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An Ultrastructural Study of the Role of Calcification Nodules in the Mineralization of Woven Bone

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Summary. Osteolathyrism has been used as an experimental model for the study of calcification nodules during the mineralization process. Periosteal exostoses developing in osteolathyrism characteristically have spherical basophilic structures (calcification nodules) in the vicinity of developing bone spicules. In thin sections, the nodules were seen scattered between collagen fibers in the intercellular matrix. Collagen fibers did not appear to be present within the nodules but sometimes were packed just outside them. Matrix vesicles were also present in areas of early mineralization.

After EDTA decalcification, the majority of the nodules consisted of a fine granular material surrounded by an electron-dense peripheral zone. The peripheral dense zone was occasionally incomplete in small nodules in areas of early mineralization. An electron-dense central area could be observed in the center of the nodules.

Evidence has been presented indicating that the calcification nodules arise from smaller mineralization foci, presumably matrix vesicles. The calcification nodules enlarge to approximately 1.0 μm in size, at which point development is slowed or halted allowing the formation of the peripheral dense zone.

Although coalescence of nodules was observed, this was more a random event. The further mineralization of the trabeculae was achieved by the calcification of the collagen fibers. The mineralized trabeculae reflected this pattern of nodular and collagenous calcification. It is suggested that this pattern of calcification is characteristic of rapidly developing woven bone.

Key words: Bone — Bone formation — Calcification — Calcification nodule — EM.

Introduction

Early studies of bone mineralization indicated that the mineral phase was deposited on or within the collagen fibers (1-4). More recent studies (5-7) suggest two additional structures may be implicated in the early calcification process. These are matrix vesicles (8, 9) and bone nodules (5). Matrix vesicles have been extensively studied (9-11) but bone nodules have received less attention. Bernard and Pease (5) described bone nodules as discrete spheres, composed of hydroxyapatite crystals which grew radially into the surrounding matrix. Decalcified, the organic substructure of the nodule was revealed by Bernard and Pease (5) to contain (a) a central dense zone, thought to be the initial calcification locus; (b) an outer marginal or peripheral zone, thought to be decomplexing collagen; and (c) an intermediate zone, composed of altered colla-

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gen. These nodules, when coalesced, formed spicules of bone.

Not all descriptions of bone formation include all three structures as being involved in the mineralization process. Further, not everyone agrees on the importance of matrix vesicles (12) or on the composition and morphology of the bone nodules (5-7).

In the present report, osteolathyrism has been used as a model for the study of the nodules. Osteolathyrism is characterized by the rapid formation of periosteal exostoses at the sites of muscle attachment on long bones (13, 14), where the various stages of bone formation can readily be observed. Studies (13, 14) have shown that the various stages of bone formation within the exostosis are not formed at one time but that they appear in sequence, and that by the seventh day all stages of bone formation are represented.

Selye (15) noticed that large nodules (basophilic globules) were so prominent in osteolathyrism that they might be pathognomonic for lathyrism. Electron microscopic studies of bone formation in lathyritic rats (16) have revealed that all three structures, matrix vesicles, bone nodules, and collagen fibers, are involved in the formation of woven bone. The following report describes the nodules and outlines their possible role in the calcification process of woven bone.

Materials and Methods

Weanling rats were given a diet of commercial powdered food containing 0.4% beta-aminopropionitrile (BAPN). After 7 or 8 days on the diet, the animals were given an intraperitoneal injection of sodium pentobarbital (5-7 mg/100 g body weight), perfused with 4.0% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) alone or with 0.2% ruthenium red according to Luft (17). After perfusion, the middle third of the femora with the adductor longus and pectineus muscles attached were removed, the marrow cavities cleaned, and the femora further fixed for 2 to 4 h at 4° C.

Femora were decalcified in 0.1M ethylenediamine tetraacetic acid (EDTA) in the glutaraldehyde fixative at pH 7.4 for at least 10 days at 4° C. The femora were then washed overnight in 0.1M cacodylate buffer with 5% sucrose added. The exostoses were removed and cut into blocks approximately 1 mm^3. These blocks were post-fixed in 1.0% osmium tetroxide in 0.1M cacodylate buffer for 2 h at room temperature. Some of the femora were processed undecalcified.

For those tissues treated with ruthenium red in the glutaraldehyde fixative, ruthenium red was also included in the EDTA, the buffer wash, and the osmium tetroxide.

All the tissue blocks were dehydrated in ascending series of ethanols, followed by propylene oxide, embedded in Epon and polymerized at 60° C for 24 h. Epon sections approximately 1 μm thick were stained with toluidine blue for orientation purposes. For the detection of mineralization, semithin sections of undecalcified tissue were softened in xylene, then incubated in von Kossa's silver nitrate for calcium and counterstained with 2% safranin 0.

Silver to gray thin sections were obtained and placed on copper grids. To ensure minimal loss of calcium while cutting blocks of undecalcified tissue, we adjusted the water in the cutting trough with ammonium hydroxide to pH 7.6, and did not allow undecalcified sections to remain floating on the water more than 20 sec.

Ultrathin sections were further contrasted with either uranyl acetate for 30 min followed by 5 min in lead citrate, or with 5% phosphotungstic acid in distilled water at pH 1.2 for 30 min, followed by rinsing in 1% phosphotungstic acid in distilled water (5). The sections from tissue prepared with ruthenium red were viewed without additional staining. All sections were viewed in an RCA 3G electron microscope at an operating voltage of 50 kV.

Results

The zonation of the lathyritic exostosis described by Yeager and Hamre (14) and Yeager and Gubler (13) was observed in this study. Using their criteria we could identify three zones: an outer proliferative zone, a middle zone characterized by large amounts of intercellular material, and an inner deep zone containing trabeculae of woven bone. The region of transition between the middle and deep zones contained the structures involved in the mineralization of bone.

Light microscopy revealed round nodules approximately 1.0 to 1.5 μm in diameter in the matrix of this transition zone (Fig. 1). The nodules had no orientation with respect to cells. In the von Kossa stained sections the nodules were heavily covered with silver. The nodules in toluidine blue stained, decalcified sections had a doughnut-like appearance, with the central portion of the nodule appearing lighter than the outer ring. An occasional nodule had a central dark area so that three zones were distinguished. In both decalcified and undecalcified sections, the nodules were observed to coalesce and form larger islands which resembled the woven bone of trabeculae.

Electron micrographs revealed vesicular structures, presumed to be matrix vesicles (9, 10), within the intercellular matrix of the transitional zone (Figs. 2, 4, and 7). These membrane-bound matrix vesicles were approximately 0.1 μm in diameter. Occasionally, crystals of hydroxyapatite were observed within the matrix vesicles from undecalcified sections (Fig. 2).

The most striking feature of the transitional zone was the presence of nodules of calcification. The size of the nodules ranged from 0.5 to 1.5 μm in diameter. In undecalcified sections, they were com-

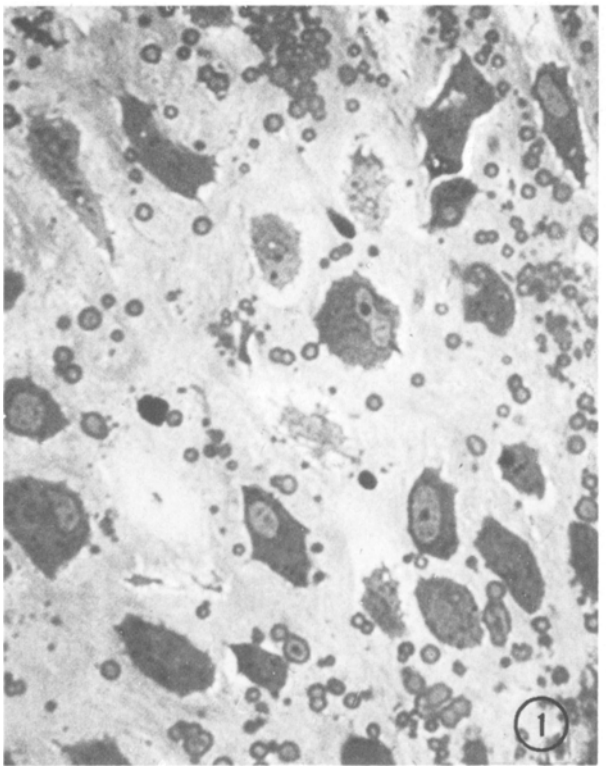

Fig. 1. A toluidine blue stained section from an area of a trabecula undergoing mineralization. Calcification nodules appear as spherical structures, with a doughnut-like appearance. × 1600

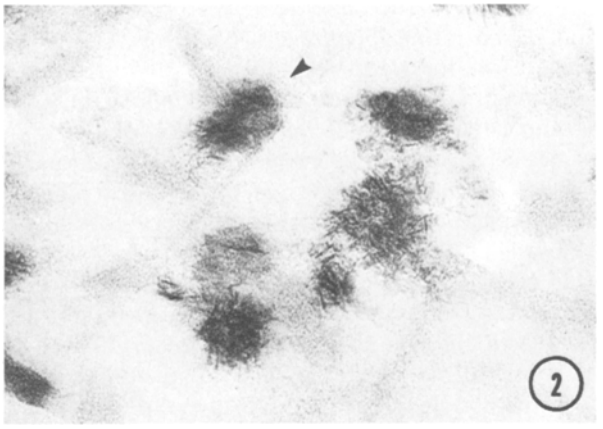

Fig. 2. An electron micrograph of matrix vesicles during initial stages of calcification. The vesicles contain varied amounts of apatite. The remnant of a trilaminar membrane is evident (arrow). Unstained, × 100,000

posed of clusters of calcium crystals. After EDTA decalcification the nodules appeared as spherical structures within the matrix (Fig. 3).

The typical nodules showed three zones. A distinctive peripheral zone of high electron density

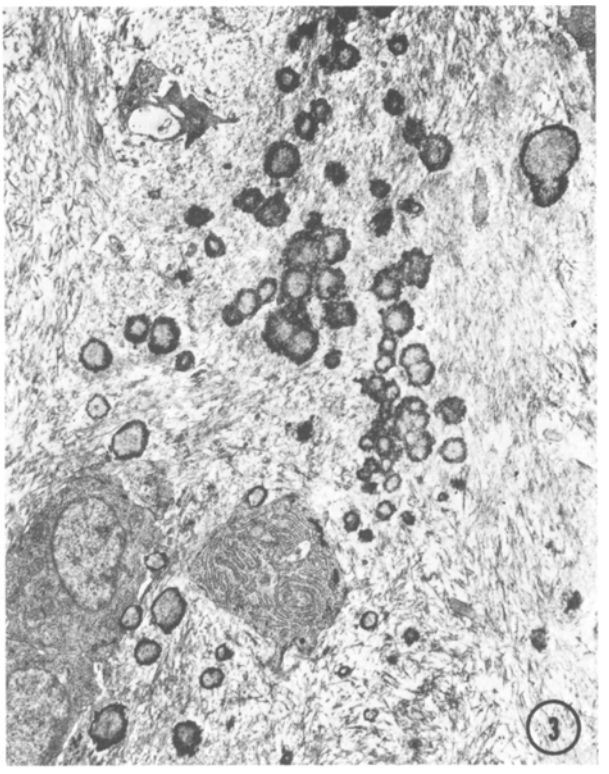

Fig. 3. An electron micrograph from a trabecula undergoing mineralization. Numerous calcification nodules are present, Single isolated nodules as well as clusters of nodules are evident. EDTA—decalcified, uranyl acetate and lead citrate, × 4000

was observed around the nodules. This peripheral zone appeared to separate the nodules from the surrounding matrix. Uranyl acetate and lead citrate (Fig. 4) showed this outer electron-dense zone to be composed of densely packed granules. No distinct limiting membrane could be identified, although at times the granular material appeared in rows. Ruthenium red preparations greatly enhanced the peripheral zone of the nodules (Fig. 5). The zone did not appear to be composed of granules with this stain, but rather was composed of an amorphous material of intense ruthenium red reaction. With the phosphotungstic acid technique (Fig. 6), the peripheral zone was not stained. However, the surrounding collagen stained intensely with phosphotungstic acid.

Internal to this peripheral zone was an intermediate zone of less dense material. After uranyl acetate and lead citrate staining (Fig. 4), this material was composed of a fine electron-dense granular component and a less dense filamentous material. The material was not uniformly dispersed. Ruthenium red (Fig. 5) did not enhance the material, which was of intermediate electron density when compared to the uranyl acetate-lead citrate (Fig. 4) and phosphotungstic acid (Fig. 6) stained material.

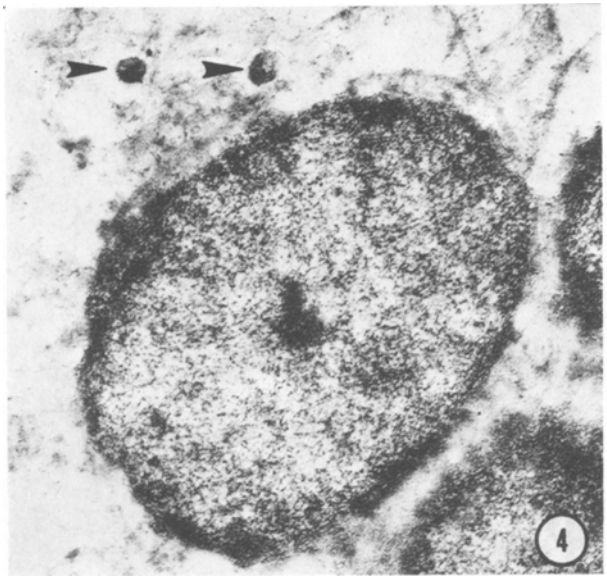

Fig. 4. A calcification nodule as observed with uranyl acetate and lead citrate staining. Note the matrix vesicles isolated within the matrix (arrows). EDTA—decalcified, uranyl acetate and lead citrate, × 40,000

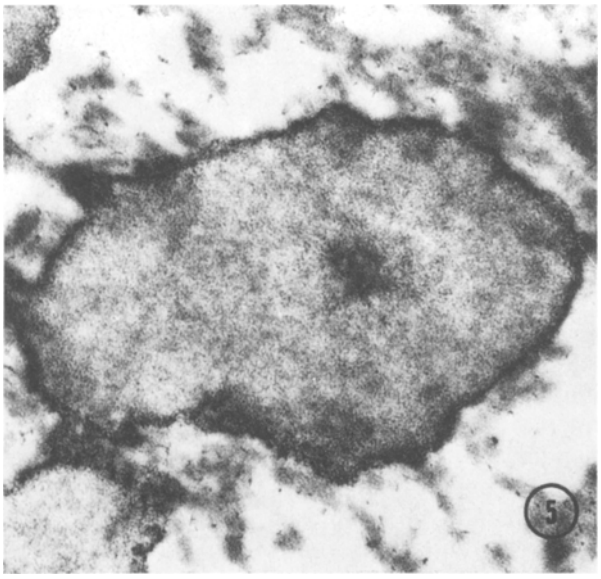

Fig. 5. A calcification nodule as observed with ruthenium red staining. The peripheral zone as well as the central area are strongly stained. EDTA—decalcified, ruthenium red, × 40,000

There was no evidence of phosphotungstic acid stainable material within the nodule (Fig. 6). Collagen fibers could not be recognized within the nodules.

If the nodule was sectioned through the central portion, an electron-dense area was observed in the center of the nodule (Fig. 4 through 6). This area was observed with all the techniques employed. It was amorphous in shape and measured approximately 0.1 μm. No limiting membrane was associated with this central dense area.

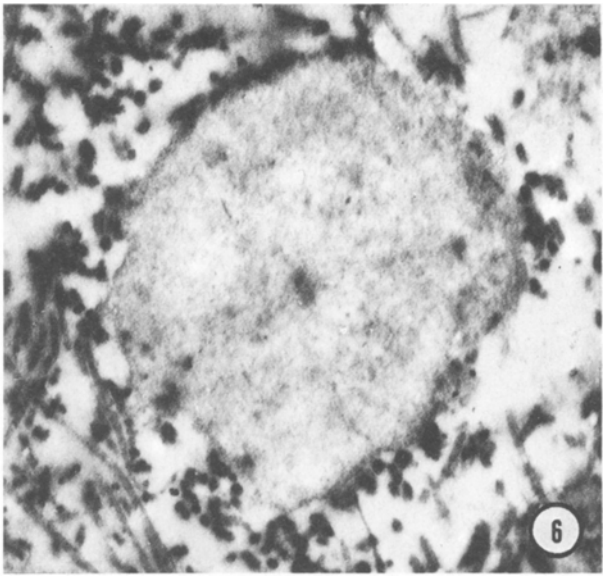

Fig. 6. A calcification nodule as observed with phosphotungstic acid staining. The peripheral zone is not as evident as with the previous stains (Figs. 4 and 5). EDTA—decalcified, phosphotungstic acid, × 40,000

Occasionally smaller nodules were seen in which the dense peripheral zone was incomplete or absent. In these nodules no sharp demarcation between the nodule and the surrounding matrix existed (Fig. 7). The central dense area could also be observed in these nodules. It appeared similar to those observed in the large nodules.

Occasionally the nodules were clustered in groups, and often appeared to coalesce. This coalescing of nodules appeared to be a random event, occurring only when nodules formed in proximity to one another. Additional mineralization of the matrix occurred as elongated projections of crystals extended from the nodules to the collagen fibers. The calcification of the collagen fibers continued forming islands of mineralized matrix.

After EDTA decalcification, these islands were observed to be composed of coalesced nodules and interwoven collagen fibers (Fig. 8). Individual nodules were still recognizable. The collagen fibers between the nodules appeared densely matted, and individual fibers were barely visible, making it easy to differentiate mineralized from unmineralized areas (Fig. 8).

The islands continued to enlarge and fuse, and became continuous with the trabeculae. Cells in the area became entrapped within the trabeculae (Fig. 9). The less dense internal material of the nodules gave a mottled appearance to the trabeculae (Fig. 9). In some nodules the electron-dense central regions could still be observed.

Deeper into the inner zone some evidence of remodeling of the trabeculae was observed. In these

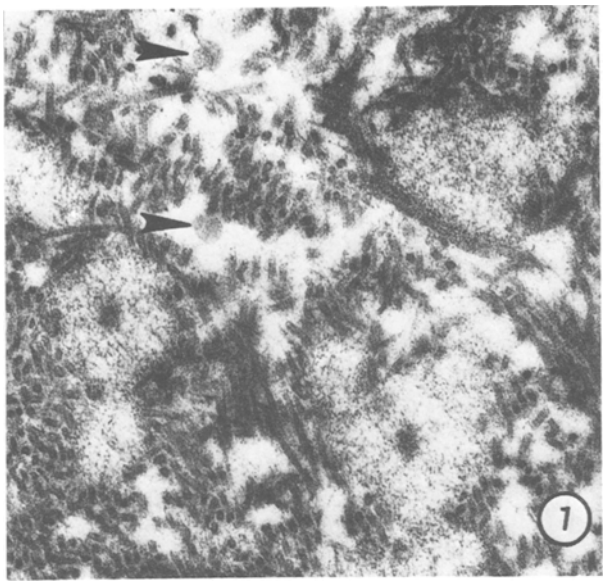

Fig. 7. Calcification nodules from a trabecula where mineralization is in early stages. The nodules are smaller and lack the peripheral dense zones. Note the presence of the central dense zones within the nodules and the matrix vesicles in the surrounding matrix (arrows). EDTA—decalcified, uranyl acetate and lead citrate, × 25,000

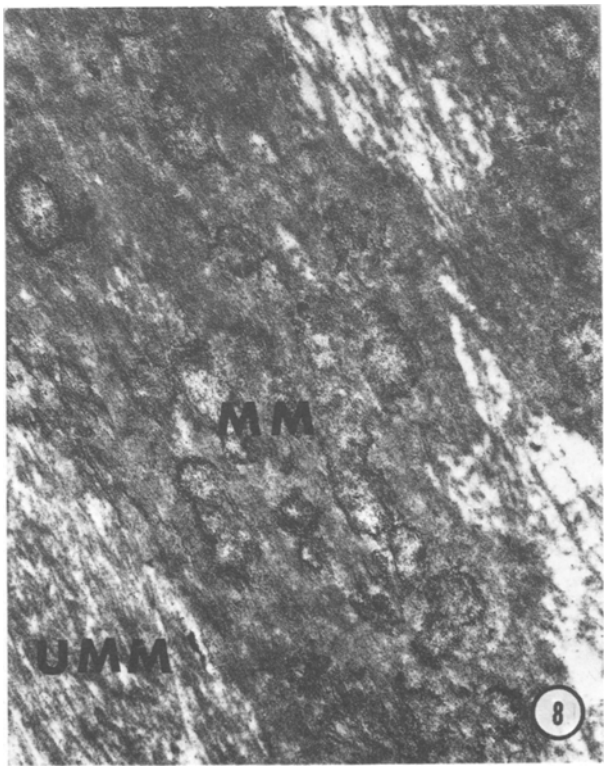

Fig. 8. A trabecula where mineralization is advanced. The mineralized matrix (MM) is composed of nodular elements and matted collagen fibers. There is a clear distinction between the mineralized matrix and the unmineralized matrix (UMM). EDTA—decalcified, ruthenium red, × 12,000

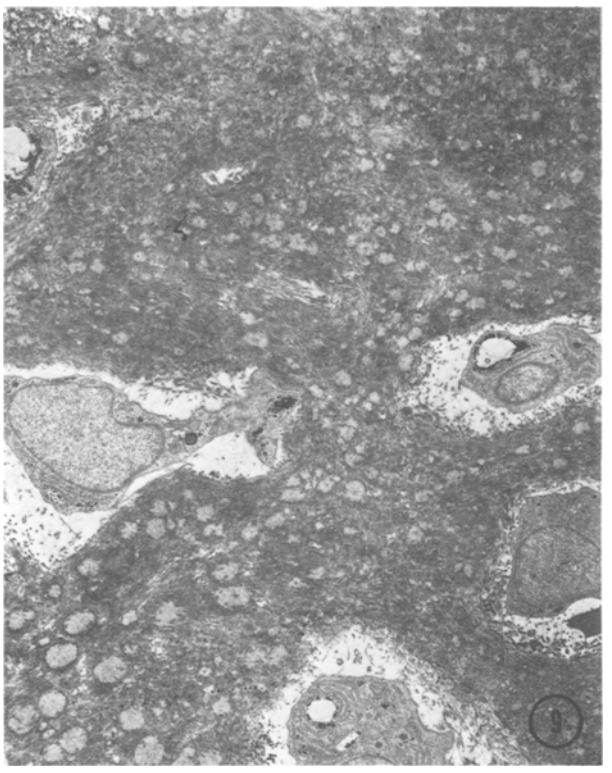

Fig. 9. A trabecula which was completely mineralized. The mineralized matrix appears mottled due to the clusters of nodules and interwoven matted collagen fibers. Occasional electron-dense central areas can be seen in some nodules. EDTA—decalcified, uranyl acetate and lead citrate, × 4000

areas, lamellar bone was added to the surface of the trabeculae. Neither matrix vesicles nor calcification nodules were observed between the osteoblasts and the lamellar bone. Mineralization involved only the collagen fibers.

Discussion

Large spherical crystalline structures have been described in membranous bone by many investigators (5, 6, 18, 19). Bernard and Pease (5) called these clusters "bone nodules," and considered the calcification of bone to be the outcome of their coalescence. However, it should be pointed out that the term "bone nodule" for these structures is misleading. Similar radial foci of crystals are seen in calcifying cartilage (6, 8, 20–22), and therefore are not peculiar to bone mineralization. Since these structures represent foci of calcification in tissues other than bone, it is proposed that they be called "calcification nodules."

Evidence has accumulated showing that the first identifiable crystals of hydroxyapatite are deposited within small (0.1 μm in diameter), extracellular, membrane-bound matrix vesicles (9, 10). The present study confirms the presence of matrix vesicles

during the early stages of mineralization and suggests that mineralization begins within these vesicles.

In outlining the role of matrix vesicles during the calcification process, Anderson (9) and Anderson and Sajdera (11) proposed that calcium hydroxyapatite crystals form within the membrane-bound vesicles. This is followed by increased crystal growth and development until the crystals rupture the vesicular membrane and become exposed to the extravesicular matrix. The exposed crystals "seed" further crystal growth and development in the extracellular spaces. We suggest that once the matrix vesicle ruptures, and its contents are exposed to the intercellular matrix, it no longer be called a matrix vesicle. The further growth and development of hydroxyapatite crystals within the matrix is the calcification nodule. It represents the spread of calcification within the ground substance, and is composed of inorganic components.

The central dense area observed within the nodule might represent the organic remnants of the ruptured matrix vesicles. Bernard and Pease (5) explained the existence of these central regions as remnants of osteoblast extrusions, which function as initial calcification loci. They believed this central area to represent the first site of hydroxyapatite deposition within the interfibrillary matrix. Further crystal growth then proceeds from this site. Such an explanation would be consistent with experimental findings that matrix vesicles contain crystals of hydroxyapatite, which when released locally into the extracellular space permit further nucleation of mineral apatite (9-11).

The material comprising the interior of the nodule is visible only after removal of the calcium salts. Bonucci (6, 20, 21) termed the underlying organic matrix of hydroxyapatite crystals as "crystal ghosts," since the underlying material had the same shape, size, and arrangement as undecalcified crystals. Thyberg (23) reported that after removal of all hydroxyapatite crystals by EDTA, crystal ghosts were not present; nor were they observed in this investigation. The term crystal ghost is therefore misleading. It does not always appear as an actual organic template of the hydroxyapatite crystal, and use of the term should be avoided.

Bernard and Pease (5) described these internal regions of the nodules as globular material with filamentous strands of degraded or partially solubilized collagenous material. Since collagen fibers were not observed to be directly associated with the formation of the nodules, and based on the lack of any appreciable phosphotungstic acid stainable material within the nodule, the material present would appear to be noncollagenous in nature. Bonucci (6) believes that the material does not contain collagen but rather represents proteins of the ground substance. There is ample evidence of protein polysaccharide interaction with hydroxyapatite crystals (24). Further, Scherft (7) showed that the organic matrix of developing islands of mineralization contains Thorotrast-stainable mucopolysaccharides. In the present study, the material was not greatly enhanced after ruthenium red staining.

A striking feature of the underlying organic structure of the calcification nodules is the presence of the peripheral dense zone. This zone is at the junction between the mineralized matrix of the nodule and the surrounding unmineralized matrix. Scherft (7) examined the borders around mineralization foci and called these borders lamina limitans. He defined lamina limitans as a prominent histological border or demarcation at the junction of mineralized and nonmineralized matrix. The peripheral dense zone associated with the nodule fits into this definition. We therefore suggest that the term "lamina limitans" be used in describing the peripheral dense zone of the calcification nodule.

Scherft (7) hypothesizes that the lamina limitans could originate by accretion or adsorption of organic material on the surface between the mineralized matrix and the unmineralized matrix. The purpose of this material would presumably be to aid the deposition of apatite. Bernard and Pease (5) believe the dense material represents the de-aggregation of collagen during the mineralization process. There is, however, no reason why collagen breakdown should be occurring at the site of mineralization. Also, collagen was not observed to be directly associated with nodules in this study. The ruthenium red positive nature of the material would indicate the presence of protein polysaccharides and possibly phospholipids (25). It has been speculated that protein polysaccharides aid in the delivery of calcium ions necessary for the nucleation of apatite crystals (24, 26, 27). Acid phospholipids have also been shown to be associated with the calcification process (28-30). Boskey and Posner (28) identified a phospholipid-phosphate complex which might serve as a means of transporting calcium to the site of hydroxyapatite formation. Thus the presence of both protein polysaccharides and phospholipids within an area of active hydroxyapatite formation would be justifiable. It is evident that further examination of the material within this peripheral zone is necessary.

Variations in the thickness and density of the peripheral zone of different nodules might represent changes in the rate of accumulation or activity of the material at the mineralizing surfaces. Scherft (31) believes that the various size differences of the

lamina limitans he observed in bone and cartilage reflect alterations in the rate of mineralization. Further, he reported that laminae limitans are lacking in regions where mineralization is actively extending and present where activity is arrested. This would account for the presence of small nodules which did not show a distinct peripheral zone or lamina limitans. In early stages of mineralization, calcification occurs so rapidly that the material is utilized before it has time to accumulate in sufficient quantities along the periphery. As mineralization progresses, extracellular fluid concentrations are altered, as evidenced by the progressive decrease in proteoglycans associated with increasing degrees of mineralization (26, 32, 33). This change of the matrix fluid in the vicinity of the nodules could slow or halt the rate of calcification, allowing the accumulation of the material at the periphery of the nodule. The fact that all the nodules exhibiting the peripheral dense zone were approximately the same size indicates that the nodules expand to a certain size before development is halted. This may indicate that further growth of the nodule is limited by either the quantities of material contained within the matrix vesicle or the local environment.

In cartilage, calcification of the matrix is accomplished by the radial increase in size of round crystal clusters (6, 22). The crystal clusters coalesce, eventually completely obscuring the matrix as the cartilage becomes entirely calcified. In the present investigation, the coalescence of nodules appears to be a random event, occurring only when nodules happen to form in proximity to one another. The further mineralization of the matrix involves calcification of the collagen fibers. Elongated projections of crystals along the collagen fibers have been reported in membranous bone during early mineralization (1, 2, 6, 18, 19). The presence of hydroxyapatite along the collagen does occur in bone.

Mineralization spreads across the matrix by the calcification of the collagen fibers adjacent to the nodules. This spreading of apatite crystals along the collagen fibers between nodules interconnects isolated nodules and results in the formation of mineralized islands. The calcification of the collagen appears to alter the morphology of the collagen fibers. This is apparent from the amorphous matted appearance of the fibers in decalcified sections. At present, the cause for the matted appearance of the collagen fibers in the mineralized matrix is not known. Perhaps the appearance of the collagen fibers is a reflection of the lathyritic collagen, which is not stabilized by a full complement of intermolecular cross-links (34).

The mottled appearance of the decalcified trabeculae is noteworthy. Amemiya (35) observed a mottled appearance of the decalcified matrix in osteolathyrism, similar to that observed in this investigation. He believed that this mottled pattern of mineralization was due to a defect in the formation of collagen fibers and ground substances, and therefore considered this to be characteristic only of mineralization in the lathyritic periosteal exostosis. However, we contend that the mottled appearance of the matrix simply represents the presence of nodules in the matrix. This is characteristic of very rapidly growing woven bone (5-7). The lamellar bone seen deep in the inner zone did not have this mottled appearance, indicating that normal appearing lamellar bone can occur in lathyritic animals.

We propose that in situations demanding the rapid formation of bone, cells speed up the process by producing matrix vesicles which begin the calcification process. Their rupture results in the formation of calcification nodules. The nodules then initiate mineralization of the collagen fibers. The bone formed is immature woven bone. Other situations not requiring such rapid bone formation will result in lamellar bone, and matrix vesicles and calcification nodules do not appear essential for its formation.

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