

## Bone mineralization

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### 1. ABSTRACT

This review attempts to summarize the findings made available by the literature on the mineralization of bone. The types of bone, their structures and compositions, the nature and organization of organic and inorganic matter, the organic-inorganic relationships, and the mineralization mechanism itself, are the main topics of the present review. As in other hard tissues, bone mineralization occurs in, and is conditioned by, the components of the organic matrix. Collagen fibrils have long been considered the factor that is able to induce the deposition of apatite crystallites through

a process of heterogeneous nucleation. Interfibrillar non-collagenous proteins are now considered to be co-factors that permit crystallite deposition. The main components of these proteins are reviewed. It is hypothesized that two independent types of mineral are present in bone, one contained in the collagen fibrils and corresponding to the granular, electron-dense bands, and the other contained in the interfibrillar spaces and corresponding to needle- and filament-like crystals. The deposition mechanism of these mineral structures remains elusive. The formation

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of the crystallites through an epitaxial mechanism is discussed.

## 2. INTRODUCTION

Biom mineralization is the process through which living organisms produce specific hard tissues that are indispensable to movements and support (bones), eating (teeth), protection, defense and offense (shells, scales, scutes, spines, carapaces), holding (eggs) and other functions, not the last important being the metabolism of calcium, phosphate and other electrolytes. The term 'biomineralization' derives from the fact that tissue hardness is achieved through the deposition of various mineral ions in the context of organic substrates. According to Mann (1), 40 different minerals have been identified in living organisms so far; of these, calcium, silicon, iron, barium, strontium are the most representative. The term biocalcification, or calcification, is considered to be synonymous with biomineralization by many (discussed by Bonucci, 2) and this alternative use is certainly appropriate in the case of bone, because its inorganic constituent is a carbonated hydroxyapatite, whose predominant component is calcium. For this reason and for simplification, the terms calcification and mineralization will be considered equivalent in the pages that follow.

Probably because it is the main calcified tissue in vertebrates, many of the studies on the biomineralization process have been carried out on bone, despite the fact that, at first sight, it is not an ideal tissue for that type of studies, not only because its hardness and compactness make it difficult to handle (a disadvantage common to all calcified tissues), but also because the term 'bone' refers to distinct types of tissues (woven-fibered, parallel-fibered, lamellar), each of which has its own structure, composition and osteogenic mechanisms (3,4). Moreover, different types of bone may show different degrees of calcification, as exemplified by a comparison between primary (Figure 1A) and secondary bone (Figure 1B): the former calcifies to a maximum as soon as it is laid down, whereas the latter, after a sudden deposition of about 70% of the mineral substance, proceeds slowly in reaching the final 100% degree of calcification (5). On the other hand, this diversity, which may be expressed in adjacent areas of the same skeletal segment, could be advantageous, because it allows a direct comparison between small structural and compositional differences. Researches on bone tissue mineralization cannot, therefore, neglect a preliminary analysis of the type it pertains to and of its degree of calcification.

Bone calcification is strictly dependent on two parameters: the function of bone cells, and the properties of the bone matrix. The latter could not exist without the activity of the former, which, in their turn, are influenced by feedback stimuli deriving from the latter. Although the cells and matrix constitute a unique, integral system on which the properties of the tissue closely depend, this chapter does not take the cells into account because its chief focus is local calcification mechanisms, which depend mainly on the bone matrix and its components.

## 3. TYPES OF BONE

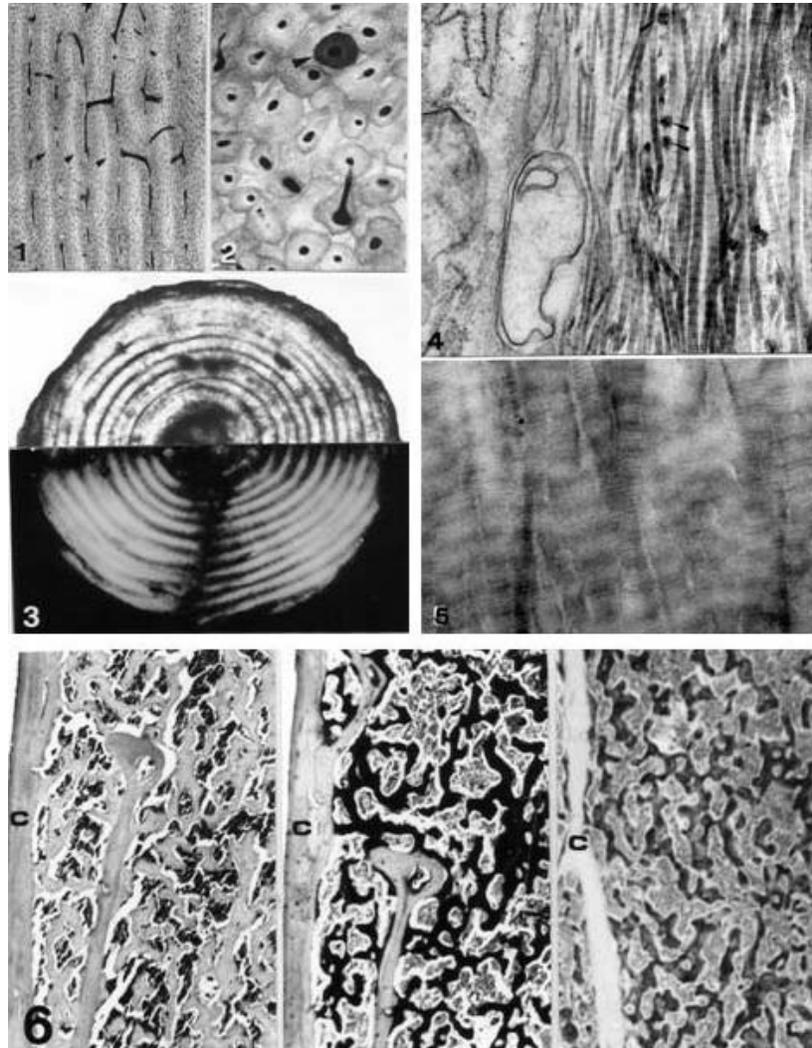
It is well known that the vertebrate skeleton comprises compact bone and trabecular bone (otherwise called spongy, or cancellous bone). The trabecular bone consists of a network of laminar and polygonal structures called trabeculae, which enclose bone marrow spaces; compact bone has a solid, dense organization. Generally speaking, spongy bone is the type of bone mainly found in the core of the short and flat bones, as well as in the epiphyseal ossification centers; compact bone is the type that constitutes the diaphyses of the long bones and the cortical shell of the short and flat bones. The two types differ not only in their structural organization (known as 'bone connectivity'), but also in the amount of bone marrow, degree of cellular functions and metabolic activity (higher in the former), and their mechanical properties (stronger in the latter).

Actually, differences in the typology of bone tissues are chiefly due to their connectivity, which reflects the diversity in their microscopic architecture consequent on the variable amount, percentage and organization of their organic constituents (6,7). On this basis, the following bone types can be distinguished: woven, or woven-fibered, also called immature, or embryonic, or primary; parallel-fibered; lamellar, secondary, mature, or osteonic.

Woven-fibered bone is chiefly characterized by the particular structure of its matrix, where coarse, irregularly arranged bundles of collagen fibrils predominate. That is why bone that is woven-fibered appears almost black (isotropic) under the polarization microscope. It is also called immature, embryonic or primary bone, because it is the first to appear during skeletal development. It is actually a component of the adult skeleton, too, and is formed in all reparative osteogenic processes. Moreover, primary bone may also have a parallel-fibered organization. In woven bone, the collagen fibrils form an irregular, relatively loose network whose interfibrillar spaces contain non-collagenous proteins, in amounts that, even if variable, exceed those in other types of bone. The medullary bone, that is, the trabecular bone that forms in the diaphyseal canal of the long bones of pigeons and other birds in preparation for egg deposition, is an example of spongy bone in which the amount of non-collagenous proteins is extremely high (8,9).

Parallel-fibered bone is distinguished by its bundles of collagen fibrils. These, although they show reciprocal interlacements, fundamentally run almost parallel to each other and, for this reason, are anisotropic (bright) under the polarization microscope. It is mainly found in the compact bone of the primary inner and outer circumferential systems (Figure 1A) and in some primary osteons, but also occurs in secondary osteons. The content of non-collagenous proteins is lower than that in woven bone.

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**Figure 1.** A. Microradiograph of parallel-fibered bone: the mineralization is uniform and at its maximum; note, however, that the interstitial bone (arrowheads) is a little more mineralized than the parallel-fibered bone. x 35 B. Microradiograph of secondary osteonic bone: the arrowhead points to an osteon that is still at an initial phase of formation and is much less mineralized than the other, completely formed osteons. x 35 C. Cross section of a manually isolated secondary osteon (lamellar bone) seen under the light (above) and the polarizing (below) microscope: note the alternation of dark and bright lamellae. x 450. D. Collagen fibrils of the osteoid border clearly showing their periodic pattern. Note that they are loosely arranged (compare with 1E). The arrowheads point to unmineralized matrix vesicles. Part of an osteoblast on left. Uranyl acetate and lead citrate, x 66,000. E. Compact bone, decalcified and stained with phosphotungstic acid. The collagen fibrils are fused side-by-side in parallel (compare with 1D). x 90,000. F. Medullary bone of pigeons: the irregularly arranged trabeculae of medullary, spongy bone are always more deeply stained than the permanent, compact bone (c), showing that it contains a highest concentration of acidic molecules. Left: hematoxylin-eosin; center: PAS-colloidal iron; right: Alcian blue, pH 3.5; x 120.

Lamellar bone is characterized by collagen fibrils organized into laminar structures called lamellae, each lamellar unit comprising collagen fibrils showing the same preferential orientation (Figure 1C). The lamellar bone is also called secondary bone, because it replaces most of the primary bone during bone remodeling, or mature bone, because it constitutes most of the adult skeleton, or osteonic bone, because it is mainly found in the osteons. These are cylindrically shaped structures whose major axis consists of a vascular canal (Haversian canal) of various diameters. Osteons can, in

fact, be viewed as the end-products of the repair of the osteoclast resorption lacunae; the diameter of their canal, initially identical with that of the lacunae, decreases as they are centripetally rebuilt.

Osteon structure depends on the way the collagen fibrils are arranged in the lamellae; these, in their turn, are arranged around the Haversian canals. In this connection, three main types of osteons can be detected when their cross-section is examined under the polarization microscope (10): completely anisotropic (uniformly bright) osteons, which are considered to have most of their

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collagen fibrils running transversally in poorly outlined lamellae; completely isotropic (black) osteons, which are considered to have most of the collagen fibrils longitudinally oriented in poorly outlined lamellae; and osteons – the most frequent – which show an alternation of bright and black lamellae under the polarizing microscope and are considered to have collagen fibrils whose course in one lamella is more or less perpendicular to the course in the neighboring lamellae (Figure 1C). The mechanical properties of osteons closely depend on their lamellar organization (11) which, however, is still partly controversial (discussed by Bonucci, 7). On the basis of scanning electron microscope studies, it has, in fact, been reported that their lamellar appearance is due to the alternation of two types of lamellae, one collagen-rich and dense, another collagen-poor and loose, all having an interwoven arrangement of their fibers (12,13). On the basis of polarizing light microscopy, synchrotron X-ray diffraction, and confocal microscopy studies on isolated lamellae, both the bright and dark lamellae showed axial unidirectional collagen bundles and two overlapping oblique bundles (14). In reality, bone types differ not only in their micro-architecture, but also in the structure and composition of their organic matrix and in their degree of calcification.

### 4. THE ORGANIC MATRIX OF BONE

The organic matrix of all types of bone consists of collagen fibrils and non-collagenous proteins, with small amounts of phospholipids and traces of serum proteins. Generally speaking, it can be stated that, with few exceptions concerning non-collagenous proteins, the matrix composition is always the same in the various types of bone, the only differences being quantitative rather than qualitative (reviewed by Bonucci, 2). The percentages reported by McLean and Urist (15) for the collagen and non-collagenous proteins of compact bone (about 90% and 10%, respectively) are susceptible to considerable variations, especially as far as the latter are concerned.

#### 4.1. Collagen fibrils

The collagen fibrils in bone mainly consist of type I collagen, which is a member of the wide collagen superfamily. It is represented by fibrils about 7,8 nm in diameter showing a characteristic periodic banding ( $D = 68-70$  nm) under the transmission electron microscope (Figure 1D). Although its ultrastructural pattern changes with the treatments applied for its visualization (16), the periodic banding reflects the way the collagen molecules and the reacting groups of their amino acid are arranged in the fibrils (17).

The collagen molecules are rod-like structures 280-300 nm in length and 1.5 nm in diameter, which derive from the aggregation of three polypeptide chains into a left-handed helical configuration, a process that requires the cleavage of their N- and C-propeptide domains by specific procollagen proteinases (for the synthesis, molecular assembly, secretion and aggregation of collagen molecules into fibrils, see Veis, 18). The vertebrate amino acid chains have the characteristic triplets *gly-x-y*, in which *gly*

is glycine, and *x* and *y* are often proline and hydroxyproline. The sequence in one polypeptide chain may be similar to that in the other two chains (homotrimeric units) or may be different (heterotrimeric units). The molecules of the type I collagen consist of two equal chains, called  $\alpha 1(I)$ , and one different chain, called  $\alpha 2(I)$ .

The mechanisms through which the collagen molecules assemble into fibrils, and the way they give rise to the characteristic periodic banding of 68-70 nm, are still uncertain. According to the bidimensional model of Hodge and Petruska (19), a spontaneous alignment of collagen molecules occurs in such a way as to give rise to a quarter-staggered overlap array. The molecules have a length equivalent to 4.4 times the D-period; they are considered to be divided into 5 consecutive segments (numbered from 1 to 5) in the N to C direction, 4 of which (1, 2, 3, 4) are 1-D in length, whereas the 5<sup>th</sup>, corresponding to the C-terminal telopeptide, is only 0.4 D long. The molecules aggregate in parallel array, in such a way that the minimum cross-section of a microfibril contains 5 of them; their side-by-side interaction, however, does not occur in an end-to-end way, because they are mutually displaced along their axial plane. This induces the alternation along the native collagen fibrils between zones where all 5 molecules overlap ('dense' zones), and zones where the 5<sup>th</sup> segment gives rise to a minor molecular density (only 4 molecules overlap) and induces the formation of 'gap' or 'hole zones' 0.6 D long. Intra- and inter-molecular cross links give these fibrils their high degree of stability and insolubility.

The model suggested by Hodge and Petruska is not easily adaptable to a three-dimensional fibrillar structure; this explains why several different assemblages have been suggested (discussed by Bonucci, 2), either based on a side-by-side aggregation of microfibrils, with their overlap and gap zones in register, or without the intervention of microfibrils, or even supposing that a liquid crystalline order takes place before the telopeptide cleavage induces the deposition of insoluble fibrils.

The internal organization of the fibrils plays an important role in the process of mineralization, because on one hand it may induce the initial nucleation of the inorganic substance and, on the other, it should provide the spaces in which the inorganic crystals are held (see below, p. 00). According to Schiffmann *et al.* (20), the length of the overlap zones and hole zones is 25-30 nm and 40-45 nm, respectively, and the diameter of a hole is equal to that of a collagen molecule, that is, according to Glimcher and Krane (21), about 1.5 nm. The intermolecular spaces are variable to some extent (22); the collagen equatorial reflections obtained by Bonar *et al.* (23) using X-ray and neutron diffraction analysis gave values of 1.24 and 1.53 nm in the wet state and 1.16 and 1.12 nm in the dry state, for the mineralized and demineralized ox compact bone, respectively. Atomic force microscopy and Fourier analysis of rat calcaneal collagen have given a value of 1.43 nm for the molecular diameter and 2.21 nm for the intermolecular distance (24). According to Katz and Li (25), the average intermolecular gap, either of bone or of dentin fibrils, is 0.6

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nm, approximately twice that of either tail or achilles tendon (about 0.3 nm), and the intermolecular distance in the former is about 1.9 nm. In reconstituted collagen fibrils, Katz and Li (26) have calculated – assuming that the molecules are assembled in a quasi-hexagonal configuration – that, for 1 g of collagen, intrafibrillar spaces include 0.13 ml/g which pertain to the helical groove of the molecules, 1.01 ml/g which are interstitial, 0.66 ml/g due to pores, and 0.48 ml/g due to holes. Moreover, these Authors, as well as Weiner and Traub (27), have stressed the possibility that, as a consequence of the in register aggregation of the microfibrils, the lateral, parallel alignment of the ‘gaps’ can give rise to channels (‘grooves’) throughout the fibrils.

Measurements of intra- or inter-fibrillar spaces in the calcified collagen fibrils of bone meet with several difficulties. First, only indirect evaluations are possible, because the mineral masks the collagen structure and prevents a direct examination of the collagen fibrils; on the other hand, the removal of inorganic substance by decalcification induces severe changes in the collagen ultrastructure and organization (2,28). Second, the mineralization itself may induce changes in the fibril organization. This is exemplified by the low density and loose arrangement of the uncalcified collagen fibrils in the osteoid tissue (29 and Figure 1D), which fuse side by side, in parallel, as the degree of calcification increases and attain to the greatest compactness where calcification is complete (30 and Figure 1E). The solubility of the collagen matrix decreases, in fact, as the degree of mineralization increases, so that it becomes insoluble under conditions that readily solubilize soft tissue collagen (31). In this connection, the compactness of the collagen fibrils is not the same in all types of bone: it is the highest in compact bone, but is low in woven bone, where the amount of interfibrillar non-collagenous proteins is consequently high (32), reaching the lowest degree in special types of bone, like the medullary bone of birds (9,33).

### 4.2. Non-collagenous proteins

The denomination of these proteins as non-collagenous is quite generic and means only that they are distinct from the collagen component, although the two moieties may be linked so closely that they are inseparable even under highly dissociative solvent conditions (34). Even if they represent a minor and quantitatively variable proportion of the organic components of the calcified matrices, they play a major role in inducing and regulating the mineralization process. This is indirectly indicated by the close link that exists between them and the inorganic substance – a link which allows them to stay intact in fossil bones for thousands of years and implies that they can be extracted from the calcified matrix only after decalcification. Just because of the primary role they may play in the induction and regulation of the biomineralization process, the non-collagenous proteins are treated and discussed in practically all chapters of this volume, including one that is specifically dedicated to the topic. For this reason, only the non-collagenous proteins that are supposed to be more directly involved in bone mineralization are considered in the following pages. They include well-defined molecules whose function in bone, although not always

definitively clarified, appears to have a fundamental role in the induction and regulation of the calcification process.

#### 4.2.1. Proteoglycans

This name refers to long-known components of the bone matrix (35) whose specific functions are still being investigated. They are found in the extracellular matrix of soft and hard tissues, as well as in the cellular membrane and organelles. Their name derives from the fact that, with the exception of hyaluronan, their molecule consists of a proteic core to which glycosaminoglycan (GAG) chains are covalently linked. The GAG chains are linear polymers of disaccharide units (for reviews see 36-38) that may be sulfated, and correspond to chondroitin sulfate (sulfated hexosamine-hexuronic acid), keratan sulfate (sulfated hexosamine-galactose), heparin and heparan sulfate (sulfated hexosamine-sulfated hexuronic acid), or may be non-sulfated, as in hyaluronan (glucuronic acid-N-acetyl glucosamine).

The amounts of proteoglycans change with bone types: the highest occur in bone types with the lowest compactness of the organic matrix and degree of aggregation of collagen fibrils. This is particularly evident in the medullary bone of birds, whose matrix contains much higher quantities of acid proteoglycans (8,39), and is consequently much more deeply stained by Alcian blue and colloidal iron methods (9), than the cortical bone (Figure 1F). In addition, amounts of proteoglycans appear to decrease with, and to be roughly inversely related to the degree of, matrix calcification. This behaviour, which can have significant implications in the mechanism of calcification (see below, p. 00), now seems to be neglected, although it was repeatedly emphasized in the past. It has been documented in isolated osteons at different degrees of calcification: the hexosamine concentration was 0.61% of dry weight in the uncalcified osteoid tissue, only to fall to 0.31% in low calcified osteons and 0.28% in osteons at the highest degree of calcification (40). The total amount and the molecular size of glycosaminoglycans fell with increasing bone calcification (41), and Prince *et al.* (42) showed that about 45% of proteoglycans were removed during bone calcification. Using histochemical methods, Baylink *et al.* (43) showed that the osteoid tissue, but not the mature calcified matrix, stained with Alcian blue or Toluidine blue for acid proteoglycans, and that at the calcification front the sulfate concentration dropped as that of calcium increased. Takagi *et al.* (44) obtained similar results using the high-iron diamine-thiocarbohydrazide-silver proteinate method. Using ultrastructural histochemical methods, Hoshi *et al.* (45) showed that cuprolinic blue-stainable proteoglycan granules were abundant in the osteoid tissue of embryonic rat calvaria with their size falling as calcification proceeded. Nefussi *et al.* (46) observed dense filamentous reticular patches stained by cuprolinic blue between the collagen fibrils of the calcified matrix. Bonucci and Silvestrini (47) found chondroitin-4-sulfate at the periphery, but not in the calcified inner portion, of the areas of early calcification (i.e., the calcification

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nodules) that give rise to the calcification front. Nakamura *et al.* (48) found that keratan sulfate proteoglycans could be detected in calcification nodules of the membranous ossification in rat calvaria and that their immunoreaction was stronger at their periphery than in their calcified central zone. Ultrastructural changes in proteoglycans during the calcification process have been documented several times (44,49-53).

The variable structure of the GAG chains and the numerous combinations of core proteins with which they may be covalently linked explain why the proteoglycans, for long considered practically inert molecules only contributing to structural integrity, osmotic pressure and the degree of hydration of the intercellular matrix, now prove to have a variety of biological functions (reviewed by 54,55). On the basis of their molecular characteristics, size and location, they have been classified as belonging to three main groups: small leucine-rich proteoglycans, modular proteoglycans and cell-surface proteoglycans (55). Members of the first two groups have been found in the bone matrix and may play a role in its calcification.

### 4.2.1.1. Small leucine-rich proteoglycans

The small leucine-rich proteoglycans (SLRPs) are small molecules marked out by their proteic core which displays leucine-rich repeats, N-terminal cysteine clusters and at least one GAG chain. They can be divided into five classes (56). All five are “matricellular proteins”, that is, extracellular proteins that are able to modulate cell-matrix interactions and cell functions (57,58). All are members of a signaling cascade and can modulate a number of biological processes (59); some of them, which are briefly considered below, have a recognized function in regulating bone structure and mineralization.

#### 4.2.1.1.1. Decorin

Decorin (class I) is the most representative SLRP in tendons (36) and bones (60). It consists of a leucine-rich core protein and one chondroitin or dermatan sulfate chain. It is associated with almost all types of collagen and in tendons it is localized near the *d* and *e* bands of the fibrils (61). Its main role is that of assembling collagen fibrils (62,63), as confirmed by observations that rats with disruption of the decorin gene had abnormal collagen structure and, consequently, fragility of the skin (64,65), and that in cultures of MC3T3-E1 cells expressing higher (sense) or lower (antisense) levels of decorin the collagen fibrils were thinner and thicker, respectively, than in controls (60). In addition, stable osteoblastic cell clones expressing high and low levels of decorin induced delay or acceleration, respectively, in the onset of mineralization (60,66), suggesting a decorin inhibitory effect. In line with this possibility, Hoshi *et al.* (30) have shown that decorin is abundantly localized near the collagen fibrils of the osteoid border and that, with the onset of calcification, these fuse side by side, forming fibrils measuring as much as 400 nm in thickness, or even more; at the same time, decorin, which was detected by means of ultrastructural immunohistochemistry, decreases around these fibrils. Decorin has also been detected in human heterotopic bone (67,68).

#### 4.2.1.1.2. Biglycan

Biglycan is another representative of class I of the SLRP family. It consists of a leucine-rich core protein and two chondroitin or dermatan sulfate chains, and is highly expressed both in the extracellular bone matrix and at the cell surface (69). Mice with biglycan deficiency develop collagen fibril abnormalities (65), have delayed osteogenesis after marrow ablation (70), and develop an age-related osteoporosis-like phenotype (71,72). According to studies by Chen *et al.* (73) and Parisuthiman *et al.* (74), biglycan modulates osteoblast differentiation and its deficiency affects BMP-4 signal transduction, reducing the Cbfa1 transcription factor and the osteoblast differentiation. Biglycan-deficient female mice, however, do not develop osteopenia after ovariectomy, suggesting that biglycan may have a dual role in bone, modulating both its formation and resorption (75).

#### 4.2.1.1.3. Asporin

Asporin is another member of class I of the SLRP family; in its case there is no glycosaminoglycan attachment site. It competes with decorin in binding to collagen and its polyaspartate domain binds calcium while contributing to the regulation of collagen mineralization (76).

#### 4.2.1.1.4. Fibromodulin

Fibromodulin and lumican (class II) are widely distributed in connective tissues and both play a role in collagen fibrillogenesis. Fibromodulin is expressed by osteoblasts during fetal membranous ossification (77); lumican is secreted *in vitro* by differentiating and mature osteoblasts only, and becomes a major component of the bone matrix (78). Fibromodulin- and lumican-null mice have reduced tendon stiffness and osteoarthritis (79).

#### 4.2.1.1.5. Osteoadherin

Osteoadherin, a class II, keratan sulfate SLRP, isolated from bovine and rat bone, binds to hydroxyapatite crystals and shows the same localization as that of bone sialoprotein at the calcification front (80). Moreover, it can enhance osteoblast differentiation and the degree of *in vitro* mineralization (81).

### 4.2.1.2. Modular proteoglycans

The group of modular proteoglycans, also called lecticans, gathers together large, heterogeneous, often highly glycosylated molecules, which can be divided into two subgroups: the hyalectans, whose N-terminal domain binds hyaluronan (hyaluronic acid; HA), and the non-hyalectans (55). Hyalectans include aggrecan, versican, neurocan and brevican; the non-HA-binding lecticans comprise several molecules, one of which, perlecan, has been found in cartilage and other connective tissues.

#### 4.2.1.2.1. Hyaluronan

Hyaluronan differs from other proteoglycans because its GAG chains are not linked to a core protein; moreover, it is not sulfated, with its repeating disaccharide units consisting of N-acetylglucosamine and glucuronic acid. Hyaluronan can bind a number of molecules, giving rise to hyalectans, which include aggrecan, versican,

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neurocan and brevican. Aggrecan appears to be the most interesting of the group, especially because of its abundance in cartilage. It consists of many GAG chains that are covalently linked to a core protein and mainly comprise chondroitin sulfate and a smaller amount of keratan sulfate. In the extracellular space, aggrecan exists exclusively as macromolecular aggregates composed of hyaluronan molecules, stabilized by a link protein, which bind together a number of aggrecan molecules (reviewed by 82,83). While its role in cartilage, especially its contribution in regulating the cartilage mechanical properties, is relatively well known (reviewed by 37), its function in bone is less clear. It has been detected in normal and in ectopic bone, its amounts falling as bone matures (68). The antibody (Mab)5D5, which recognizes the core proteins of large proteoglycans such as versican and brevican, gives a positive immunohistochemical reaction in the calcification nodules (84); during immunostaining these had been decalcified on the grids, where they appeared as structures with an electron-dense periphery surrounding fine filamentous and granular material.

### 4.2.2. Glycoproteins

This denomination refers to acidic molecules rich in glutamic, aspartic and sialic acids and mostly containing *o*-phosphoserine and *o*-phosphothreonine, so they are also known as phosphoproteins, most of which are covalently bound to collagen (85). Histochemically (PAS method), they are detectable in the osteoid tissue and at the calcification front (86,87), as also shown by immunohistochemistry (88). Bone glycoproteins comprise osteonectin, bone sialoprotein, osteopontin, dentin matrix protein 1, matrix extracellular phosphoglycoprotein and acidic glycoprotein-75. With the exception of osteonectin and acidic glycoprotein-75, these glycoproteins are grouped under the acronym SIBLING, which derives from Small Integrin-Binding Ligand, N-linked Glycoprotein, a family of genetically related proteins clustered on human chromosome 4 (89,90). Another glycoprotein –  $\alpha_2$ -Heremans-Schmid glycoprotein, or  $\alpha_2$  HS glycoprotein, AHSG, fetuin-A – is synthesized in the liver and accumulates in the bone matrix (91).

#### 4.2.2.1. Osteonectin

Osteonectin (ON), which is homologous with BM-40 protein and with SPARC (Secreted Protein, Acid and Rich in Cysteine), is a glycoprotein that can bind to both collagen fibrils and hydroxyapatite (92). It is not, however, a component of bone tissue alone: it is, in fact, expressed by all uncalcified connective tissues and by a number of soft tissues (93,94). Moreover, its concentration in bone matrix is variable, with higher levels recorded in adult human trabecular bone than in lamellar bone (95) and appears to be inversely correlated with the degree of calcification (96). In agreement with this observation, Bianco *et al.* (97) reported strong ON immunoreactivity in uncalcified osteoid tissue; Nefussi *et al.* (98) found in cell culture a uniform ON distribution through the osteoid tissue and the calcified matrix. Romberg *et al.* (99) observed the inhibition of crystal growth by ON *in vitro* and Hunter *et al.* (100) found a lack of ON nucleating properties even at concentrations of up to 100  $\mu$ g/ml. In osteonectin-null

mice, the mineral contents and the degree of crystallinity (crystal size and perfection) were higher than in the age-matched wildtype controls, while collagen maturity was greater in both the cortical and trabecular bone (101). The role of osteonectin in bone remains uncertain: it is a “matricellular protein” and, as such, has a general multifactorial function with particular reference to the regulation of calcium-mediated processes (102), cell-matrix interactions (103), hydroxyapatite binding sites (104) and regulation of bone remodeling (105).

#### 4.2.2.2. Bone sialoprotein

Bone sialoprotein (BSP) is another matricellular protein. It belongs to the SIBLING family and is a glycosylated, sulfated, phosphorylated, sialic acid-rich protein that can bind both hydroxyapatite and cell-surface integrins through the Arg-Gly-Asp motif (for review see 106). It is expressed by differentiated osteoblastic cells (107) and is immunohistochemically detectable in their Golgi and post-Golgi secretory structures (108); its mRNA has been found in mature osteoblasts, not in their precursors (109).

BSP is mainly localized in the bone matrix, including cement lines and “laminae limitantes” (110). Its expression, however, extends to osteoclasts, fetal epiphyseal cartilage cells and trophoblastic cells of the placenta (111), ameloblasts (112), and osteotropic cancers, especially those that develop microcalcifications (113). Its amounts change with the type of bone (3) and with the degree of matrix calcification (96). Anyway, it seems to play an important role in bone mineralization, not only because of its close relationship with the collagen fibers (114), but also because it is prominently localized in the early aggregates of crystals (“calcification nodules”) at the calcification front (98,115), as well as in their matrix. Moreover, the overexpression of BSP heightens osteoblast-related gene expression as well as calcium incorporation and nodule formation by osteoblast cultures (116). These findings, and the proven capacity of nucleating hydroxyapatite (100,117), suggest that BSP, with the co-operation of collagen fibrils (114), plays a primary role in the early stages of calcification. Bone sialoprotein knockout mice show impaired bone growth and defective mineralization, with a reduction in bone formation (118) and the delayed repair of cortical defects (119,120). BSP overexpression in mice leads to osteopenia and mild dwarfism, as a consequence of the enhancement of osteoclastic resorption, and to a shrinking of the osteoblast population (121).

#### 4.2.2.3. Osteopontin

Osteopontin (OPN) has many points of contact with bone sialoprotein: it is a glycosylated, phosphorylated, sulfated sialoprotein and contains the Arg-Gly-Asp motif and the polyacidic amino acid sequence which allow it to bind to hydroxyapatite and cells. Although OPN is a component of the calcified bone matrix, it is mainly found at the bone surfaces facing single cells, especially osteoclasts; moreover, it shares with the bone sialoprotein a location at the calcification nodules (for review see 122). On the other hand, it is expressed in a variety of hard and

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soft tissues and, unlike BSP, it inhibits hydroxyapatite nucleation and crystal formation (100,123,124). Mice poor in osteopontin have a normal bone structure, but display an altered osteoclast formation *in vitro* (125) and, after ovariectomy, show a fall in bone volume lower than in controls (126). It is now clear that OPN, besides its adhesive properties and role in the inhibition of the calcification process, has other functions. It is mainly a cytokine that is involved in tissue inflammation and repair and, besides regulating bone resorption and bone calcification, is active in diverse biological processes, such as wound healing, angiogenesis, immunological reactions, tumorigenesis and atherosclerosis (reviews in 96,127-131).

### 4.2.2.4. Dentin matrix protein 1

Dentin matrix protein 1 (DMP1) was originally identified from a rat incisor DNA library and was considered to be specific to dentin. Actually, it has been detected in bone, where it is mainly expressed by osteocytes, its expression being intensified by mechanical loading (132,133). Besides this, DMP1 is expressed in non-calcifying tissues (134). It is an acidic, highly phosphorylated protein whose molecule is cleaved into three distinct segments: the N-terminal and the C-terminal fragments (135), which are localized in different compartments, the former occurring as a proteoglycan, the latter being highly phosphorylated (136), with both acting as promoters of mineralization, plus a third chondroitinsulfate-linked N-terminal fragment (DMP1-PG), which is an inhibitor. These three work together in controlling the mineralization process (137). DMP1 can nucleate hydroxyapatite *in vitro* when immobilized on type I collagen fibrils (138,139) and is considered a key regulator of matrix mineralization (140); its mRNA is expressed in coincidence with the start of mineral deposition (141). According to Tartaix *et al.* (142), DMP1 in its native form inhibits mineralization, whereas it initiates the process when it is cleaved or dephosphorylated. DMP1 deficiency results in hypomineralized matrix (143); it induces defective osteocyte maturation and heightened fibroblast growth factor 23 (FGF23) expression (144), leading to autosomal-recessive hypophosphatemic rickets in humans (135).

### 4.2.2.5. Matrix extracellular phosphoglycoprotein

Matrix extracellular phosphoglycoprotein (MEPE; also called osteoblast/osteocyte factor 45, OF45, or Osteoregulin) is expressed by osteoblasts in association with mineralization (145); in normal human bone, however, it is mainly expressed by osteocytes (146). It is a member of the phosphatonin group, a class of phosphate-regulating factors. Its intraperitoneal injection induces dose-dependent hyperphosphaturia and hypophosphatemia in mice (147). MEPE is markedly expressed in cells of the tumors that cause hypophosphatemic osteomalacia as well as in osteoblasts in X-linked hypophosphatemic rickets (reviews in 148,149). The phosphorylated intact protein is a promoter of the mineralization process (150); a MEPE-derived, 23-amino-acid peptide (dentonin), promotes stem cell proliferation in dental pulp (151,152). Specific binding of MEPE to PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome)

regulates the degradation of MEPE and the release of small peptides (ASARM-peptides; acidic serine-aspartate-rich MEPE-associated motif) that play a role in phosphate regulation and inhibit mineralization (minhibins; 153) by binding to hydroxyapatite, an inhibition that is regulated by PHEX cleavage of ASARM (154). Because of these properties, MEPE has been considered a bone-renal hormone (155).

### 4.2.2.6. Acidic glycoprotein-75

Acidic glycoprotein-75 (BAG-75) is a sialic acid-rich phosphoglycoprotein that has limited structural homology to osteopontin and is found in bone matrix in 75 and/or 50 kDa forms (115). It is expressed very early during intramembranous ossification, when it is found, together with BSP, in microfibrillar complexes *in vitro* and *in vivo* that have the potential to sequester phosphate ions (156) and to become mineralized (157,158). The mineralization of these complexes seems to be dependent on the cleavage of BAG-75 and BSP by an AEBSE-sensitive (AEBSE: 4-(2-aminoethyl)benzenesulfonyl fluoride HCl), osteoblast-derived serine protease (159).

### 4.2.2.7. $\alpha$ 2-HS glycoprotein /Fetuin

$\alpha$ 2-HS glycoprotein /Fetuin (AHSG) is also known as  $\alpha$ 2-Heremans-Schmid glycoprotein. It is a protein synthesized in the liver and secondarily accumulated in bone, to a point such that it comes to be a major non-collagenous component of mineralized bone matrix in mammals. It inhibits the transforming growth factors/bone morphogenetic proteins and, together with matrix Gla protein, gives rise to calcium-phosphate complexes that prevent calcium and phosphate deposition in soft tissues (160). This effect appears to be obtained through the formation of "calciprotein particles", that is, soluble, colloidal spheres, 30-150 nm in diameter, which contain AHSG, calcium and phosphate, and are initially amorphous and soluble, only to become progressively more crystalline and, finally, insoluble (161). The inhibition of calcification by AHSG is confirmed by *in vitro* studies (162) and by the occurrence of diffuse calcifications in various soft tissues and organs of AHSG-deficient mice fed on a mineral and vitamin D-rich diet (163).

### 4.2.2.8. Alkaline phosphatase

Alkaline phosphatase can be added at the end of this chapter, because this enzyme is a Ca-binding glycoprotein (164) and is present not only on the cell membrane and in matrix vesicles, but also in the mineralizing matrices of cartilage and bone (165). Its tissue non-specific isoenzyme, or tissue non-specific alkaline phosphatase (TNSALP), is certainly critical for the occurrence of a normal mineralization process, as shown by the skeletal defects that develop in congenital hypophosphatasia and in TNSALP knockout mice. In both cases, in fact, rickets- and osteomalacia-like changes develop, characterized in bone by excessive amounts of osteoid tissue (166). In spite of the severe defects of matrix mineralization, the formation of inorganic crystals in matrix vesicles is not impaired; rather than a primary calcification

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defect, the lack of alkaline phosphatase appears to induce a retarded propagation of crystals from the calcified matrix vesicles into the surrounding matrix (167). These and other findings have led to the suggestion that TNSALP activity could induce the removal of inhibitors of the calcification process, more specifically of the matrix inorganic pyrophosphate, which can be removed through its hydrolysis (168) and by modulation of the expression of the plasma cell membrane glycoprotein-1 (PC-1) that is needed for its synthesis (169). The Ca-binding properties of the alkaline phosphatase molecule might have a basic role in the mineralization process, besides that connected with its enzymatic activity: hypothetically, the link of calcium ions to its molecule might promote the formation of organic-inorganic hybrids that, as discussed below (p. 000), might represent the first step of crystal formation.

### 4.2.3. Gla-proteins

These proteins are named after the observation that the vitamin K-dependent,  $\text{Ca}^{2+}$ -binding,  $\gamma$ -carboxyglutamic acid (Gla) amino acid is a constituent of their molecule. They include bone Gla-protein and matrix Gla protein.

#### 4.2.3.1. Bone Gla-protein

Bone Gla-protein (BGP), better known as osteocalcin (OC), is synthesized by the cells of the osteoblastic lineage (reviewed by 170,171). It is localized in bone cells and calcified bone matrix, its concentration proving to be higher in compact than in trabecular bone. It is not contained, or its concentration is very low, in uncalcified osteoid tissue (172,173), whereas it is localized in calcification nodules (174), which, after decalcification, appear as filamentous 'grey patches' (175). Levels of circulating OC rise with bone turnover (as happens, for instance, in hyperparathyroidism; 176) and fall when osteoblastic activity falls (as after glucocorticoid administration; 177). The role of osteocalcin in bone formation and calcification is still, however, uncertain. *In vitro* studies have shown that calcium phosphate precipitation is delayed by OC in solution, and that this effect disappears if OC is immobilized on sepharose beads (178). Inhibition by warfarin of vitamin K, which functions as a cofactor in the carboxylation of glutamate residues, induced a fall in bone OC content, but did not cause significant bone changes in rats (179) and did not alter bone mineral density or the markers of bone turnover in monkeys (180). Warfarin administration in rats did, however, prevent the formation of calcification nodules in the osteoid tissue and resulted in the dispersion through it of crystalline particles (181). In warfarin-treated rats, ovariectomy induced a significant decrease in the diaphyseal bone mineral content, bone mineral density, cortical thickness, and maximum load, changes that were not observed in the control, ovariectomized animals (182). Impairment of the OC function due to incomplete carboxylation leads to a rise in the risk of osteoporosis development and vascular calcification (183); osteocalcin-deficient mice have increased bone formation, higher bone mass and improvement in the functional qualities of bone (184) but show reduced crystal size and perfection (185). In reviewing the topic, Krueger *et al.* (186) have emphasized the role of osteocalcin as a regulator of the mineralization

process. Fukumoto and Martin (187) have expressed the concept that bone is an endocrine-like organ because its cells synthesize FGF23, which regulates phosphate excretion (see also 188), and osteocalcin, which acts on the  $\beta$ -cells of the pancreas to enhance insulin production.

#### 4.2.3.2. Matrix Gla-protein

Matrix Gla protein (MGP) shares many features with osteocalcin, but MGP and OC do not cross-react and they must be kept distinct (reviewed by Price, 170). Moreover, OC is only found in calcified tissues, while all soft tissues, not only the calcified ones, contain MPG. Its mechanism of action is not fully understood, but MPG is now considered an inhibitor of calcification. This conclusion is chiefly based on studies on vascular calcifications, especially those that develop in chronic uremia (189-192), and is confirmed by studies on MPG-deficient mice, which show widespread vascular calcifications and die from aortic rupture (193-195). The possibility that MGP gives rise to the formation of a high molecular weight complex of calcium, phosphate and fetuin has been discussed above (p. 00).

A vitamin K-dependent protein, named Gla-rich protein (GRP) because it contains 16 gamma-carboxyglutamic acid (Gla) residues in its 74-residue sequence, has recently been described in sturgeons (196). GRP accumulates at sites of pathological calcifications (197).

### 4.2.4. Lipids

Lipids are intrinsic components of the bone matrix (198,199), from which they can only be completely extracted after removal of the inorganic substance (200). They are also found at the calcification front in calcification nodules, as shown histochemically by their staining after glutaraldehyde-malachite green fixation (201) and immunohistochemically by their reaction with the antibody MC22-33F, which is specific to phosphatidylcholine, sphingomyelin and dimethylphosphatidylethanolamine (202). Lipids are contained in matrix vesicles, too (203). A significant proportion of the lipids found in the calcified matrix, especially that of young bone, corresponds to calcium-phospholipid-phosphate complexes (204). Vogel and Boyan-Salyers (205) and Dziak (206) have discussed the possible involvement of acidic lipids in calcification. Xu and Yu (207) have suggested that lipid particles distributed along collagen fibrils may mediate their calcification.

### 4.2.5. Function of non-collagenous proteins

Although the importance of non-collagenous proteins in bone physiology is not debatable, and the specific function of each of them has achieved consensus on a general perspective, the effects that they play together on bone mineralization are poorly known and are open to further research. This openness depends on several factors. First, a number of different non-collagenous proteins have been found in the osteoid tissue and at the calcification front (88,98), as well as in isolated calcospherites (208). Their heterogeneity does not allow any earmarking of one or a group of them as directly responsible for inducing

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and/or regulating the mineralization process. On the other hand, although the location of a protein in the mineralization sites is a prerequisite to their being active in the mineralization process, it is obviously inadequate to conclude that they are involved in, or are promoters or inhibitors of, the mineral formation. Secondly, most of the information on the properties and activities of single non-collagenous proteins has been obtained by means of *in vitro* research, when it is known that they change according to whether the proteins are free in solution or attached to a substrate. Thus, osteonectin, osteocalcin and dentin phosphoprotein in solution delay calcium phosphate precipitation, whereas this effect disappears when the proteins are immobilized on sepharose beads (178). Third, the action of the proteins on the induction of mineral formation changes with their concentration and this, in its turn, can change during the mineralization process. As already discussed, the concentration of acid proteoglycans, which changes with the bone type, falls during mineralization (40-45), and their quality, and, possibly conformation, change too (49-53). Removal of inhibitors can result from these processes, as exemplified by the PHEX cleavage of ASARM (154). Alternatively, the fall of protein concentration may be closely related to the necessity that they be processed to become active or to permit the activity of other molecules. Post-translational modifications, such as phosphorylation, glycosylation, and proteolytic processing, can themselves induce variations in the activity of specific molecules (150). Still another possibility, hypothesized by Bonucci (2,209,210), can be taken into account (discussed below, p. 000): the enzymatic proteolytic process could be a necessity indispensable to the transformation of the earliest inorganic structures into definitive apatite crystals. Ultrastructural studies on PEDS-decalcified bone and cartilage sections have, in fact, shown that the early mineral particles are organic-inorganic hybrids whose organic component, which is visualized as crystal ghosts under the electron microscope, becomes less and less evident as the formation of the calcification nodules proceeds and the maturation of inorganic crystals goes forward, that is, as the crystal diffractograms turn from amorphous into crystalline (discussed by 2, 209). The disappearance of crystal ghosts has been interpreted as due to their enzymatic removal, a hypothesis that, on one hand, is supported by the demonstration that the cleavage of BSP and BAG-75 is associated with crystal nucleation and appears to be a permissive step for mineral progression (159), and on the other is in agreement with the loss of organic material that occurs during mineralization. Taking all these comments into account, two proteins appear to be chiefly involved in the early phase of the mineralization process: BSP and BAG-75. They, in fact, are co-localized, and seem to be the first to appear in the earliest calcification nodules (108,158,208,211); they seem to be expressed before, or together with, the first mineral particles, and their proteolytic degradation appears to be a preliminary condition for the completion of the mineralization process (159). DMP1 has also been considered a key regulator in mineralized matrix formation (140); it is, however, expressed in a number of non-mineralized tissues (134). Interestingly, because the extracellular matrix of bone contains DMP1 fragments, the proteolysis of DMP1 has been supposed to play an important role during dentinogenesis and osteogenesis (212).

## 5. THE INORGANIC PHASE OF BONE

Most of the physiological functions of bone are due to the inorganic substance that permeates the components of the organic matrix. The hardness and strength of the tissue, its resistance to mechanical forces, and its protective effect on soft tissues all depend on the amount of inorganic substance whose abnormalities inevitably induce osteopathies of various types. An understanding of the properties of the inorganic substance and of the mechanism of its deposition are essential to a knowledge of the physiology and pathology of bone.

### 5.1. Composition

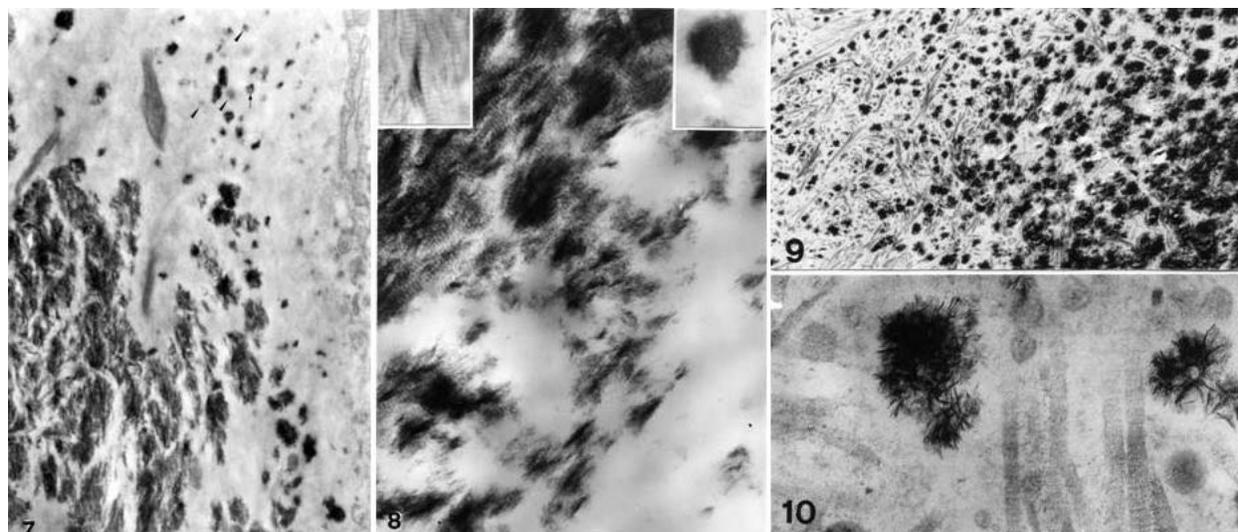
It was pioneering X-ray diffraction studies that led to a conception of the inorganic phase of compact bone as a polycrystalline hydroxyapatite (213), an inference later confirmed several times (reviewed by Posner, 214; Glimcher, 215): the bone mineral was considered to have the formula  $\text{Ca}_5(\text{PO}_4)_3\text{OH}$  and to consist of a rhombic unit cell with an *a*-axis of 943 nm and a *c*-axis of 688 nm. Actually, besides calcium and phosphorus, the composition of bone apatite includes several other ions, above all carbonate, which accounts for about 5% of the weight of the bone ash (216). On the other hand, the Ca/P molar ratio is not that of natural hydroxyapatite (equal to 1.67) but ranges from 1.57 to 1.71 (217) and changes with mineral "maturation", increasing from 1.35 in early calcification nodules to 1.60 in fully calcified areas according to Wergedal and Baylink (218), or from 1.60-1.70 to 1.81-1.97, respectively, according to Landis and Glimcher (219). Several constituent ions may be substituted in the crystal lattice of bone apatite by other elements (214) and the values of the *a* and *c* axes, as well as the *a/c* ratio, may change accordingly (220).

In spite of a great number of investigations, the composition of the inorganic bone substance, although rather close to that of hydroxyapatite, is still an unresolved issue. The problem is further complicated by the possibility that the formation of hydroxyapatite is preceded by that of a precursor phase. In this connection, great attention has been addressed, in spite of the problem of great instability, to amorphous calcium phosphate in vertebrates (221) and amorphous calcium carbonate in lower organisms (222). Both have been found *in vivo* in areas of early mineralization, where they have been reported as amorphous or fine granular, electron-dense material (discussed by Bonucci, 2). Both are unstable and perhaps stabilized by specialized organic molecules (discussed below, p. 00).

### 5.2. Organization

A number of studies have been dedicated to determining the morphology, dimensions and organization of the inorganic substance in bone. These parameters are, in fact, not simply descriptive, but may well turn out to be fundamental to an understanding of the process that leads to their formation and to matrix calcification. The electron microscope examination of the inorganic substance in the fully mineralized areas of bone shows two distinct, although

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**Figure 2.** A. Detail of calcification front: the degree of calcification increases going from right to left, as isolated calcification nodules increase in size and coalesce. Arrowheads point to some uncalcified matrix vesicles, the arrow points to a matrix vesicles undergoing mineralization. Part of an osteoblast on right. Unstained, x 70,000. B. Detail of calcification front: degree of mineralization increasing from the lower right to the upper left corner. Note that the inorganic substance forms granular, electron-dense bands, and strengthens the collagen periodic binding, only where the degree of mineralization is high and the calcification nodules have coalesced. The area with low mineralization only shows calcification islands (one shown at the upper left corner) and calcification nodules (one shown at the upper right corner). Unstained, x 90,000. C. Medullary bone of pigeons: the wide calcification front consists of numbers of calcification nodules that increase in size and coalesce (right) as the degree of mineralization increases. Uranyl acetate and lead citrate, x 7,500. D. Detail of calcification nodules and uncalcified matrix vesicles at the calcification front, where a few uncalcified collagen fibrils are evident too. Uranyl acetate and lead citrate, x 110,000.

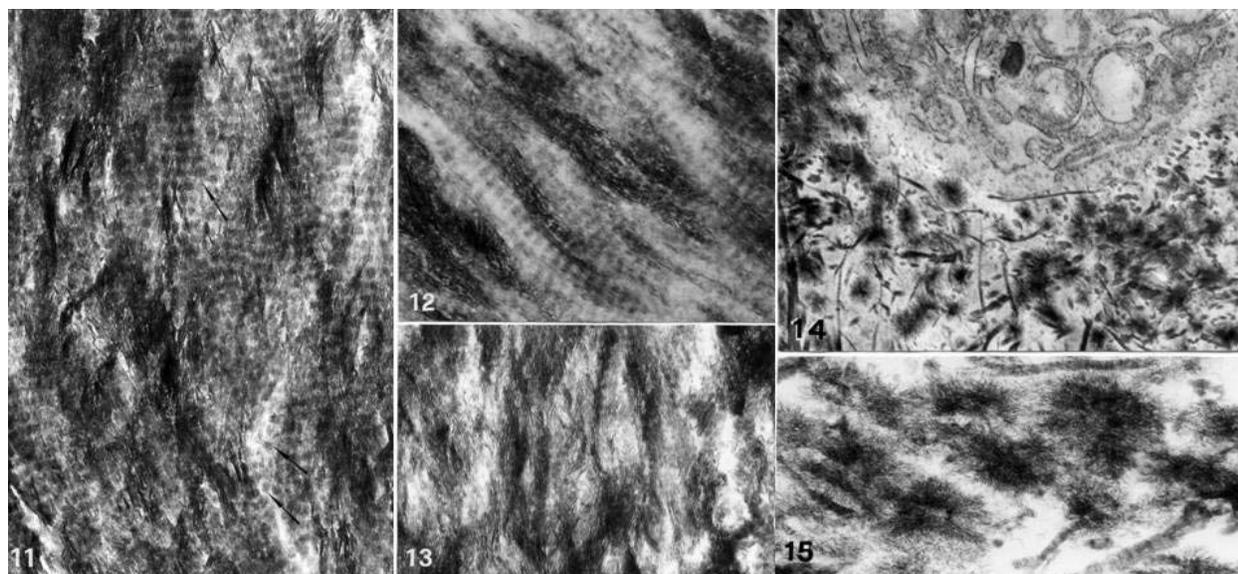
Studies that aim to establish how the inorganic substance in bone is organized and structured must take into consideration its appearance not only at sites where the calcification process has reached completion, but, above all, at those where it is only at an early or intermediate phases of development. The early deposition of inorganic substance occurs at the calcification front, that is, at the limit between the uncalcified collagen fibrils of the osteoid tissue and the calcified matrix (Figure 2A). The prevalent picture found under the electron microscope at this site corresponds to roundish or elongated aggregates of filament-like crystals that are called “calcification nodules” and “calcification islands”, respectively (Figs. 2A-D). The former are located in the interfibrillar spaces; they are of variable size; their constituent crystallites increase in numbers with the degree of calcification, so that they eventually coalesce. The calcification islands are elongated in shape, with their constituent crystallites often running parallel to the axis of the collagen fibrils, so that their formation seems to be related to the fibril surface.

Besides calcification nodules and islands, roundish bodies, 25-250 nm in diameter and consisting of an amorphous, osmiophilic matrix surrounded by a membrane, are recognizable at the calcification front (Figs. 2A, 2D). They correspond to matrix vesicles, and a description of them can be found in dedicated reviews (2,223-227), as well as in a chapter of this volume. Many of

them can be found in the osteoid tissue and in the calcification front of bones that, like woven bone, have a loose collagen texture, whereas relatively few can be traced in compact bone. Many of them contain the earliest aggregates of filament-like crystals. As these increase in numbers, they fill the vesicles, disrupt the vesicle membrane and spread into the surrounding matrix, so forming calcification nodules. As a result, these predominate at the calcification front of bones that present a loose texture of collagen fibrils.

Besides the filament-like crystals collected in calcification nodules and islands, the inorganic substance in bone gives rise to another characteristic electron microscope picture consisting of granular, electron-dense bands. These have a characteristic relationship with the collagen fibrils – they run transversally through adjacent, side-by-side aggregated collagen fibrils and seem to accentuate their periodic pattern (Figure 3A). Several Authors (for instance, 21, 219) have considered the electron-dense bands as the first step in the mineralization process of bone. In reality, they constitute an early event in that process, but are not recognizable in areas showing the onset of calcification, where the calcification nodules predominate: they become evident, and their relationships with collagen periodic banding become clear, a little later, as soon as the degree of calcification induces the earliest coalescence of the calcification nodules; here the collagen

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**Figure 3.** A. Secondary bone, showing the typical granular, electron-dense bands that characterize the incompletely mineralized bone matrix. The arrows point to areas where the bands seem to have been disrupted and to have given rise to isolated platelets. Unstained, x 95,000. B. Secondary osteonic bone at an early degree of mineralization: the granular, electron-dense bands are evident (compare with fig13.jpg). Unstained, x 85,000. C. Secondary osteonic bone at a high degree of mineralization: the granular, electron-dense bands are masked by numerous filament-like crystallites. Compare with fig12.jpg. Unstained, x 80,000. D. Bone calcification front in a section treated with the PEDS method: aggregates of crystal ghosts have replaced the calcification nodules; the former are so similar to the latter that the section seems undecalcified. An osteoblast partly visible above. PEDS: Formic acid decalcification, uranyl acetate and lead citrate staining, x 30,000. E. Details of aggregates of crystal ghosts. PEDS: Formic acid decalcification, uranyl acetate and lead citrate staining, x 75,000.

calcified osteons and can be recognized as having been almost completely obliterated when calcification nears completion (228 and Figs. 3B-C).

From its onset to its completion, the calcification process seems to present the following sequence of events: first of all, matrix vesicles are produced by osteoblasts; secondly, calcification nodules develop in vesicles simultaneously with the formation of calcification islands, either in the vesicles and/or on the surface of collagen fibrils; nodules and islands then enlarge, lose their outline and coalesce; collagen fibrils aggregate with one another and electron-dense bands become apparent; the filament-like crystals of calcification nodules and islands increase in number, gather in strips and take on the same orientation as the collagen fibrils through which they are scattered; at this point, the electron-dense bands become less and less obvious.

These electron microscope observations, which point to the existence of two types of mineral structures in bone – filament-like crystals and electron-dense bands –, have been repeatedly confirmed using different methods of preparation (reviewed by 2). It cannot be neglected, though, that several reports support the view that bone crystals have a platelet-like shape and that the filament-like crystals are no more than platelets seen from one side. Even if this view has not been substantiated under the electron microscope, which shows that in well-preserved preparations the platelet-like figures are extremely rare with respect to the

high numbers of filament-like structures, the platelet-like shape of the bone crystals has been supported by studies relying on ultracryomicrotomy (229) and by stereoscopic analysis using goniometers that make it feasible to examine single crystals from different angles (230-231). These, and other studies that support the same view (232-236), have been mostly carried out on crystals that had been isolated from bone by means of mechanical and chemical methods, all of which have the potential to induce changes in crystal shape and structure. It should be borne in mind that the bone crystals are nanostructures whose physical state can be easily altered, especially when they are removed from their biological environment. In reviewing the problem of crystal morphology, Bonucci (2) pointed out that in intact, well-preserved bone specimens the filament-like crystals and the electron-dense bands are recognizable under the electron microscope much more frequently than the platelet-like crystals, which are mostly restricted to damaged areas (Figure 3A) and to the margins of fractures. He also showed that the single bone crystallites dissociated by the activity of the osteoclasts, that is, by a physiological process, probably less dangerous than the physical or chemical *in vitro* methods, have a filament- and needle-like ultrastructural shape. Bonucci (2) suggested that the structures believed to be platelet-like crystals might be no more than fragments of the granular, electron-dense bands rather than true crystals. This suggestion is in agreement with the findings of a goniometric study carried out on whole bone by Lees *et al.* (237), who showed that most of the platelet-like crystals were intrafibrillar and that only a

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few of the interfibrillar filament-like crystals turned into a platelet-like configuration under the goniometer. It also agrees with the results of a study carried out by atomic force microscopy on crystals isolated by non-aqueous methods at a low temperature, which showed structures, named 'mineralites', that display poor stoichiometry and a low degree of crystallinity due to their being only one or two unit cells thick, that is, with values  $(0.61 \pm 0.19 \times 10 \pm 2 \times 12 \pm 2 \text{ nm})$  in mature bovine bone and  $2 \times 6 \times 9 \text{ nm}$  in young post-natal bovine bone) too small to allow them to be classified as crystals, and such that they are able to fit into the 'channels' formed by laterally adjacent hole zones across the collagen fibrils (238,239). The truth is that the dimensions of the bone crystals can hardly even be measured (discussed by Bonucci, 2). The values reported in the literature, drawn from X-ray or electron diffractograms or from direct measurement under the electron microscope of whole vertebrate bone, are exceedingly variable and can only be taken as approximations. This is especially true of crystal length whose reported values range from a minimum of 17 nm to a maximum of over 350 nm.

The doubts about the conformation of the bone inorganic nanoparticles extend to their crystalline organization. The first indication that inorganic substance in bone is polycrystalline came from early polarized light microscopy and X-ray diffraction studies; this has been followed up by a number of confirmations (reviewed by Posner, 214; Glimcher, 215). It therefore became habitual to designate the inorganic particles of bone as hydroxyapatite "crystals" or "crystallites". With reference to developing bone salt, however, Arnott and Pautard (240) made the objection that it was usually referred to as a "crystal" without proof that any portion of it is specifically crystalline. Posner *et al.* (241), on the other hand, had shown that the bone crystals change in size and composition with age, a finding later confirmed by Burnell *et al.* (242). Landis and Glimcher (219), using electron microscopy and electron diffraction, found that, while the older regions of bone show diffractograms indicative of poorly crystalline hydroxyapatite, the early inorganic deposits do not provide any electron diffraction pattern of a specific calcium phosphate solid phase, a property that is only acquired with their maturation. Wheeler and Lewis (243) reported on the paracrystalline nature of bone apatite, and Arnold *et al.* (244), using energy-filtering transmission electron microscopy in the selected area electron diffraction mode, observed that primary crystallites have an apatite-like structure but show crystal lattice distortions representing an intermediate state between amorphous and fully crystalline, that the early-formed crystallites have a paracrystalline character comparable to biopolymers, and that the lattice fluctuations of the crystallites decrease with maturation. These findings are fundamental in gaining an understanding of the mechanism of bone calcification, as reported below (p. 00).

The amorphous or poorly crystalline organization of the early inorganic structures in bone could not be appreciated using X-ray diffraction, because this method of analysis can only be carried out on the bulk of the calcified matrix of relatively wide bone specimens, hence,

predominantly on fully calcified bone. The small, comparatively rare areas of initial or incomplete calcification could only be appreciated after the electron microscope made it possible to examine different phases of the calcification process, from its beginning to its completion. It then became clear that, in agreement with the results reported above for the Ca/P ratio, bone mineral undergoes a process of transformation from an initial, non-crystalline phase (characterized by electron diffractograms of amorphous type), through a poorly crystalline phase to a final, apatite-like, almost crystalline phase. It is obvious, therefore, that the terms "crystal" and "crystallite" are not fully adequate and cannot be taken as having the meaning they have in mineralogy. They have, however, been retained in the following pages from force of habit.

An important problem concerns the relationships, if any, between the filament-like crystals and the electron-dense bands and whether the former might grow from nuclei present in the latter. This possibility seems to be in line with observations on developing osteons: as reported above (p. 00), osteon calcification occurs in two stages: first a rapid phase of calcification, amounting to about 70% of the total, and then a second slower phase that leads calcification to completion. The amounts of filament-like crystals seem to increase during the slow phase, whereas those of the electron-dense bands seem to fall proportionally (228), as if the former derived from the latter. Actually, the same effect could be due to an independent increase in the numbers of filament-like crystals, which could progressively mask the electron-dense bands. Moreover, the intrafibrillar formation of filament-like crystals finds an insurmountable obstacle in the fibril structure itself, as discussed below (p. 00). The available findings do not support the view that the filament-like crystals might derive from nuclei of the electron-dense bands, and point to the existence in bone of two different and independent aggregation states of the inorganic substance, the first consisting of filament-like, predominantly interfibrillar crystals, the second of electron-dense, predominantly intrafibrillar granular bands. The study of these organic-inorganic relationships could contribute a great deal to a full understanding of the mechanism of bone calcification.

## 6. THE ORGANIC-INORGANIC RELATIONSHIP IN BONE

The organic-inorganic relationships in bone can be studied using various methods. Of these, the electron microscope ones might appear at first sight have unique advantages, because they should lead to a direct visualization of the inorganic and organic components, and of their ultrastructural relationships, in the intact bone matrix. The reality is more complex: the inorganic crystals are electron-dense and mask the organic components of the calcified matrix they are directly associated with, so that the organic-inorganic relationships are hard to detect, except for the granular, electron-dense bands that are clearly related to the collagen periodic banding. That is why the organic structures of the bone matrix can only be studied morphologically after decalcification of the tissue.

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As discussed in detail by Bonucci (2), the usual decalcification procedure, consisting in soaking the bone specimen in a decalcifying solution, causes not only the solubilization of the inorganic substance, but also of an unpredictable number of organic components. This can deeply change the ultrastructure of the matrix and is a cause of morphological artifacts: the collagen fibers are more or less dissociated, no, or very few, interfibrillar materials are recognizable, and the previously calcified areas appear to be almost empty. These artifacts can be avoided by using the Post-Embedding Decalcification and Staining (PEDS) method: the tissue is decalcified after it has been embedded in a resin that, without preventing the solubilization of the inorganic ions, immobilizes the organic molecules, so reducing their extraction to a minimum, and permitting their 'staining' with heavy metals (28,245). The discussion that follows on the organic-inorganic relationships in bone is mainly based on the results obtained using the PEDS method.

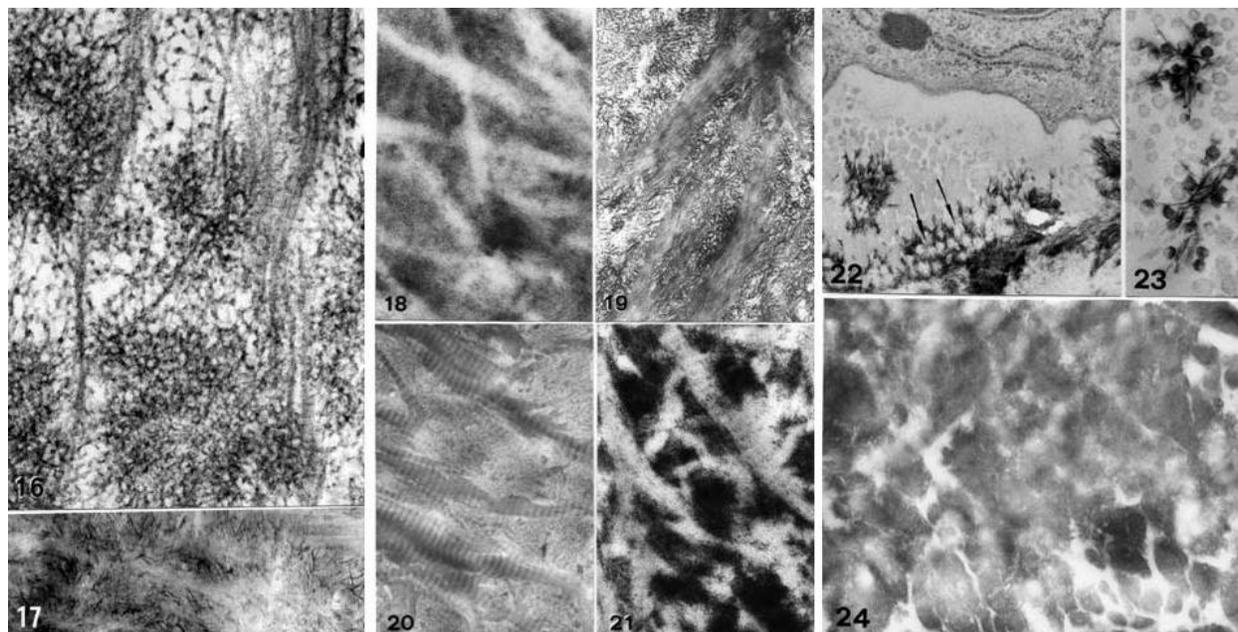
As reported above, the inorganic substance in bone shows two prevalent patterns: filament-like crystals and electron-dense bands. These patterns persist during calcification, but their reciprocal frequency changes with the type of bone, the site in the bone matrix, and, above all, the phase of the calcification process and the degree of calcification. As a result, their relationship with the organic components of the matrix changes too.

The first filament-like crystals appear in bone with the formation of the calcification nodules and the calcification islands that together make up the calcification front in the osteoid tissue. Most of these crystal aggregates form in matrix vesicles and are, therefore, contained in interfibrillar spaces. If the decalcification procedure is carried out before embedding, they appear as empty interfibrillar spaces. By contrast, after implementing the PEDS method, they appear as aggregates of filament-like structures that have an ultrastructural morphology very similar to that of the untreated crystals, the main difference being that the latter are intrinsically electron-dense and can be seen under the electron microscope even if unstained, whereas the former have no electron-density and are visible under the electron microscope only after they have reacted with heavy metals (Figs. 3D-E). This explains why they have been called "crystal ghosts" (245; reviewed by 2,209,210). Aggregates of crystal ghosts are easily recognizable in place of small calcification nodules and calcification islands. As these enlarge with the rising degree of calcification, their central, fully calcified zone, after applying the PEDS method, takes on a granular appearance and the crystal ghosts become poorly recognizable or not recognizable at all, although they can still be made out at the periphery of the aggregates as filament-, crystal-like structures protruding into the surrounding matrix.

As reviewed by Bonucci (2,209,210), crystal ghosts are found not only in the calcification zones of bone, but also in those of cartilage, dentin, enamel and other vertebrate hard tissues. It might be thought that they are artifacts produced by the PEDS method. They can,

however, be visualized under the electron microscope using other methods of preparation. An *in situ* method has been used by Prostack and Lees (246): they first selected and photographed under the electron microscope specific areas of calcification in bone and tendons, then decalcified the sections on the grids with a drop of 1N HCl, and lastly re-photographed the selected areas after staining with vanadyl sulfate; they found a one to one correspondence between the inorganic crystals and their demineralized crystal "ghosts". Sections from epoxy resin- or glycolmethacrylate-embedded tissues can be treated with phosphotungstic acid (PTA) at very low pH, which at the same time decalcifies and stains, while revealing polysaccharides and glycoproteins (247-249). This method yields results very similar to those obtained with the PEDS method and give a first indication that the crystal ghosts have a glycidic component. Another method, based on the stabilization of acidic molecules with cationic dyes, consists in treating the bone tissue with cationic substances (250) and then decalcifying it before embedding in a resin (251). This system, which eliminates any possible artifacts that might be due to the embedding resin in the PEDS method, shows aggregates of organic crystal-like structures, reminiscent of crystal ghosts, in place of calcification nodules (209, 252 and Figure 4A; this figure refers to calcifying cartilage, in which the organic crystal-like structures and their relationships with the acid proteoglycans of the matrix are specially obvious). The stabilization of organic crystal-like structures by the treatment with cations is, by the way, an indication that they are acidic structures. All these results suggest that these crystal ghosts comprise acid proteoglycans, a view supported, under the light microscope, by the positive reaction of the areas of initial calcification with Alcian blue or colloidal iron at acidic pH and by the methylation-saponification reaction, and, under the electron microscope, by their staining with colloidal thorium dioxide, ruthenium red (Figure 4B) or cuproinic blue and, to some extent, by their ultrastructural morphology, which shows a transition between crystal ghosts and the granular structures formed by collapsed proteoglycans (252 and Figure 4A).

The granular, electron-dense bands represent the other ultrastructural picture that distinguishes calcification of the bone matrix. They are contained in the hole zones of the collagen fibrils; they become fewer and tend to disappear as calcification proceeds. These results, and those reported by Lees *et al.* (237), support the conclusion that in compact bone the inorganic substance shows two different ultrastructural patterns: first, filament-like crystals, which are formed in matrix vesicles, are initially collected in interfibrillar calcification nodules and calcification islands, are later located between, and more or less parallel to, the bundles of collagen fibrils, and are closely related to organic, crystal-like, acidic structures (crystal ghosts), so that they can be considered organic-inorganic hybrids; second, granular, electron-dense bands, contained in the collagen fibrils and located in their hole zones. These inorganic patterns appear to be mutually independent, as discussed in the following chapter.



**Figure 4.** A. Section of calcifying epiphyseal cartilage fixed in the presence of acridine orange, decalcified with EDTA, and embedded in Araldite. The calcification nodules are replaced by roundish aggregates of filamentous structures that on one hand are similar to the crystal ghosts shown by the PEDS method, on the other are in contact with, and to some extent resemble, the granular structures which in the uncalcified matrix correspond to collapsed proteoglycans. X 160,000. B. Area of initial calcification in trabecular bone: the crystal ghosts are stained by ruthenium red, showing that they are acidic structures. PEDS, ruthenium red, x 60,000. C. Trabecular bone of chick embryo: the crystallites occupy the spaces between the collagen fibrils, which appear as empty (unmineralized) stripes. Unstained. x 90,000. D. Trabecular bone of chick embryo: staining with uranyl acetate and lead citrate confirms that most of the crystals are contained between the collagen fibrils, which are almost unmineralized. x 105,000. E. Trabecular bone of chick embryo stained *en bloc* with aqueous, acidic phosphotungstic acid, which at the same time decalcifies and stains: the crystals are replaced by filament-like structures that are reminiscent of crystal ghosts and are located between collagen fibrils, some of which show lateral aggregation. x 60,000. F. Trabecular bone of chick embryo stained *en bloc* with alcoholic-phosphotungstic acid, which decalcifies and mainly stains proteins: the picture is similar to that shown in Figure 4E but the electron density is much greater; it is also similar to the picture shown in Figure 4C. x 78,000. G. Detail of an area of initial mineralization (arrows) showing cross sectioned collagen fibrils appearing as roundish empty spaces surrounded by mineral substance. An osteoblast partly visible above. Uranyl acetate and lead citrate, x 60,000. H. Area of initial mineralization: filament like crystals join, and run between, cross-sectioned, collagen fibrils some of which are electron-dense and appear to be calcified. Uranyl acetate and lead citrate, x 70,000. I. Compact bone decalcified and stained with acidic phosphotungstic acid (PEDS method): the cross-sectioned collagen fibrils are homogeneous and do not show any space where crystallites could be allocated (see also Figure 1E). x 120,000.

## 7. THE MECHANISM(S) OF BONE CALCIFICATION

Among the theories put forward to explain the mechanism of bone calcification, the one proposed by Glimcher (253) received almost universal consent. Chiefly on the basis of the close ultrastructural relationships between collagen periodic banding and the inorganic substance, which give rise to the “electron-dense bands” described above, Glimcher suggested that the mineral substance is contained in the hole zones of the fibrils, where atomic groups of the collagen molecules are arranged in such a way as to allow the heterogeneous nucleation of inorganic nuclei, and that the definitive crystals arise from these nuclei through the addition to them of further inorganic ions (reviewed by Glimcher and Krane, 21). This theory was supported not only by the

ultrastructural finding that the granular, electron-dense bands are in relationship with the collagen period and are contained in the hole zones, but also by the observation that they represent a precocious phase of calcification, that they characterize the low degree of calcification of the bone matrix, and that they become less and less evident as the degree of calcification rises and numbers of filament-like crystals rise too. These concepts were widely accepted even though controversies developed around the idea that the electron-dense bands represent the very earliest phase of calcification and, above all, that inorganic nuclei pertaining to them can develop into crystals within the limited space of the hole zones without disrupting the molecular organization of fibrils.

First, as discussed above (p. 000), the earliest phase of the calcification process in bone does not consist

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in the formation of the electron-dense bands, which are not recognizable either in relationship to the loose, irregularly woven collagen fibrils of the osteoid border or at the calcification front. Only aggregates of filament-like crystals (calcification nodules and calcification islands) are found at these sites. The granular, electron-dense bands first appear some distance away, where the collagen fibrils begin to aggregate in parallel bundles (Figure 2B). Second, although the final size of the filament-like crystals is poorly known (see above), at least their length is long enough to span both the hole and the overlap zones of the fibrils (228,254); it follows that they are longer than the collagen holes, and their thickness must be greater than the intermolecular spacing of collagen molecules (see also Lees and Probst, 255). Consequently, if their development originated from nuclei belonging to the electron-dense bands, they would inevitably disrupt the walls of the holes within which they would have to grow and would break the fibril intermolecular links to reach their final dimensions. The solubility of the bone matrix would increase as a result, whereas it actually falls during calcification, to the point that bone collagen is essentially insoluble in reagents such as NaCl and acetic acid under conditions that solubilize the collagen from a wide variety of soft tissues (31).

To overcome these discrepancies, it has been suggested that the crystals might be accommodated, without disrupting the fibril structure, in pre-existing pores, channels and grooves resulting from the confluence of contiguous holes (23,26,27,256-258). In this case, however, the filament-like crystals should have a transversal course with respect to the fibrils axis, as the electron-dense bands have, whereas they prevalently appear to run parallel to them. The obvious conclusion seems to be that the filament-like crystals and the electron-dense bands develop separately and independently, and that their shape depends on the space in which they develop. There are no doubts, in any case, that a large proportion of crystals are located in the interfibrillar spaces (see also Lees *et al.*, 237; Lees and Probst, 255), although their percentage changes with the type of bone, being the highest in the medullary bone of birds, but very high in woven bone, too (32, and Figs. 4C-F). Using acoustic microscopy, Pidaparti *et al.* (259) have calculated that about 75% of mineral crystals reside outside the collagen fibrils in osteons, a percentage perhaps excessive for a tissue in which the collagen fibrils are rather compact. Rough measurements carried out in areas like that shown in Figure 3B (calcification front of osteonic bone) give values of about 37% occupied by filament-like, interfibrillar crystals and 48% by electron-dense bands, being the rest still uncalcified; similar measurements in trabecular bone of chick embryo (like those shown in Figure 4E and Figure 4F) give values of about 65% occupied by filament-like crystals. The conclusion that these crystals are located between and on the collagen fibrils and are in relationship with non-collagenous material is supported not only by electron microscope findings showing that a lot of them are located in the interfibrillar spaces (Figs. 4C-D) and that these contain non-collagenous material that reacts strongly with alcoholic PTA (hence, it is proteic; Figure 4F) and corresponds to crystal ghosts stained by aqueous PTA (Figure 4E), but also by the

observation that in areas of initial or incomplete calcification the inorganic substance is often located on the surface of still uncalcified collagen fibrils, so that these appear in cross-section as empty spaces outlined by electron-dense rings (Figure 4G), that filament-like crystals, which are clearly located in the interfibrillar space, sometimes join adjacent calcified and uncalcified collagen fibrils (Figure 4H), and that cross-sections of calcified collagen fibrils, decalcified and stained by acidic PTA, appear homogeneous and do not show the empty spaces where the removed crystals would have had been located (Figure 4I).

The findings reported above strongly suggest that two different, not necessarily interdependent, mineral phases do exist in bone, one consisting of the filament-like crystals located in the interfibrillar spaces, the other consisting of the granular, electron-dense bands contained in the hole zones of the collagen fibrils. What remains to be understood is whether they have the same or different mechanism(s) of formation.

If the filament-like crystals are located in the interfibrillar spaces, as they are, they must necessarily develop in relation to non-collagenous components of the matrix. Provided that suitable methods for identifying them are used (for instance, the PEDS method), these components can in fact be shown in areas of early calcification – i.e., calcification nodules and calcification islands – as filament- and crystal-like, acidic structures called “crystal ghosts” (245). The very close similarity between crystal ghosts and untreated crystals strongly suggests that the former may be templates for the formation of the latter. In this case the filament-like crystals would not develop through a process of heterogeneous nucleation but through an epitaxial mechanism that includes a chemical reaction between inorganic cations and acidic groups of templates. These would become to be more or less completely occluded by the inorganic ions, so leading to the formation of organic-inorganic hybrids (discussed by Bonucci, 2,209,210). The filament-like shape of the crystals would simply reflect the shape of their organic ghosts. This arrangement would explain why, as reported above (p. 000), the early inorganic deposits provide an amorphous electron diffraction pattern, have a paracrystalline character and show crystal lattice distortion.

An inorganic-organic reaction might also be responsible for the formation of the electron-dense bands. As reported above, the mineral substance that goes to make up these bands is contained in the hole zones of the collagen fibrils; the bands themselves are an outcome of the alignment of the hole zones consequent on the parallel aggregation of the collagen fibrils. After decalcification according to the PEDS method, the treatment with aqueous phosphotungstic acids shows lightly stained bands of organic material that replace – while showing the same location and morphology as – the electron-dense bands seen in undecalcified sections (Figure 4E). It seems that the inorganic material that gives rise to the electron-dense bands simply reacts with, and repeats the ultrastructural pattern of, an organic material pre-existing in the collagen

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holes. Veis (18) had already proposed a similar concept, suggesting that a fine filamentous organic material contained in the fibril compartments could react with, and could be occluded by, the inorganic material as this gradually fills the available space. The nature of this material is not known. As mentioned above, a number of phosphoproteins are covalently linked to, and are intrinsic components of, the collagen fibrils. It is noteworthy that reconstituted collagen fibrils did not induce mineral formation, whereas crystals were formed when acidic proteins (phosphoproteins) were added to the fibrils (260). Reconstituted collagen fibrils in the presence of poly-L-aspartic acid induced the intrafibrillar deposition of ribbon-shaped apatite crystals with their *c*-axis co-aligned with the fibril axis, closely resembling the bone mineralization pattern (261). Another point of interest is that decorin is localized near the *d* and *e* bands of the collagen fibrils and that its amount decreases with calcification.

The organic-inorganic relationships in bone need to be better known to allow a complete understanding of how the early calcification process occurs. In particular, knowledge of the composition of the crystal ghosts is mandatory. They are acidic molecules and contain sulfate groups, as if they pertain to acid proteoglycans. The possible involvement of these molecules, either as promoters or as inhibitors of inorganic substance deposition, has long been suggested (262,263). Other acidic substances, however, are located in the early calcification nodules and calcification islands, first of all the bone sialoprotein which, as reported above (p. 000), in association with collagen, promotes apatite nucleation (114) and is a protein that is glycosylated, sulfated, phosphorylated and rich in sialic acid protein. The BAG-75, a biomarker of the areas of initial calcification (264), is another sialic acid-rich phosphoglycoprotein. The role of the collagen-phosphoprotein complexes in inducing the calcification of bone and other hard tissues has been repeatedly emphasized (260,264-269). In this connection, it is remarkable that acidic amino acids and acidic proteins, if adsorbed or immobilized on matrix structures, become hydroxyapatite nucleation sites and regulate the crystal growth (270-273). The role of aspartic acid-rich proteins in inducing and regulating the biomineralization process in a number of calcifying tissues has been often reported (274-280).

The calcification process is not limited to the formation of the early organic-inorganic hybrids, improperly called crystals, but includes their 'maturation' into nanostructures having a definitely crystalline character (discussed by Bonucci, 2,209). As calcification increases, in fact, the amorphous diffractograms of the early mineral aggregates turn crystalline (219) and the lattice fluctuations disappear (244). The acquisition of a quasi-perfect hydroxyapatite reticulum seems to occur through the loss of components of the organic matrix, a process very well documented in enamel (see Nanci, 281), but also demonstrated in bone and other hard tissues, at least as far as acid proteoglycans are concerned (see above, p. 000). This loss, which probably has the dual aim of permitting further inorganic accretion and of creating space available

to the growing crystals, is also documented under the electron microscope by the progressive disappearance of crystal ghosts in the central zones of the enlarging and coalescing calcification nodules (Bonucci, 2,209,210).

## 8. CONCLUSIONS AND PERSPECTIVES

The calcification of bone, as well as that of other hard tissues, is under the control and regulation of the organic matrix. This comprises two sets of components, one whose main function is that of support, and another that is directly engaged in the calcification process. The former can be identified in the collagen fibrils, whose arrangement not only distinguishes the various types of bone and their architecture, but is also responsible for their mechanical properties; the second is located in the interfibrillar spaces, but is also an interfibrillar component, and consists of various non-collagenous, mostly acidic molecules. In close relation to these matrix components, two ultrastructural patterns characterize the inorganic substance within bone: electron-dense bands and filament-like crystals. The former are due to the deposition of inorganic substance in the holes of the collagen fibrils, so that their amount is directly proportional to the compactness of the fibrils; the latter are located in the interfibrillar spaces, give rise to calcification nodules and calcification islands at the calcification front, and develop in connection with non-collagenous molecules. Even if their structure, composition, organization and specific function in calcification are not yet fully understood, most of these molecules are acidic, which allows them to sequester calcium ions and play a template function, so leading to the epitaxial formation of structures that, improperly called crystals, are organic-inorganic hybrids whose filament-like shape repeats that of the template. These hybrids only acquire a quasi-perfect hydroxyapatite structure with a maturation process, which implies the almost complete removal of their organic constituent. The different phases of this process and its mechanism are among the most important topics to be faced by future investigations. Another urgent need is that of establishing whether the electron-dense bands and filament-like crystals are truly different structures formed through the same basic process, and if their shape and organization depend in the first case on those of the collagen holes in which they form, in the second case on those of the interfibrillar, filament-like, non-collagenous molecules that behave as their templates.

The concepts outlined above may need to be improved and corrected, but they do offer an explanation for the available findings and represent an attempt to identify a mineralization mechanism that may be valid for every hard tissue. Several pinnacles must probably still be climbed, but the summit does not seem too far away.

## 9. ACKNOWLEDGEMENTS

The Author is indebted to all his co-workers for their continuous support and technical assistance. The more personal studies have been supported by grants from the "La Sapienza" University of Rome, the

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Italian Ministry of Education and the Italian National Research Council.

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**Abbreviations:** AEBSF: 4-(2-aminoethyl)benzenesulfonylfluoride HCl, AHSg: a2-Heremans-Schmid Glycoprotein, Fetuin, ASARM: Acidic Serine-Aspartate-Rich MEPE-associated motif, BAG-75: Bone Acidic Glycoprotein-75, BGP: Bone Gla-Protein, BMP: Bone Morphogenetic Protein, BSP: Bone Sialoprotein, DMP1: Dentin Matrix Protein 1, FGF23: Fibroblast Growth Factor 23, GAG: Glycosaminoglycans, Gla: Carboxyglutamic acid, GRP: Gla-Rich Protein, HA: Hyaluronic Acid, MEPE: Matrix Extracellular Phosphoglycoprotein, MGP: Matrix Gla-Protein, OC: Osteocalcin, OF45: Osteoblast/osteocyte Factor 45, ON: Osteonectin, OPN: Osteopontin, PAS: Periodic Acid-Schiff, PC-1: Plasma Cell membrane Glycoprotein-1, PEDS: Post-Embedding Decalcification and Staining, PHEX: Phosphate-regulating gene with Homologies to Endopeptidases on the X chromosome, PTA: Phosphotungstic Acid, SPARC: Secreted Protein, Acid and Rich in Cysteine, SIBLING: Small Integrin-Binding Ligand, N-linked Glycoprotein, SLRP: Small Leucine-Rich Proteoglycans, TNSALP: Tissue Non-Specific Alkaline Phosphatase

**Key Words:** Bone; Bone Mineralization, Bone Matrix, Organic-Inorganic Relationships, Mineralization Mechanism, Review

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