. R.: Bone formation by autoinduction. *Science* 1965: **150**: 893–899.
49. URIST, M. R.: Biochemistry of calcification. In: BOURNE, G. H. (ed.): *The biochemistry and physiology of bone.* Vol. IV. Academic Press, New York 1976, pp. 11–24.
50. WERGEDAL, J.: Characterization of bone acid phosphatase activity. *Proc. Soc. Exp. Biol. Med.* 1970: **134**: 244–247.
51. WUTHIER, R. E.: The role of phospholipids in biological calcification distribution of phospholipase activity in calcifying epiphyseal cartilage. *Clin. Orthop.* 1973: **90**: 191–200.