Chapter 6

# The Mineralization of Bone and Its Analogies with Other Hard Tissues

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## 1. Introduction

Bone is one of the many biological tissues characterized by the still poorly defined process of mineralization, that is, by the deposition of inorganic substance in their organic matrix, and is one that, chiefly due to its important biological functions, but also because of its widespread presence in primates and its easy availability, has been investigated for hundred of years by thousand of investigators (see reviews by [1-3]). Its study, however, is a demanding task, because of the complexity and variability of the tissue – factors that derive mainly from differences in its histological types, biochemical composition, phases of maturation, and degree of mineralization (the terms 'mineralization', 'biomineralization', and 'calcification' are treated as equivalents in this paper; see Bonucci [2]). These differences, although recently emphasized [4-5], have not always received due recognition. In this connection, revealing indicative examples include, for instance, the differences between the compact, lamellar bone and spongy, woven bone [4], or between the dense rostrum bone of toothed whales [6] and the medullary bone of birds [7]. These differences may lead to incorrect interpretations of apparently contrasting results, but may also lead to new insights if they are recognized to derive from distinctive traits of tissues belonging to the same broad type, which is what they are. The concepts that follow, which chiefly focus on the local mechanism of matrix calcification, mainly refer to woven bone, which raises fewer technical issues than compact bone, but they are valid for, and can be extended to, all other types of bone and hard tissue.

## 2. Historical notes

So much research has been carried out on bone mineralization that it is hard to provide an inclusive summary; moreover, research of this kind is often interlaced with that on other cal-

cifying tissues, especially cartilage, enamel and mollusk shells, so that, even if references are limited to the main studies closely connected with the subject of this review, i.e., the local mechanisms of matrix calcification, their selection may appear subjective, and omissions are hard to avoid. With these limitations in mind, the first important investigations and theories on the calcification of bone and other hard tissues date back to the first quarter of the twentieth century. In 1923, Robison [8], considering that the concentration of phosphate ions in the bone matrix is too low for allowing calcium phosphate precipitation, suggested that it could be locally increased by the hydrolysis of phosphate esters promoted by an enzyme, alkaline phosphatase. This theory was later challenged, chiefly because the local amounts of phosphate esters are insufficient to produce concentrations of phosphate ions high enough to exceed the solubility product of calcium phosphate, nor could the problem be solved by resorting to the consideration of phosphate sources such as glycogen or adenosine triphosphate (ATP). It did, however, have the great merit of drawing attention to an enzyme that is actually fundamental to calcification [9], while stressing the primary role of organic molecules in biological calcifications. This was also the important indication given by Freudenberg and György [10] in the same year, 1923. They noted that cartilage calcification occurs in vitro if the tissue is first treated with calcium chloride and then with phosphate, but not the reverse, and concluded that this was due to the binding of calcium to a colloid, with the subsequent binding of phosphate and formation of calcium-phosphoprotein complexes. In line with this concept, Robison and Rosenheim [11] suggested that an enzymatic 'second mechanism' should constitute a 'local factor' that could induce the precipitation of calcium and phosphate ions even when their concentration is too low. On the basis of the correlation between the degree of matrix metachromasia and the calcification process, Sobel [12] suggested that chondroitin sulfate, or the collagen-chondroitin sulfate complex, were possible constituents of the local factor, which was, in any case, supposed to be a component of the organic matrix. Again early in the twentieth century, DeJong [13] first observed that bone powder gives a crystalline X-ray diffraction pattern similar to that of inorganic apatite, an observation which is fundamental to an understanding of the organization of the calcium phosphate that forms the bone mineral, but is, at the same time, the basis for a number of misinterpretations of its crystalline nature, as discussed below (the word 'crystals' or 'crystallites' used in this paper in referring to the bone mineral has been retained for historical reasons, even if it may lead to misconceptions about their true structure and nature). On the basis of the coincidence of their X-ray diffractograms, Caglioti [14] suggested that the inorganic substance and components of the organic matrix were closely connected, and Dawson [15], noticing that trypsin digestion increases the sharpness of the diffractograms, supposed that this was due to the binding of the inorganic substance to the organic matrix. Dallemagne and Melon [16] and Ascenzi [17], on the basis of bone birefringence, hypothesized the existence of organic-inorganic bonds.

The concepts summarized above prompted a number of studies. The histochemical demonstration that in bone and cartilage the matrix that calcifies contains glycoproteins and acid proteoglycans (i.e., is PAS-positive and metachromatic; Pritchard [18]) drew attention to these substances and supported the suggestion that the local factor could be chondroitin sulfate [12]). At almost the same time, Di Stefano et al. [19] suggested that the formation of

crystals in the matrix was a process catalyzed by a template, and Neuman and Neuman [20] discussed the possibility that it might be the result of the stereotactic properties of a component of the organic matrix: in their opinion, calcium and phosphate ions could bind to an organic template according to the space relationships in the apatite lattice, so giving rise to apatite nuclei that would grow into definitive crystals by further ion aggregation. Specific steric relationships between amino-acid side-chains groups in the collagen fibrils were then considered the possible nucleation centers within the fibrils [21] (see below). A close relationship between the inorganic substance and the collagen fibrils was shown and repeatedly confirmed by electron microscopy, starting with the pioneering studies of Robinson and Watson [22] and Ascenzi and Chiozzotto [23]. Another fundamental observation was obtained by microradiography: Amprino and Engström [24] first showed that the degree of bone mineralization is not constant, because that of primary bone is always higher than that of secondary bone; moreover, they found that osteon calcification occurs in two stages, of which the first is characterized by quick deposition of about 70% of the final mineral content, and the second by the slow completion of this process.

## 3. Bone organization

Although, as reported above, the structure and composition of bone vary with its type, it is organized according to well-defined characteristics that allow an easy recognition of the nature and type of the tissue (reviewed by [1]). In this connection, and with reference to the local mechanism of bone mineralization, three main components must be considered, two of which, the collagen fibrils and the interfibrillar, non-collagenous proteins, constitute the bone organic matrix, while the third is the inorganic substance. Obviously, a fourth component consists of the whole complex of osteogenic and osteoclastic cells, as well as osteocytes and bone marrow cells, and cellular products – including matrix vesicles – which play a primary role in bone formation, maintenance and metabolism; they are only considered indirectly in this review, and reference is made to them when needed. In particular, the matrix vesicles, in spite of their fundamental role, are not considered in detail because the complexity and importance of the topic would excessively broaden the text and because excellent reviews are available [25-27].

### 3.1. Collagen fibrils

Collagen fibrils are the most representative bone constituents, amounting to 18.64% by weight [28] and about 90% [29] of the organic matrix of compact bone. They are type I collagen fibrils, i.e., fibrils about 78 nm in diameter characterized under the electron microscope by an axial periodic structure consisting of the repetition of two consecutive 'bands': an electron-dense band about 0.4D in length and a less electron-dense band about 0.6D in length, the D value being 68-70 nm [30,31]. This ultrastructural feature depends on the spatial arrangement of the collagen molecules, which are rich in glycine (33% of the chain), proline and hydroxyproline (22% of the chains), are 280-300 nm long and 1.4 nm thick, and are formed by three polypeptide chains aggregated in a left-handed helical

configuration and twisted around a common axis to form a supercoil [32,33]. The three polypeptide chains are characteristic of type I collagen: two of them have the same amino acid sequence and are called $\alpha$1(I), one has a different amino acid sequence and is called $\alpha$2(I) [34]. The way molecules are assembled into fibrils is still uncertain. Hodge and Petruska [35] have suggested that the parallel alignment of the molecules occurs in such a way that they are staggered by approximately a quarter of their length (depicted in Fig. 1), so generating regions where the molecules alternately overlap (dense zones) and are separated by gaps (hole zones) that correspond to the interval between the 'tail' and the 'hand' of successive molecules in the same plane. The fibril structure is stabilized by intra- and intermolecular cross-links [36]. These are also responsible for the low degree of solubility of the bone collagen, which is essentially insoluble in reagents such as NaCl and acetic acid under conditions that solubilize collagen from a wide variety of soft tissues [37]. The three-dimensional assembly of the collagen fibrils is still uncertain; the various theories on this topic have been discussed in several reviews [32,38].

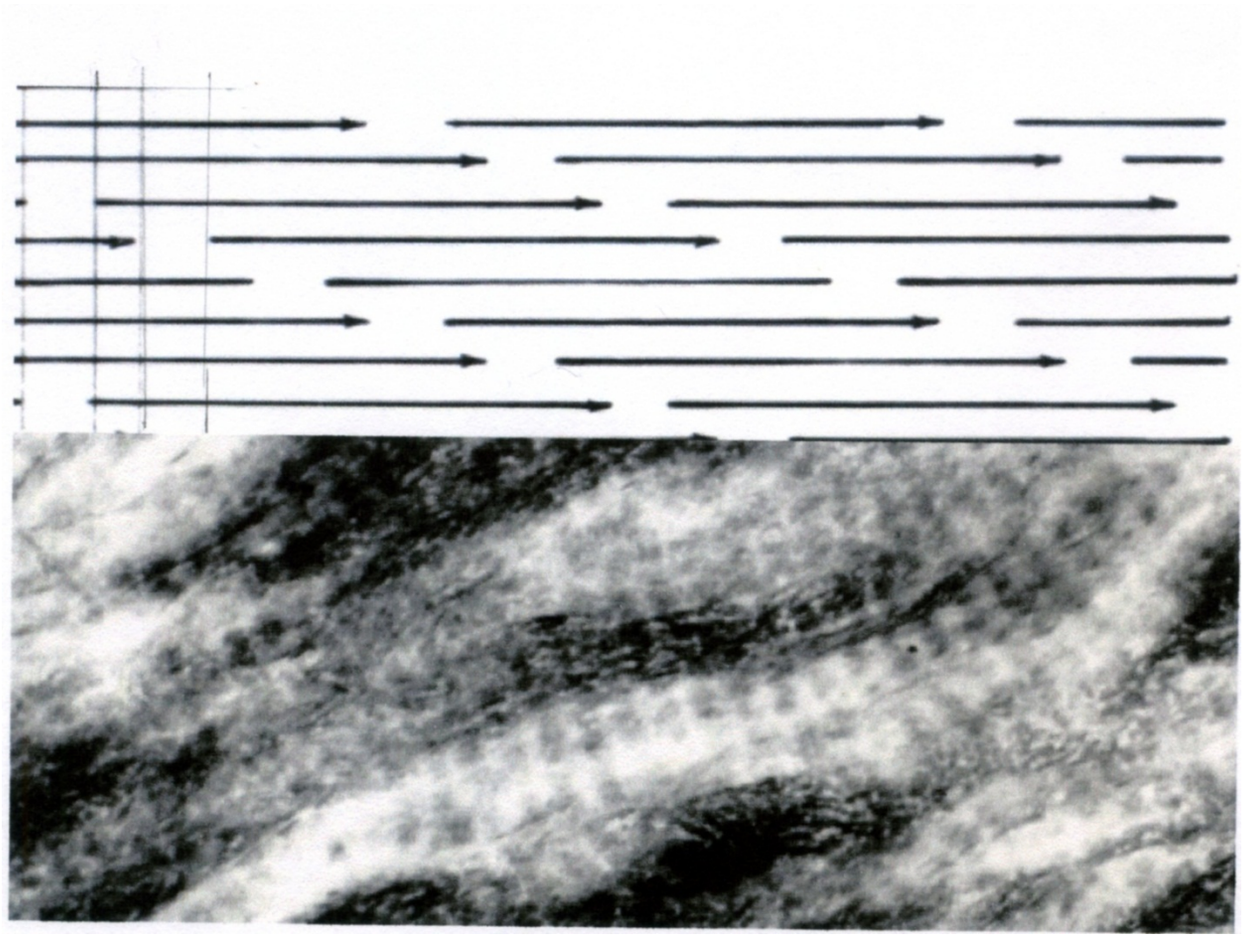

**Figure 1.** Above: diagram depicting the suggested arrangement of collagen molecules in a fibril: they are shifted in such a way as to generate overlapping (d) and holes (h) zones. Below: in low calcified bone matrix, the inorganic substance is collected in electron-dense bands that cross the collagen fibrils and emphasize their periodic banding. Unstained, x 60,000.

The amount and arrangement of collagen fibrils in bone are not constant and depend on the type of bone (reviewed by [1]). The highest collagen concentration is found in the compact bone of the diaphysis of long bones, where the fibrils are closely packed in lateral register and give rise to the so-called lamellar or parallel-fibered bone. The interfibrillar spaces are reduced to a minimum in this type of bone and the non-collagenous components of the matrix are also at a minimum. A loose arrangement of fibrils is found in woven bone, where irregular interfibrillar spaces contain abundant but variable amounts of non-collagenous proteins [4]. This arrangement is particularly evident in embryonic bone [39] and in the medullary bone of pigeons and other birds [7].

Although type I collagen fibrils are the most abundant component of the bone matrix, and are intimately connected with the inorganic substance (see below), they are not components of all the tissues that calcify and do not appear, therefore, to be essential for the biomineralization process to take place [40]. Thus, the matrix of the calcifying epiphyseal cartilage contains collagen type II, which is thinner than type I and lacks a clearly recognizable periodic pattern; a number of tissues that calcify do not contain collagen fibers at all, as typically occurs in the case of dental enamel. This does not, of course, exclude the possibility that collagen fibrils can induce the calcification process, especially if in combination with phosphoproteins, the collagen-phosphoproteins complex facilitating calcification better than collagen alone [41].

### 3.2. Non-collagenous components

Besides collagen fibrils, the bone matrix contains a mixture of substances, most of which are permanent components of its structure, while others are transitory cellular products (alkaline phosphatase, growth factors) and molecules present in the circulating fluids (albumin, $\alpha_2$HS-glycoprotein) [42]. As already mentioned, these substances, roughly indicated as 'non-collagenous components' or 'non-collagenous proteins', vary qualitatively and quantitatively, depending on the type of bone (reviewed by [1,2,43]). Qualitatively, they include proteoglycans, so-called Gla-proteins, phosphoproteins and phospholipids. Quantitatively, their local concentration correlates directly with the speed of formation of the bone tissue and indirectly with the packing density of its collagen fibrils [44].

**Proteoglycans.** As indicated by their name, these molecules consist of a core protein and a variable number of glycosaminoglycan chains, their properties depending on the composition and arrangement of both these components [45]. There are plenty of them in the cartilage matrix but they are much less frequent in the bone matrix, where their concentration reflects the relatively low amounts of non-collagenous components. They are, in any case, better represented in the uncalcified, osteoid tissue, which is PAS-positive and metachromatic [46], than in the calcified matrix [47], suggesting that they are partly lost during calcification. This has been confirmed by the biochemical analyses of isolated osteons at different degrees of calcification carried out by Pugliarello et al. [48], who found values for exosamines of 0.61% of dry weight in the osteoid tissue, 0.31% in osteons at the lowest degree of calcification, and 0.28% in osteons at the highest degree of calcification (see below for further discussion of this important topic).

The proteoglycan molecules have been divided into three groups on the basis of their properties – the small leucine-rich proteoglycans, the modular proteoglycans, and the cell-surface proteoglycans [49] – the first two groups being components of the bone matrix.

The **small leucine-rich proteoglycans** (SLRPs) are characterized by leucine-rich repeats in their proteic core; they have N-terminal cysteine clusters and at least one GAG chain. Divided into 5 classes [50], they are able to modulate cell-matrix interactions and cell functions, and for this reason are also known as 'matricellular proteins' [51]. The most representative SLRP in bone appears to be decorin (DCN), which modulates collagen matrix assembly and mineralization. It may have an inhibitory role, as suggested by the observation that a high or a low expression of DCN by osteoblasts causes delay or acceleration, respectively, of the calcification process. Studies by Hoshi et al. [52] are in agreement with this possibility: using ultrastructural histochemistry, they have shown that DCN is localized near the collagen fibrils of the osteoid border and that it is removed whenever the fibril fusion occurs at the onset of calcification.

Another member of the SLRP family is biglycan. Its role in bone calcification is not known exactly: however, biglycan deficiency, probably by affecting BMP-4 signal transduction and reducing the Cbfa1 transcription factor [53], causes abnormalities in collagen fibrils [54], induces delayed reparative osteogenesis and leads to an osteoporosis-like phenotype [55].

Other SRLPs (asporin, fibromodulin, lumican, osteoaherin) have been isolated from bone. Although they seem to be involved in collagen fibrillogenesis, their precise function is poorly known.

The **modular proteoglycans** (also called lecticans) form a heterogeneous group of large, often highly glycosylated molecules divided into hyalectans, which bind hyaluronan (hyaluronic acid, HA) and non-hyalectans [49]. The former include aggregan, versican, neurocan and brevican; perlecan is the most representative molecule of the latter. These molecules are poorly represented in bone matrix and their role in calcification, if any, is uncertain.

Aggrecan, which consists of a core protein and many glycosaminoglycans, especially chondroitin sulfate (reviewed by [56]), is abundant in cartilage, where it contributes to the regulation of the mechanical properties. It has been found in the matrix of normal and ectopic bone, where its concentration falls with tissue maturation [57].

**Gla-proteins.** This name refers to substances whose molecules contain the amino acid γ-carboxyglutamic acid (Gla). They include the bone Gla-protein and the matrix Gla protein.

**Bone Gla-protein** (BGP), also known as osteocalcin (OC), is contained in the calcified bone matrix, whereas its concentration is very low in the uncalcified osteoid tissue [58]. Its role in bone mineralization is uncertain. When in solution, it causes a delay in calcium phosphate precipitation, an effect that disappears if it is immobilized on sepharose beads [59]; inhibition by warfarin of vitamin K, a cofactor in the carboxylation of glutamate residues, induces a reduction in bone osteocalcin content without altering the bone structure of the rat [60]. Warfarin does, however, disrupt the assembly of the calcification nodules in the rat [61] and osteocalcin-deficient mice show a fall in crystal size and perfection [62]. Krueger et al. [63],

in reviewing the topic, stress the role of osteocalcin as a regulator of the mineralization process and suggest that osteocalcin deficiency due to prolonged warfarin treatment may contribute to bone demineralization and vascular calcification (known as the calcification paradox).

**Matrix Gla protein** (MGP; reviewed by [64]) is found not only in bone, but in all soft tissues too. It probably plays an inhibitory role in calcification. MPG-deficient mice show chondrocyte metaplasia of vascular smooth muscle cells and widespread vascular calcification.

**Glycoproteins** (phosphoproteins). The bone matrix contains acidic molecules that are rich in glutamic, aspartic and sialic acids, but mostly display *o*-phosphoserine and *o*-phosphothreonine, which goes to show they are phosphoproteins, and are covalently bound to collagen. Those that appear to be most involved in bone mineralization are osteonectin, acidic glycoprotein-75, bone sialoprotein, osteopontin, dentin matrix protein 1, and matrix extracellular phosphoglycoprotein. Apart from the first two, they are grouped under the acronym SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) which represents a family of genetically related proteins clustered on human chromosome 4 [65]. They can also be grouped as intrinsically disordered proteins (IDP), that is, proteins with an irregular, extended conformation and acidic character that facilitate interaction with counter ions and biomineralization [66].

**Osteonectin** (ON), also called SPARC (Secreted Protein, Acid and Rich in Cysteine) or BM-40 protein, is a glycoprotein expressed in all connective tissues and a number of other soft tissues. Its concentration in bone appears to be inversely correlated with the degree of calcification, with a maximum immunohistochemical reactivity in osteoid tissue [67]. ON-null mice show a higher mineral content and degree of crystallinity than the age-matched wildtype controls [68]. Crystal growth appears to be inhibited by ON in vitro [69], whereas even high concentrations of ON seem to lack nucleating activity. The cDNA sequence of ON reveals potential binding regions for calcium and hydroxyapatite; as it is a matricellular protein, ON may have several functions, such as the regulation of calcium-mediated processes, cell-matrix interactions, and the regulation of bone remodeling [51]. However, its role in bone calcification remains elusive.

**Acidic glycoprotein-75** (BAG-75) is a sialic acid-rich phosphoglycoprotein that has been found as 75 and/or 50 kDa forms in mineralized phases of bone and epiphyseal cartilage and in serum [70]. In bone its localization predicts the limits of subsequent mineralization; it delineates condensed mesenchyme regions that accumulate bone sialoprotein and nucleate hydroxyapatite, forming macromolecular complexes that have the potential to sequester phosphate ions [71]. A specific proteolytic processing of bone sialoprotein and bone acidic glycoprotein-75 in these complexes by an osteoblast-derived serine protease seems to be a prerequisite for their mineralization [72].

**Bone sialoprotein** (BSP) is a glycosylated, sulfated, phosphorylated, sialic acid-rich protein that can bind both hydroxyapatite and cell-surface integrins through the Arg-Gly-Asp motif (reviewed by [73]). Mainly localized in the bone matrix [44], where its concentration varies with the bone type and the degree of calcification, BSP is also expressed by osteoclasts, fetal

epiphyseal chondrocytes and the trophoblastic cells of the placenta [74]. It has a close relationship with the collagen fibrils [75] and with the calcification nodules at the calcification front [76]. Its overexpression enhances osteoblast differentiation, calcium incorporation and the formation of calcification nodules by osteoblast cultures [77]. BSP-null mice show abnormal bone growth and defective mineralization that lead to reduced bone formation and the delayed repair of cortical defects [78], whereas BSP overexpression leads to osteopenia and mild dwarfism in mice with an increase in bone resorption and reduction in osteoblast numbers [79]. These results point to a role of BSP in the early stages of calcification, a possibility supported by the demonstration of its capacity for nucleating hydroxyapatite [69].

**Osteopontin** (OPN), like BSP, is a glycosylated, phosphorylated, sulfated sialoprotein (reviewed by [80]). It contains the Arg-Gly-Asp motif and the amino acid sequence that allows its binding to cell surface and hydroxyapatite. Like BSP, it is contained in the calcified matrix and the calcification nodules, and is found at the bone surface of osteoclasts and other cells; it can, however, be found in many soft tissues, as well. Moreover, unlike BSP, it inhibits apatite nucleation and crystal formation, so that its deficiency increases mineral content and mineral crystallinity in mouse bone [81]. OPN appears to be a multifunctional cytokine that plays a role not only in the regulation of bone formation and resorption, but in many other processes too, such as tissue inflammation and repair, wound healing, angiogenesis, and immunological reactions [82].

**Dentin matrix protein 1** (DMP1) is not an exclusive feature of dentin, as its name suggests, but is also found in bone, where it is mainly expressed by osteocytes, and in soft tissues [83]. Its molecule is highly phosphorylated and is cleaved into three distinct segments, two of which are promoters, while the third inhibits the calcification process [84]. DMP1 can, in fact, nucleate hydroxyapatite when immobilized on collagen fibrils. On the basis of electron microscope studies showing that DMP1 induces in vitro the formation of parallel arrays of crystallites similar to those found in the DMP1-rich, collagen-devoid peritubular dentin, Beniash et al. [85] concluded that DMP1 controls the mineral organization outside the collagen fibrils. Studies in vitro by Tartaix et al. [86] suggest that the native form of DMP1 inhibits calcification, but becomes a promoter of the process when cleaved or dephosphorylated. These results, and the observation that its deficiency results in hypomineralized matrix, support the conclusion that DMP1 is a key regulator of mineralized matrix formation [87].

**Matrix extracellular phosphoglycoprotein** (MEPE; also known as osteoblast/osteocyte factor 45; OF45; Osteoregulin) is highly expressed in osteocytes in human bone [88]. It is a phosphate-regulating factor (phosphatonin) that induces dose-dependent hyperphosphaturia and hypophosphatemia in mice and is a product of the cells of the tumors that cause osteomalacia. Its specific activity is regulated by posttranslational modifications: the phosphorylated intact protein is an effective promoter of mineralization in the gelatin gel diffusion system, while the associated ASARM peptide (acidic serine-aspartate-rich MEPE-associated motif) is an effective inhibitor, and neither MEPE nor ASARM have any effect on mineralization once they have been dephosphorylated [89]. Moreover, dentonin (or AC-100), a synthetic 23-amino-acid peptide derived from MEPE, can stimulate stem cell proliferation in dental pulp [90] and can therefore be active in osteogenesis.

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**α2-HS glycoprotein/Fetuin**, also known as α2-Heremans-Schmid glycoprotein (AHSG), is a prominent, non-collagenous component of the bone matrix, although it is a serum protein synthesized in the liver and is only secondarily accumulated in bone. It plays an inhibitory role on calcification by forming fetuin-mineral complexes, corresponding to high molecular mass complexes of calcium phosphate mineral, fetuin and matrix Gla protein [91]. According to Heiss et al. [92], these fetuin-mineral aggregates give rise to 'calciprotein particles', i.e., colloidal spheres, 30-150 nm in diameter, which are initially amorphous and soluble, but then become progressively more crystalline and insoluble. Their inhibitory effect on calcification is confirmed by studies in cell culture and in the test tube [93] and by the diffuse ectopic calcifications that develop in AHSG-deficient mice on a mineral and vitamin D rich diet [94]. This inhibitory effect might also be effective in regulating collagen mineralization: in vitro studies by Price et al. [95] have shown that the homogeneous nucleation of calcium phosphate occurs within the collagen fibrils in the presence of fetuin, whereas without it mineral grows outside the fibrils. On the basis of these and other results, they have advanced the hypothesis that calcification occurs by inhibitor exclusion ('mineralization by inhibitor exclusion'), that is, the selective mineralization of a matrix using a macromolecular inhibitor of mineral growth.

The function of the enzyme **alkaline phosphatase** (AP) in mineralization has been discussed by Orimo [96] in a recent review, to which the reader can refer. AP is a glycoprotein with calcium-binding properties [97]. It is a component of the bone matrix [98], although it is mainly distributed on the cell membrane and in matrix vesicles. Its tissue isoenzyme TNSALP (tissue non-specific alkaline phosphatase) is critical for mineralization, as is shown by the skeletal rickets-like changes that develop in congenital hypophosphatasia and in TNSALP-knockout mice [99]. Interestingly, even in these conditions, the matrix vesicles give rise to the formation of apatite crystals; these, however, do not spread through the surrounding matrix [100], suggesting that TNSALP removes an inhibitor of crystal diffusion, a process that might involve the matrix inorganic pyrophosphate and the expression of the plasma cell membrane glycoprotein-1 (PC-1) that is needed for its synthesis [101]. The Ca-binding property of alkaline phosphatase suggests that another function is feasible, that is, the formation of organic-inorganic hybrids that would constitute the first step in crystal formation (see below).

**Phospholipids.** Observing that calcification areas are stained by Sudan black B after hot pyridine extraction, Irving [102] first suggested that areas of early calcification contain lipidic material. He subsequently reported that this material was very resistant to extraction before decalcification and consisted predominantly of phosphatidylserine and phosphatidylinositol [103]. Lipids are, in fact, intrinsic components of bone, a calcium-phospholipid-phosphate complex has been extracted from bone, and lipids can be demonstrated histochemically and immunohistochemically [104] in calcification nodules and in matrix vesicles [105]. Lipid involvement in bone calcification has been supported by the results of several studies (reviewed by [106]).

## 4. The inorganic substance

**Composition.** It has long been known that the inorganic substance of bone is a calcium phosphate with about 5% by weight of carbonate and traces of other elements (reviewed by [107]), a composition that makes uncertain the true nature of the bone mineral, even if it has been regarded as an hydroxyapatite with the formula $Ca_{10}(PO_4)_6(OH)_2$ [108]. This uncertainty is increased by the variability of values for the Ca/P molar ratio, which are reported to range between 1.57 and 1.71 [109] and, for the weight ratio, between 2.09 and 2.25 in adult human bone and between 1.82 and 1.98 in fetal human bone [110]. In reality, the size and composition of bone apatite changes with age, so that the Ca/P molar ratio varies too. It increases, in fact, from a mean value of 1.35 in the calcification nodules found in the osteoid tissue (i.e., the earliest mineral deposits) to 1.60 in the fully calcified areas [111], or from 1.60-1.70 to 1.81-1.97, respectively [112].

The problem of the nature and composition of bone mineral has been only partly solved by X-ray and electron-diffraction. The early studies by deJong [13]) had shown that the X-ray diffractograms of the compact bone are similar to those of a polycrystalline hydroxyapatite, a conclusion that has been repeatedly confirmed (see reviews by [107,108,113]). This observation has greatly helped to clear up the nature of the bone mineral but, at the same time, has often led to considering it from a purely mineralogical point of view. Actually, the concept that the bone mineral is polycrystalline, and that the crystals are the same as those of the natural hydroxyapatite, has been challenged in several studies. Arnott and Pautard [114] were the first to stress that inorganic particles in bone are defined 'crystals' without any proof that any portion of the mineralized area actually consists of crystals. Arnold et al. [115], by energy-filtering transmission electron microscopy in the selected area electron diffraction mode of dentine, bone, enamel and inorganic apatite mineral, found that the early formed crystallites have a paracrystalline character comparable to biopolymers and that, with the maturation of, and the consequent fall in, the organic proportion in the matrix, the lattice fluctuations of the crystallites diminish, so allowing them to acquire a typical (para)crystalline character. Wheeler and Lewis [116] showed that the crystalline apatite content of untreated mature cortical bovine bone has, in fact, a paracrystalline structure (i.e., no long-range order) and calculated the paracrystalline mean distance fluctuations. Several diffraction studies have shown that the structure, composition and crystallinity of the bone mineral and of the first apatite crystals formed in solution are not constant, and that crystallinity, in agreement with the Ca/P changes reported above, rises with age and degree of maturation [117-121]. In this connection, Landis and Glimcher [112] found that no electron diffraction pattern of poorly crystalline hydroxyapatite, of the type shown by the diffractograms of heavily calcified regions, was generated from the early bone mineral deposits, that the absence of diffraction rings was not due to an insufficient mass of the solid phase, and that there was a progressive change in this pattern towards crystalline diffractograms as the matrix became fully calcified. Moreover, neutron spectroscopic studies by Loong et al. [122] have shown that the bone mineral differs significantly from hydroxyapatite, not only due to the presence of labile and stable CO3 and HPO4 groups, but also because of the predominant, if not total, absence of OH groups from the specific crystallographic lattice sites which

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they occupy in pure HA. The question is further complicated by the possibility that apatite crystal formation may be preceded by that of an unstable precursor such as amorphous calcium phosphate (for a discussion of this topic see [2]). In conclusion, bone hydroxyapatite appears to differ from natural apatite, and its structure and composition vary with its age and its relationship with organic matrix components.

**Morphology.** Morphological studies have not provided a definitive answer to the problem of the morphology, size and organization of the inorganic substance in bone. This mainly depends on the fact that electron microscope techniques, the best for visualization of inorganic particles in bone, face major issues in investigating a tissue whose hardness makes difficult to handle at the ultramicrotomic level, so that unexpected changes (for instance, decalcification) can easily be introduced in ultrastructural components [123]. On the other hand, the other physical techniques available for the study of inorganic nanostructures have a poor degree of discrimination and can hardly distinguish different ultrastructures within the same segment of bone (for instance, in areas at different degrees of calcification), because their characteristics are masked by those of the volumetrically prevailing structures. The microscopic dissection, or special physical devices and techniques, can be used to avoid this handicap but they are time-consuming, technically demanding and hard to implement.

One disagreement of great importance for the implications it may have on the understanding of the mechanism of calcification concerns the shape and the size of the mineral particles and their relationship with the components of the organic matrix. The earliest electron microscope studies had led to the conclusion that bone crystals have a platelet-like shape [22,124,125]. This finding was then repeatedly reported in bone and other calcified tissues (dentin, tendons, cartilage) whether using the electron microscope or other methods of investigation [126-139] and the dimensions of the platelets were calculated (mean values reviewed by [2]).

The concept that bone mineral particles are structured as platelets clashes with earlier results obtained from polarized light studies: as early as 1933, Schmidt [140] had shown that bone has an intrinsic birefringence deriving both from its organic and inorganic components, and that the latter has a 'form birefringence' which complies with Wiener's law for rod-like composite bodies. That bone crystals may have a rod-like shape was then confirmed by using the polarizing microscope [16,141,142] and X-ray diffraction [143-148]). It was the electron microscope that definitively showed that bone crystals, at least in part, have a rod-, needle-, or filament-like shape [149-153]), like that found in the crystals of calcified cartilage and other hard tissues (Fig. 2).

The results of the goniometric tilting under the electron microscope of single crystals that had been isolated after bone dissociation have led to the suggestion that the needle-shaped crystals could be no more than a side view of small, thin platelets [126-128]. Obviously, the fact that thin platelets may appear as needles when viewed from one side is not a proof that all needle-like structures observed in bone are due to sideways views of platelets. On the other hand, others suggested that platelet-like and rod-like crystals could be found together in the same bone areas and are different aspects of the same mineral [154] connected to different organic components of the matrix (discussed below). The question is made still more

complex by recent atomic force microscopy observations that isolated mineral particles (named 'mineralites') extracted from bovine bone measure 9 x 6 x 2 nm [155,156], values that are much smaller than those obtained with other methods of investigation. In any case, depending on the compactness of bone, crystals may appear as elongated structures shaped as straight, rigid, needle-like or rod-like structures; more often, however, they appear as bent or irregular, filament-like structures (Fig. 2). For this reason, 'filament-like crystals' will be the term used preferentially in the following pages.

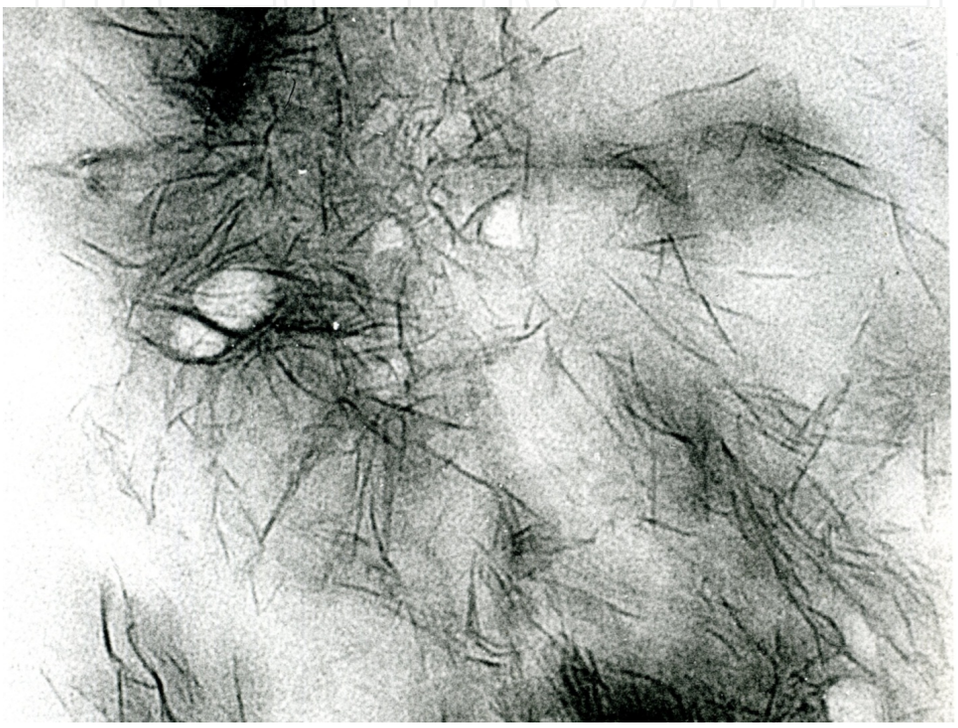

**Figure 2.** Detail of calcification nodules: crystals appears as elongated, bent, needle- and filament-like structures. Unstained, x 312,000.

**Relationships with matrix organic components.** Electron microscope studies have clearly shown that the bone mineral is only partly contained in the collagen fibrils, while it is mostly (about 75% of the total according to Pidaparti et al. [157]) located in the extrafibrillar space. This appears to be in contrast with the observation, originally reported in the earliest ultrastructural investigations on bone, that the mineral substance is closely related to the periodic banding of collagen [22,23,158], a relationship later repeatedly confirmed (reviewed by [2,3,38,159,160]. The mineral, in fact, because of its intrinsic electron density, produces a reinforcement of the collagen periodic banding (Figs. 1,3), as if it was a 'negative stain' [161]. Actually, this kind of picture is due to the location of inorganic particles within the collagen fibrils; more exactly, there is a general consensus that the mineral is contained in the 'holes' of the fibril gap-zones (reviewed by [2]. Moreover, because collagen fibrils aggregate side by side in lateral register during mineralization, their hole zones come to be in register, too, and give rise to transverse, 'electron-dense bands' that cross the collagen bundles (Figs. 1,3). One possibility is that these bands might also be located in pores, channels or grooves resulting

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from collagen fibril aggregation [132,162]. This ultrastructural pattern is above all expressed in compact bone, especially if incompletely mineralized (bone at the initial stage of mineralization, pathologically hypomineralized bone), whereas it is poorly expressed, or completely lacking, in woven bone or in bones with loose collagen fibrils (Fig. 4). Characteristically, in secondary osteons, which calcify in two phases, it is more clearly recognizable during the initial phase of formation than during the later phase of mineral completion [151]. It has been suggested that the 'dense bands', which consist of nanogranules, might give rise to platelet-like fragments if removed from the fibrils, and this might explain why platelet-like crystals have chiefly been described in studies based on bone dissolution and crystal isolation (reviewed by [2]).

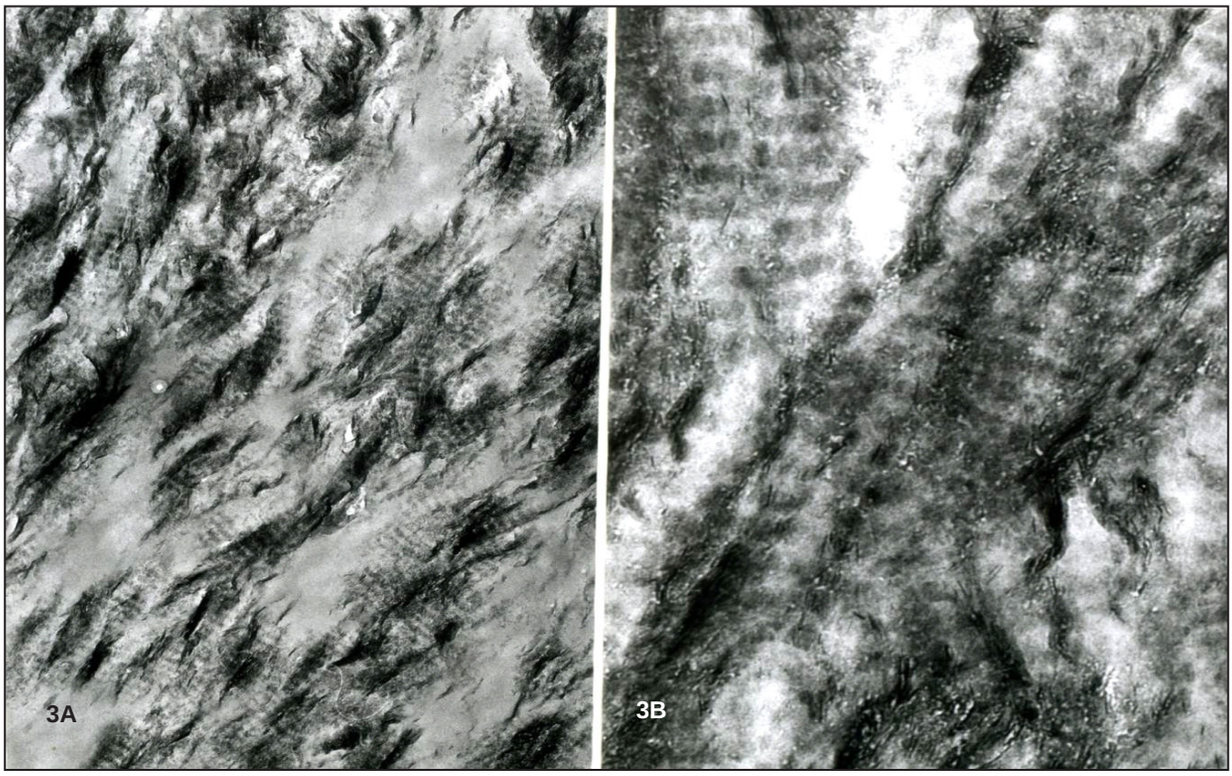


**Figure 3.** A) Area of initial calcification in compact bone, and B) osteon at the initial stage of formation: the relationship between inorganic substance and collagen periodic banding is clearly recognizable; the apparently empty areas correspond to zones of still uncalcified matrix. Unstained, x 70,000.

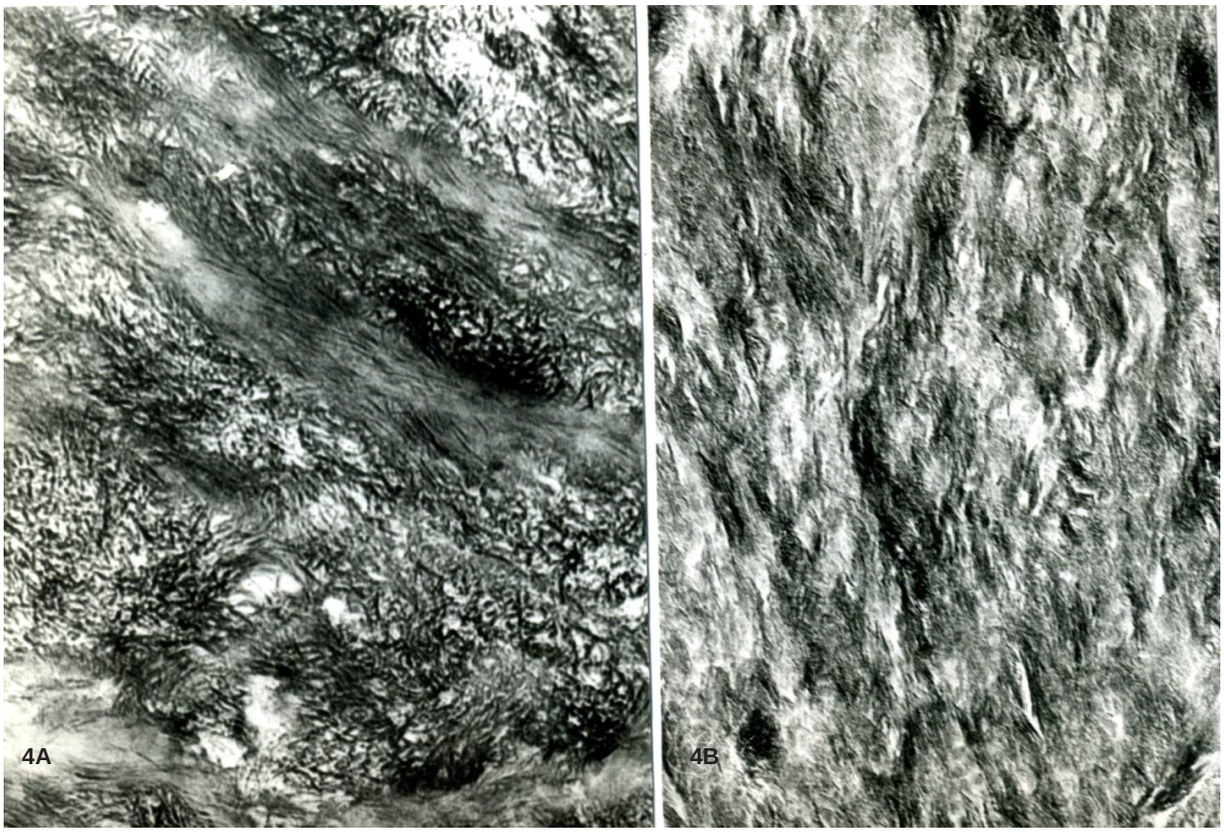


**Figure 4.** A) Embryonic avian bone: most of the inorganic substance correspond to filament-like crystals; the mineral in bands is practically unrecognizable. B) Osteon at a final stage of calcification: the inorganic substance in bands is poorly visible. Unstained, A x 90,000 and B x 75,000.

The relationship of filament-like crystals with collagen fibrils is much less clear than that of the 'dense bands', both because of the intrinsic difficulty of the problem, and because the crystal arrangement seems to change during mineralization (reviewed by [2,3,159]. At the outset, the elongated crystals form roundish (calcification nodules) or elongated (calcification islands) aggregates in the context of the still uncalcified osteoid borders (Fig. 5). The crystals of the calcification nodules very probably arise in matrix vesicles (Fig. 5, inset), then increase in numbers, acquire a roughly radial arrangement and spread from the nodules (i.e., the fully calcified matrix vesicles) into the surrounding matrix. These crystals are located in the interfibrillar spaces and fail to show any direct relationship with the collagen fibrils, which have no preferential arrangement and are still uncalcified. At this mineralization stage, the calcification nodules closely resemble those visible in the early stages of cartilage calcification; their numbers change with the type of bone, and are inversely related to the compactness of the collagen fibrils. Plenty of them can, in fact, be found in the medullary bone of birds [7]) and in embryonic bone [39], whereas they are much rare in compact bone [151].

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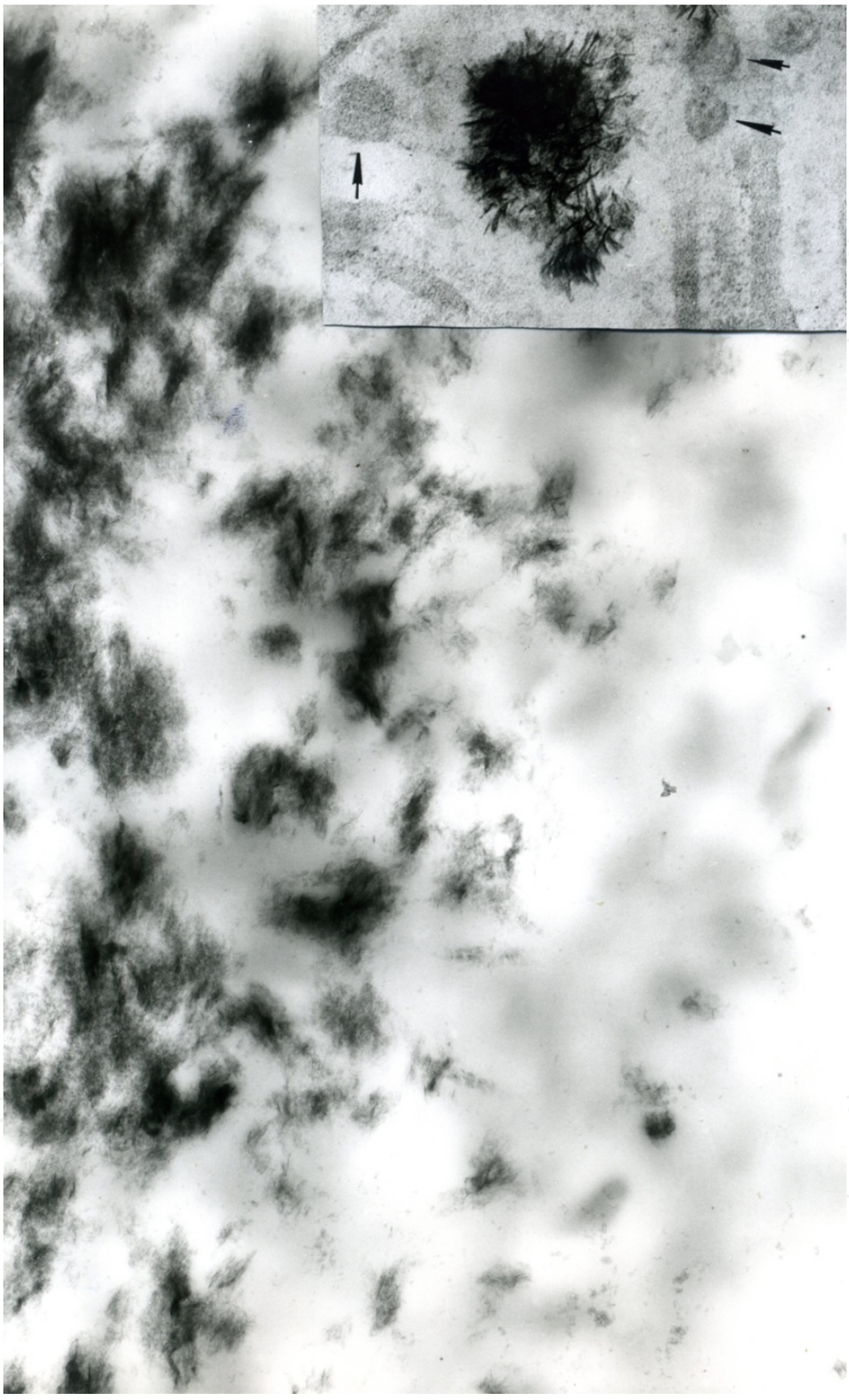

**Figure 5.** Calcification frontof spongy bone: most of inorganic substance is collected in calcification nodules; no relationship between inorganic substance and the collagen periodic banding is recognizable. Unstained, x 30,000. Inset: Detail of calcification front: a calcification nodule and a few matrix vesicles (arrows) are recognizable. Uranyl acetate and lead citrate, x 60.000.

The calcification islands are characterized by small bundles of needle-like crystals that are arranged side by side, in direct contact with collagen fibrils, and are oriented parallel to them. These crystals, in spite of their adhesion to the fibril surface, appear to be entirely located in the extrafibrillar space, as is also shown by the electron microscope observation that the cross-sectioned collagen fibrils in areas of initial calcification appear as electron-lucent circular spaces surrounded by crystals [6,138,163]. No relationships are found between the crystals of the calcification islands and the collagen period.

There seems to be little doubt that the elongated, filament-like crystals (whether in the calcification nodules or in calcification islands) are located between the collagen fibrils and on their surface, that is, in the extrafibrillar space. The problem arises, therefore, of knowing if they have a relationship with some specific organic component of the bone matrix. The problem is that the already noted intrinsic electron density of the inorganic substance masks the underlying organic structures, if any, so making any direct electron microscope study impossible; moreover, their ultrastructural resolution cannot be improved by removing the mineral through decalcification, because of the extraction artifacts that are implicit in this technique. The only solution available is that of resorting to special decalcification and staining techniques (post-embedding decalcification and staining; cationic dye stabilization) that do not affect the organic components.

The Post-Embedding Decalcification and Staining (PEDS) method relies on the decalcification of the bone matrix after embedding it in an epoxy resin (by floating ultrathin sections on the calcifying solution); the method is based on the observation that the embedding process prevents the distortion and solubilization of the organic components without blocking the removal of the mineral [164]. This has been clearly shown in epiphyseal cartilage (Fig. 6); the results recorded for this tissue are paradigmatic for the organic-inorganic relationship during the early phases of calcification in other tissues and in bone itself. As epiphyseal cartilage is easier to handle than these tissues, it is often used instead of them.

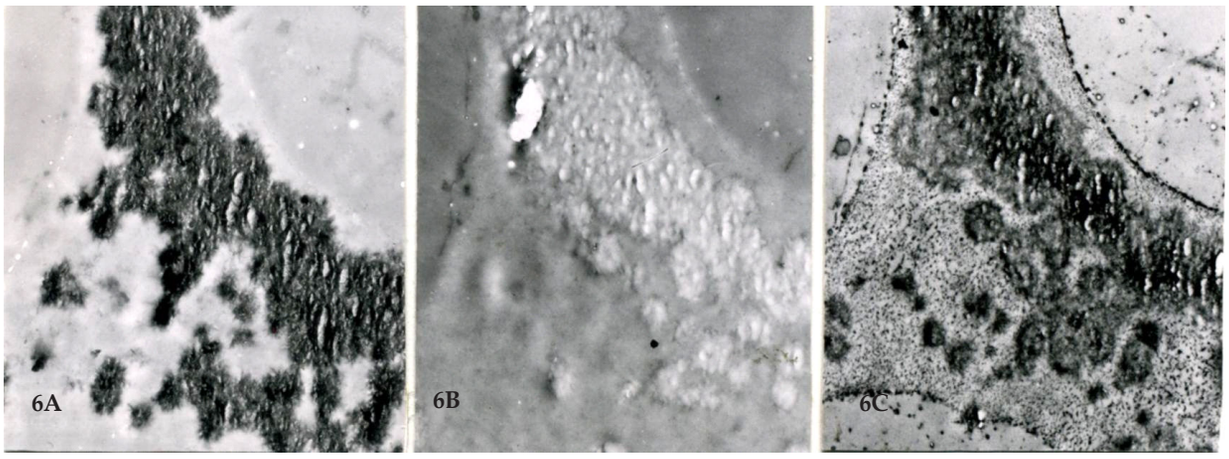


**Figure 6.** Series of sections from the epiphyseal cartilage: A) unstained; B) decalcified and unstained; C) decalcified and stained with uranyl acetate and lead citrate. PEDS method, x 18,000.

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If the PEDS method is interrupted after its first phase, that is, after floating sections on the decalcifying solution, the decalcified areas appear empty and electron-lucent, so showing that all the mineral substance has been removed (Fig. 6B). If the method is completed, that is, the decalcified sections are treated with a heavy metal (usually uranyl acetate, lead citrate, or phosphotungstic acid), the decalcified areas appear to consist of aggregates of filament-like structures (Fig. 6C) whose ultrastructure is very similar to that of untreated crystals (Fig. 7) and for this reason are called 'crystal ghosts' [165]. Crystals and crystal ghosts show such close similarity that the latter were initially considered to be crystals left undecalcified in sections. In reality, they are organic structures. It is crucially important that they are clearly recognizable where the calcification process is active, as in the smallest calcification nodules and at the border of the large but still developing ones; conversely, they disappear in the fully calcified areas (Fig. 8). They have been found in other calcifying tissues, in particular in cartilage, dentin, enamel, calcifying tendons and some pathological tissues [166-174].

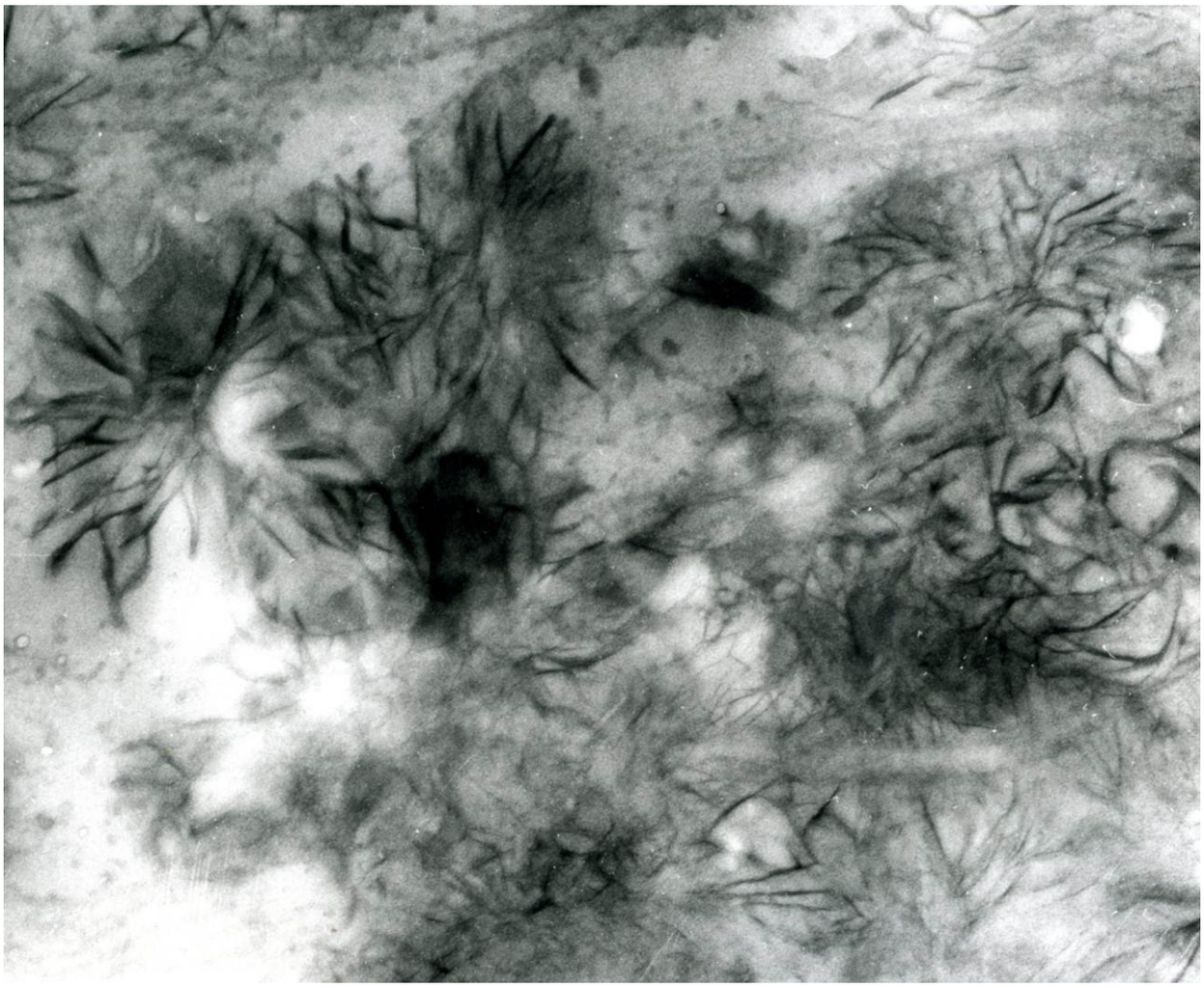

**Figure 7.** Front of calcification: detail of crystal ghosts. PEDS method (formic acid, uranyl acetate and lead citrate), x 120,000.

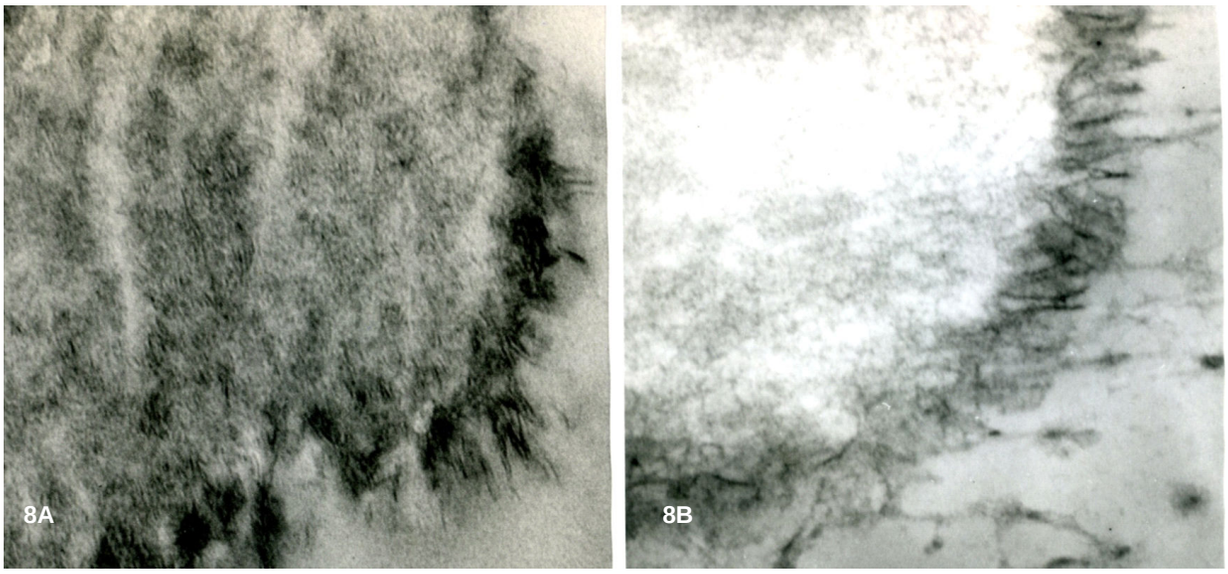


**Figure 8.** Two serial sections from rat epiphyseal cartilage. A) untreated; B) PEDS method (decalcified with formic acid and stained with uranyl acetate and lead citrate): crystal ghosts are only recognizable at the border of the calcified matrix, where the calcification process is still active, and not in the central, fully calcified area. x 70,000.

Almost the same ultrastructural pictures can be obtained using the cationic dye stabilization method (CDS). This is based on the fact that, if stabilized by cationic substances (usually cationic dyes), acidic molecules resist the extraction produced by decalcifying solutions [175,176]. When this method is used, crystal ghosts are less sharply outlined than those shown by the PEDS method; even so, they are highly reminiscent of the aggregates of untreated filament-like crystals (Fig. 9).

## 5. The mechanism of mineralization

Although our understanding of bone and other hard tissues has risen sharply in recent years (reviewed by [2]), the mechanism of mineralization is still uncertain. On the basis of the close relationship between the inorganic substance and the hole zones of the collagen period, Glimcher [21] proposed the theory that specific atomic groups located in the hole zones of collagen fibrils are arranged in such a way as to induce heterogeneous nucleation of hydroxyapatite, and that the nuclei subsequently grow by addition of further inorganic ions, so giving rise to crystals (reviewed by [113]. Most of the subsequent investigations on biomineralization, either of bone or other hard tissues, have been carried out on the basis of this theory and important improvements have been obtained. They cannot, however, be considered conclusive because of a number of experimental faults and incongruities. Several electron microscope studies have shown, for instance, that the filament-like crystals are longer than the collagen period [118,130,138,151,177-179] and are not confined to the gap zones of the fibrils but extend into the overlap zones [123,180-182]. This means that the crystals grow outside the holes and, to allow this to happen, they must pierce the intermolecular spaces and enlarge them to find the space required for them to grow. It follows that they should

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break the intermolecular cross-links – a change that should raise the solubility of bone collagen, which actually decreases during mineralization [37], and should modify the fibril periodic banding as well, which, conversely, is not appreciably affected. Moreover, according to Bachra [183], the many side-chains of the amino acid residues that point laterally at a distance of about 0.286 nm along the axis of the collagen molecules would prevent the growth of the nuclei into crystals. On the basis of neutron diffraction studies of fully mineralized ox bone, it has also been reported that less space is available within collagen fibrils than was previously assumed, and that most of mineral is therefore located outside the fibrils [184]. In line with these findings, Lees and Prostak [153] point out that the size of the crystallites is greater than the intermolecular spacing of collagen fibrils, so that crystallites could not fit in them. They report, in addition, that in fish dentin (which is similar to bone) at the initial stage of calcification the filament-like crystallites form dense strips between the collagen fibrils and practically none of them can be found within fibrils. The same authors calculate that the ratio of the weight of mineral contained in the hole zones to the total mineral content is not greater than 20% in loose bones like that of deer antlers, and this percentage falls to less than 5% for hyperdense bone like porpoise petrosal.

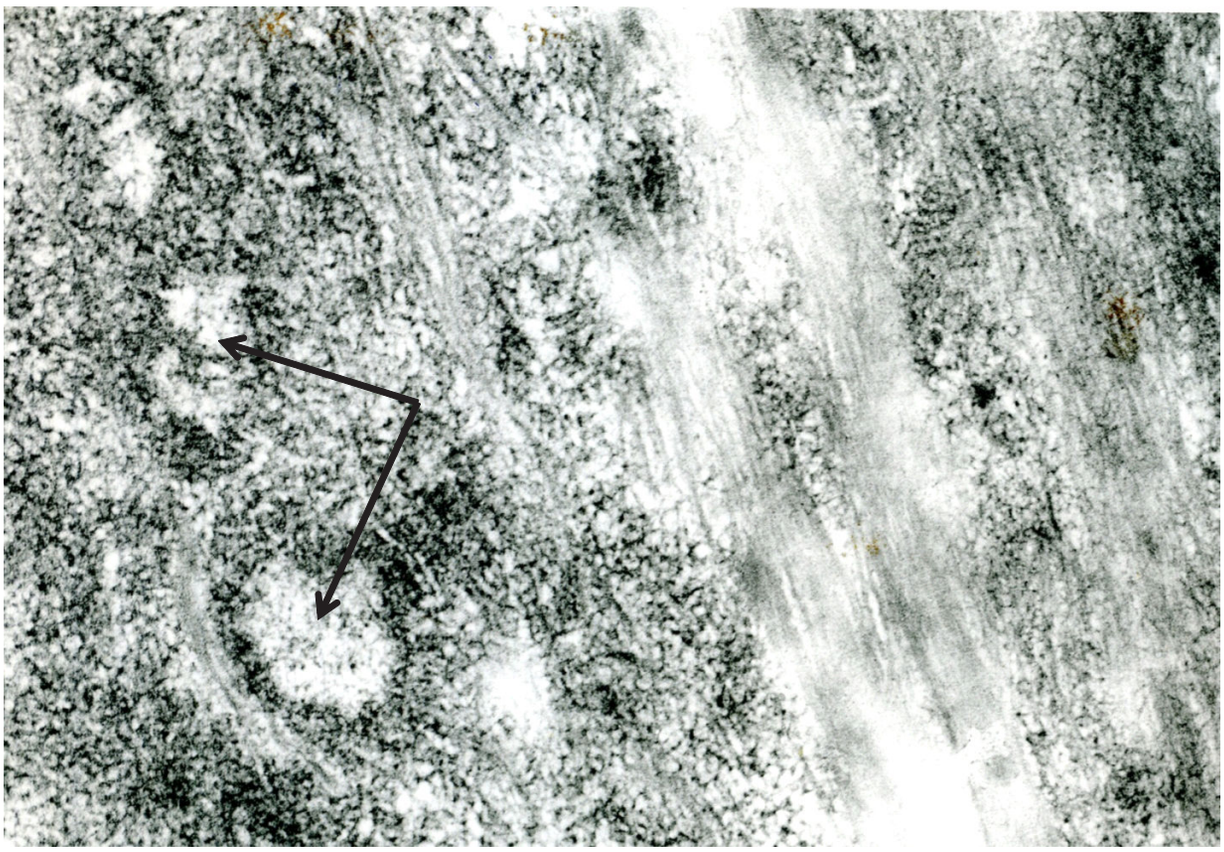

**Figure 9.** Front of calcification; section from a specimen fixed with glutaraldehyde-acridine orange, decalcified with EDTA and treated with ruthenium red; structures resembling crystal ghosts are recognizable (arrows point to the empty, central zones of the calcification nodules). x 18,000.

These observations converge in suggesting that the filament-like crystals cannot be nucleated in the holes found in collagen fibrils, and that the theory of heterogeneous nucleation must therefore be reconsidered. The electron microscope results leave little doubt that the calcified matrix of bone displays two types of mineral: electron-dense bands related to the periodic banding of collagen, and filament-shaped crystals. Probably because of the suggestion of the theory of heterogeneous nucleation, the latter have been considered to derive from the growth and development of the former. This conclusion is actually arbitrary: a number of electron microscope results have clearly shown that these two types of structure, although both consisting of hydroxyapatite, are separate entities. The aggregates of filament-like crystals, known as calcification nodules, arise in matrix vesicles and spread through the surrounding matrix; they only have secondary contact with collagen fibrils. The crystals of the calcification islands, on the other hand, seem to be in contact with the surface of the collagen fibrils, but are not contained within them. Only the electron-dense, granular bands are related to the collagen period, and are contained in the hole zones. The fact that they become less recognizable as the matrix mineralization proceeds is not due, as often arbitrarily believed, to their transformation into filament-like crystals, but to the masking effect arising from the increase in numbers of the latter.

## 6. Crystal ghosts and calcification

The obvious conclusion to be drawn from the results discussed above is that the filament-like crystals are in contact with non-collagenous organic structures contained in the interfibrillar spaces and that, as a result, their formation is probably regulated by these same structures. The finding of crystal ghosts, as described above, now takes on a new prominence, because it leads logically into a plausible explanation for the calcification mechanism (discussed by [2,3,159]). This is based on the very close ultrastructural similarity between crystal ghosts and untreated filament-like crystals – a fact that strongly suggests that they are components of the same organic-inorganic, crystal-like structures that are formed through a chemical process in which the crystal ghosts operate as templates. During the early stages of calcification, the structures called crystals would not develop through a process of heterogeneous nucleation but through an epitaxial process implying a link between inorganic cations and organic molecules (crystal ghosts) with the formation of organic- inorganic hybrids whose filament-like shape would simply derive from, and reflect, the filament-like shape of the template. It is also possible that during this initial phase of calcification crystal ghosts stabilize amorphous calcium phosphate, whose existence in bone is still, however, a controversial issue (discussed by [2]). The organic-inorganic composition of the early crystals would explain why the calcification nodules give amorphous diffraction rings at the beginning of their formation and why their crystals have a paracrystalline character and show crystal lattice distortions, as discussed above.

This possibility has been challenged because the location of organic material within the crystals appears to conflict with mineralogical laws [185]. This criticism is based on the concept that the inorganic substance of bone consists of crystals in a mineralogical sense. In reality,

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as reported above, the early crystals give amorphous diffractograms and the term "crystal" is used only as a matter of habit. If the early crystals are organic-inorganic hybrids, each of them must consist of a mixture of, and imply mutual penetration by, the two components (organic and inorganic), and it is not possible to differentiate between an inner and an outer constituent, especially considering that the filament-like crystals of bone measure less than 10 nm (from 1 to 7.7 nm; see [2]) in thickness. But even allowing the hypothesis that the organic component is contained in the inorganic one, examples of incorporation of organic material in crystal-like structures (biominerals) are quite numerous [186-199]. The organic content can induce lattice distortions [200], but at the same time it enhances the mechanical properties of crystals [192]).

At this point the need for adequate knowledge about the nature of crystal ghosts becomes a priority. So far, ultrastructural histochemical investigations, which might permit the acquisition of this knowledge, have mainly been carried out in cartilage. In this tissue, crystal ghosts are stained by acidic phosphotungstic acid, periodic acid-silver methenamine, periodic acid thiosemicarbazide-osmium; they are reactive with cations and with colloidal iron at pH 2.0 (Fig. 10), but are unreactive after methylation and saponification [201]. These results show that they correspond to acid proteoglycans, a conclusion supported by the finding of chondroitin sulfate in their molecule [202]) and by other histochemical results [167,169,203,204]. That acid proteoglycans can be involved in calcification, either promoting or inhibiting it, has long been known [12,175], and the possibility that hydroxyapatite nanocrystals can self-assemble on chondroitin sulfate templates has been put forward [205]. Of course, other types of the many non-collagenous molecules located in the bone matrix could, alternatively, constitute the crystal ghosts found in bone. In this connection, polyanionic molecules (especially phosphoglycoproteins), often containing the repetitive sequence of aspartic acid, have been described in all calcified tissues [71,206-210] and could well have the same role as that of acid proteoglycans in cartilage; anyway, independently of their nature, crystal ghosts cannot be ignored and any proposal to account for the mechanism of calcification should include their role.

In any case, the formation of crystals appears to be more complex than an epitaxial process alone. It has been stressed above that crystal ghosts disappear from the central zone of developing calcification nodules (i.e., they disappear as the degree of calcification rises; Figs. 8, 9, 10) and that at the same time the electron diffractograms, which are of amorphous type in the calcification nodules at the initial stage of calcification, change into crystalline and acquire the characteristics of hydroxyapatite. These findings show that, as calcification proceeds, the initial organic-inorganic hybrids gradually lose their organic component and change into almost pure inorganic crystals. It may be speculated that an enzymatic mechanism removes the organic component, and that at the same time the residual inorganic backbone acquires further inorganic ions and 'matures', so turning into a hydroxyapatite crystal. This hypothesis is in agreement with the well-known fact that in cartilage the calcification process is characterized by changes in the organic matrix that chiefly imply the loss of acid proteoglycans [211-224]. In this connection, it is known that the calcification process is inhibited in vitro by proteoglycans and is increased by their

degradation or removal [219,225], so that their controlled enzymatic breakdown could be considered a possible mechanism by which the calcification of growth plate cartilage might be allowed to advance in vivo [214]. In discussing this problem, Bonucci and Gomez [226] reported the observation that aggregates of filamentous structures, similar to crystal ghosts, can be found in uncalcified cartilage after staining with lanthanum chloride [227]. These structures are focal concentrations of proteoglycans that, due to a modification of their molecule, have acquired high La-binding capacity (in a broader perspective, high Ca-binding capacity) and behave as pre-crystal ghosts.

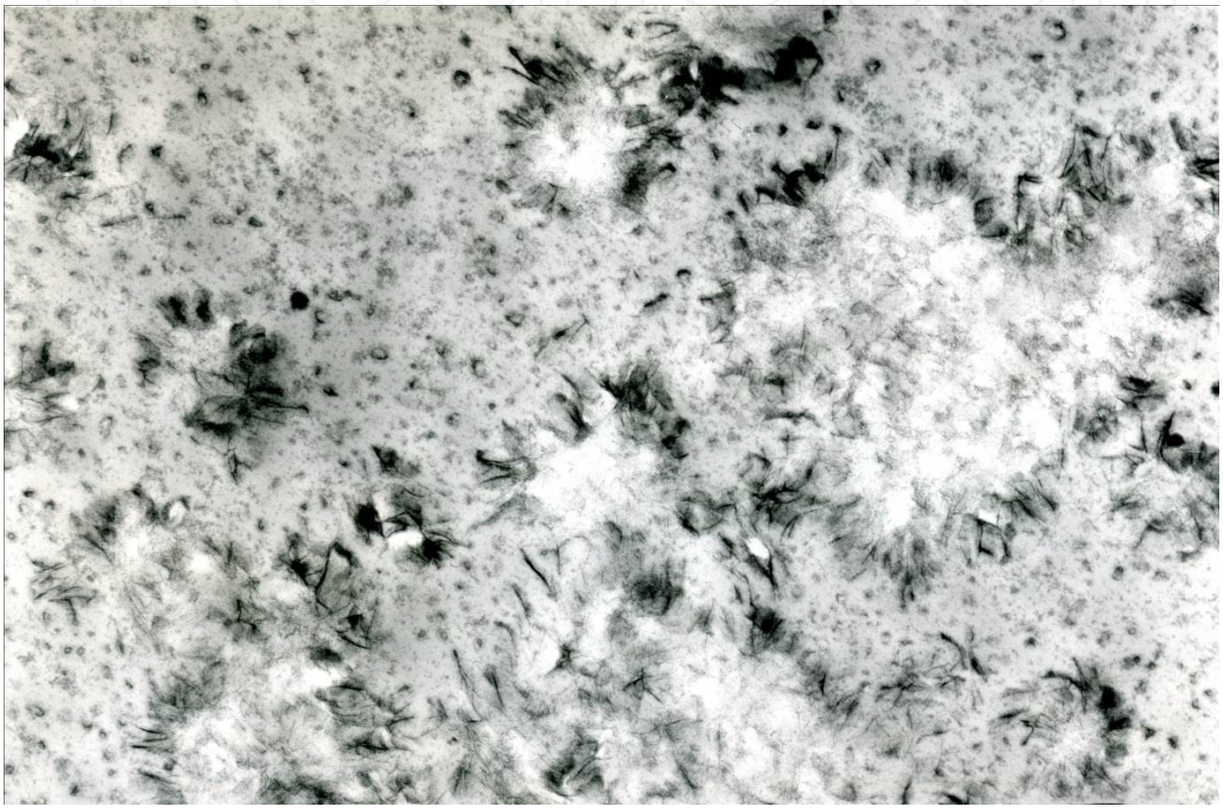

**Figure 10.** Cartilage section from a calf scapula: PEDS method (decalcified with formic acid and treated with colloidal iron at pH 2.8). Reactive crystal ghosts are recognizable at the periphery of the calcification nodules, whose central, fully calcified areas are negative. The granular structures scattered through the uncalcified matrix correspond to proteoglycan aggregates. X 15,000.

The enzymatic removal of organic material before calcification has been repeatedly demonstrated in enamel, where it is a prerequisite to calcification (reviewed by [228]) and in bone [47,48,229]. Hoshi et al. [52] have reported that bone calcification follows the removal of decorin and the fusion of collagen fibrils. Obviously, loss of other matrix, non-collagenous components may occur too.

Another issue to be resolved is whether this mechanism may also be effective in the case of the intrafibrillar calcification that gives rise to the mineral in bands. The PEDS method shows that lightly stained bands replace the inorganic electron-dense bands. In his case, too, there is a superimposition of organic and inorganic ultrastructures, as if the inorganic mate-

rial reacts with, and is occluded by, an organic material contained in the hole zone of collagen fibrils, a possibility already suggested by Veis [38].

## 7. Analogies with other hard tissues

There can be no doubt that the primary role played by the organic components in bone calcification is operative in inducing and regulating the calcification process in other hard tissues. This certainly occurs in tissues, such as dentin, cementum, and calcified tendons, whose matrix, like that of bone, consists of a meshwork of type I collagen fibrils enclosing interfibrillar non-collagenous proteins; but a number of studies shows that it also occurs in tissues that, like epiphyseal cartilage, have a different type of collagen [226,230] or no collagen at all, like tooth enamel [228], mollusk shells [231], spicules of echinoderms [232], crustacean cuticle [233], eggshell [234], and other tissues and organisms. In all these circumstances, an active role in calcification is credited to proteic material, with special reference to acid molecules. In this connection, it is worth mentioning what occurs in enamel, where the organic-inorganic relationships closely resemble those described in bone [170-172,235]: organic septa are intrinsic components of crystals during the early phase of enamel formation, only to be degraded and to disappear as enamel matures. On this topic, it is mandatory to distinguish the early from the final arrangement of the calcified areas. Technical problems often make this distinction impossible to visualize, but, whenever possible, it has shown that the early mineral consists of organic-inorganic hybrids, within which the organic phase is occluded by the inorganic one. This finding has been reported not only in vertebrate calcified tissues, but also in invertebrates and calcified non-skeletal tissues, such as eggshell and several unicellular organisms, as well as in pathologically calcified tissues (reviewed by [2]), suggesting that the initial stabilization of inorganic material by organic structures may be a process shared by all calcified tissues. This possibility is strengthened by what is known about silicification, the process that leads to the hardness of integumental hard tissues in diatom walls, sponge spicules and radiolarian micro-skeletons [236]. As in vertebrate calcification, the silicification process is characterized by a close association of silica with organic components (silicateins) which have both catalytic, enzymatic and templating functions and lead to the formation of organic-inorganic hybrids [237-239]).

## 8. Conclusions

Without considering the role of the matrix vesicles and alkaline phosphatase, which can be looked up in excellent recent reviews [27,96], this article has examined the possible role in calcification of the organic components of the bone matrix. Most remains to be discovered. The role of collagen fibrils in this tissue appears to be less necessary than previously thought: they are chiefly stromal structures and certainly their participation is not an absolute priority for calcification, which can occur in tissues where they are not found [40]; their hole zones seem to be penetrated by inorganic substance without any change in their molec-

ular arrangement, probably through the same type of mechanism that leads to extrafibrillar calcification. Most of the mineral substance is actually contained in the interfibrillar spaces (about 60% according to [153]), where it is associated with the non-collagenous components of the matrix. There are many of these, and although their individual function is known with a good degree of confidence, their mutual role is still uncertain. The electron microscope clearly shows, however, that there is an intimate contact between the inorganic substance and organic frameworks pertaining to one or several of these non-collagenous molecules. These are named 'crystal ghosts', a term justified by the close similarity between them and the structures that are called 'crystals' or 'crystallites'; their histochemical properties justify their recognition as acidic proteins, which in cartilage are mainly acid proteoglycans; and their evolution as calcification develops justifies considering them as organic templates that link inorganic ions, thus giving rise to organic-inorganic hybrids that gradually acquire a crystalline, hydroxyapatite character as the organic component is eliminated. A number of questions must still be answered: chiefly, the nature of crystal ghosts; how they acquire calcium-binding properties; how they are removed; and their relationship with matrix vesicles and collagen fibrils. They are, in any case, actively involved in the early phases of the calcification process and cannot be ignored. They may prove to be the key to opening up and entering a still partly obscure biological mechanism (biomineralization), so providing insights into the biomimetic construction of biologically inspired materials.

## Author details

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