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## Cartilage Calcification

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### 1. Introduction

Nineteenth century histologist Ranvier discovered the point of ossification and the calcification of cartilage as observed in embryos (Ranvier, 1875). By skillfully using simple methods such as hand-cutting with a razor, or with a lead-screw microtome of his own invention (still marketed as the Ranvier hand microtome), and chromic acid and carmine to stain, he observed the deposition of calcareous salts around cartilage capsules. Ranvier described chondrocytes and their distinctive arrangement in series to yield larger capsules. He observed how calcified capsules open into one another to form anfractuous cavities that become the earliest marrow spaces, and wondered what determines the resorption of the walls. After injecting Prussian blue to mark vessels in growing animals, he described wall destruction as proceeding selectively in the direction of vessel growth (Figure 1a). Soon after, Schäfer described how to prepare fresh or fixed cartilage sections, and recommended the use of osmic acid, silver nitrate and gold chloride (Schäfer, 1897). He then (Schäfer, 1907) published a series of four colour drawings comprising all the stages of ossification (Figure 1b); these drawings have been a source of inspiration for all later histology textbooks (see, for instance, Figure 1 c). Subsequent methodological advances in microscopy and microtomy allowed confirmation of all the early observations. In the first third of the twentieth century histology textbooks by Cajal, Bouin, Möllendorff, Maximow & Bloom, Cajal & Tello, Levi and Di Fiori, described endochondral ossification, the ossification centre, and the growth plate as we know them today. Interestingly, some doubts persisted about hypertrophic chondrocytes. Using silver reducing methods, Cajal and Tello described the well-developed Golgi apparatus of hypertrophic chondrocytes, and how it is reduced and fragmented in the last row of cells (Figure 1d), as well as the vacuolization of chondrocytes that makes these cells hypertrophic (Figure 1e).

The modern view has not changed, though many details have been added. Hypertrophic chondrocytes promote vascular invasion by producing a growth factor (Allerstorfer et al., 2010). They are no longer considered degenerating cells trapped within a calcified crust, but living, metabolically active cells (Farnum et al., 1990), as their environment is not hypoxic. The morphology and ultrastructure of hypertrophic chondrocytes are highly dependent on the methods used. With aqueous fixation procedures, chondrocytes appear shrunken. When the cartilage is processed by high-pressure freezing, freeze-substitution and low temperature embedding (Hunziker et al., 1984), followed by fixation in the presence of

cationic dyes such as ruthenium hexammine trichloride, its hypertrophic chondrocytes retain a configuration comprising intact membranes attached to the pericellular rim, intact organelles and mitochondria. These studies demonstrate that chondrocytes are fragile cells, and that their participation in the mineralization process requires their functional viability.

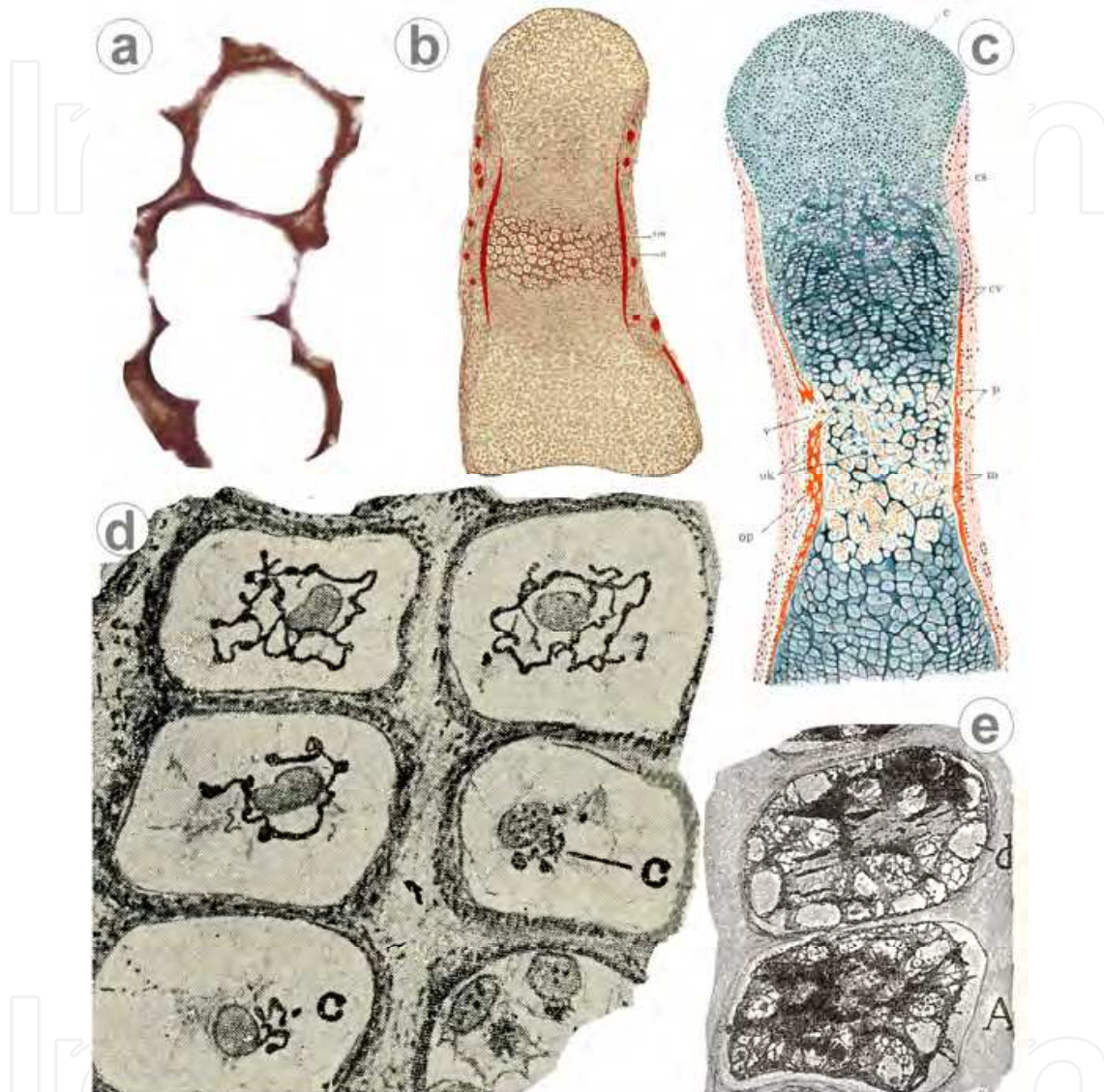


Fig. 1. (a) Ranvier's observation on the aperture of calcified capsules; (b) initial cartilage calcification in an embryo cartilage model stained with magenta spirit (from Schäfer); (c) primary ossification centre (from Möllendorff); (d) Golgi apparatus in hypertrophic chondrocytes (from Cajal & Tello); (e) vacuolization in hypertrophic chondrocytes (from Cajal & Tello).

The fate of hypertrophic chondrocytes is still a controversial issue. In many instances, especially in embryonic cartilage, they survive and dedifferentiate into osteoblasts (revised by Hall, 2005). However, other evolutions are possible, because the last row of cells is usually apoptotic. By eliminating cells, apoptosis establishes a dynamic equilibrium in the growth plate; it is not, anyway, considered to be directly involved in the mineralization process (Pourmand et al., 2007), but may interfere with it.

## 2. The chondrocyte event sequence for matrix calcification

Cartilage calcification is a regular and efficient process orchestrated by its chondrocytes, which go through a series of morphological/functional changes called the chondrocyte differentiation sequence, or, more properly, the mineralizing sequence. In histological sections treated with a general staining agent, such as toluidine blue, this sequence is easy to follow, since groups of chondrocytes show synchronous changes near the calcification zone (Figure 2a). The series comprises the proliferation, maturation, and hypertrophy zones. Staining sections with the von Kossa method allows the distribution of calcium deposits to be recognized (Figure 2b).

Mammalian and avian growth cartilages have been the subject of numerous studies as a model for cartilage calcification. Histologically, they are similar types of cartilage, but differences are found with respect to the mineralizing sequence. In the (mammalian) growth plate cartilage, the sequence is shorter, and calcium deposits are only seen around the last rows of hypertrophic chondrocytes (usually 2-3 cells); conversely, in avian cartilage scores of chondrocytes surrounded by calcified deposits are easy to find. In the former, apoptosis is observed in the last row of chondrocytes (Figure 2c), whereas, in the latter, apoptotic chondrocytes are not found within the sequence, and apoptosis only appears close to the resorption limits. As a result, the mineralizing sequence in the mammalian growth plate is shorter than in avian cartilage because it is abruptly interrupted by apoptosis.

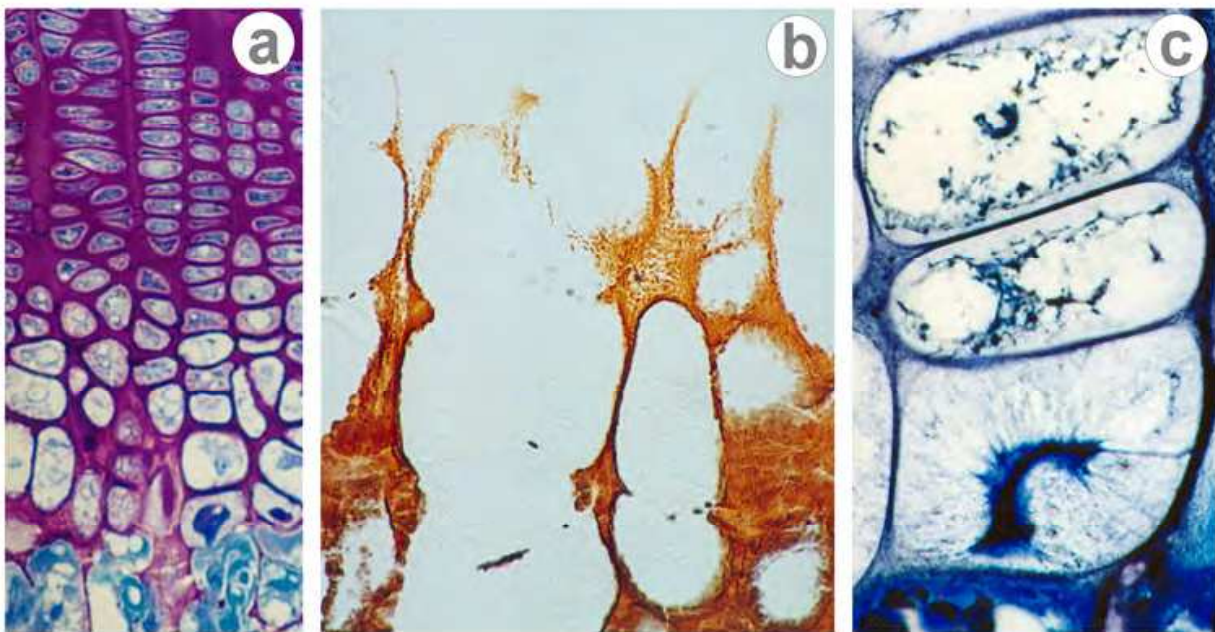


Fig. 2. Growth plate (a), mineral (b) and apoptosis in the last row of chondrocytes (c).

Whatever the type of cartilage, the best way of identifying the mineralizing potential of a given sequence is to stain its alkaline phosphatase and calcium-binding sites. Figure 3 shows the chondrocyte sequence in rat growth plate, as observed by LM methods for alkaline phosphatase and for calcium-binding sites. Figure 4 shows the results of the same LM methods, but applied to embryonic chick cartilage.

Staining the alkaline phosphatase (TNAP, tissue non-specific isoenzyme of alkaline phosphatase), using glycerophosphate or azo-dye methods, determines whether a cartilage is entering calcification, and signals the beginning of the sequence. The chondrocytes show staining of the plasma-membranes and of a thin rim of adjacent matrix. In both types of cartilage, the early maturation chondrocytes and the matrix surrounding them are TNAP-positive (Figure 3a-c; Figure 4a). This matrix is not yet calcified, and TNAP staining becomes negative wherever the matrix is calcified (Figure 3c).

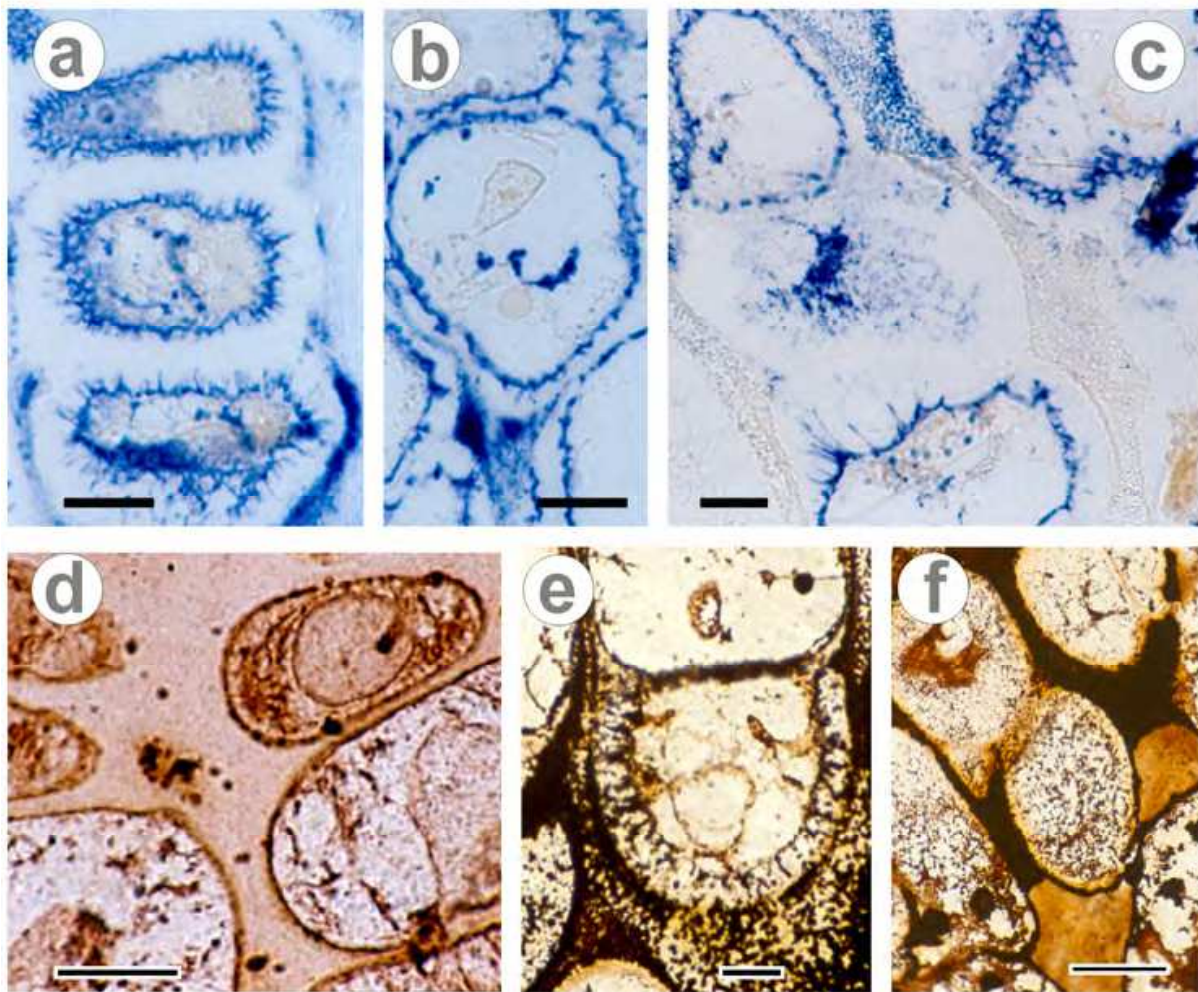


Fig. 3. TNAP (a-c); and calcium-binding staining (d-f) in rat growth plate. (Bar = 10  $\mu$ m).

Ultrastructurally, plenty of matrix vesicles (MVs; Anderson, 1967, 1969; Bonucci, 1967, 1970) are found in these zones. These are the TNAP-rich MVs first described by Matsuzawa and Anderson (1971) and later confirmed for both types of cartilage (Akisaka & Gay, 1985; Bonucci et al., 1992; Takagi & Toda, 1979; Takechi & Itakura, 1995 a, 1995b). When these TNAP-rich MVs are isolated and cultured in mineralizing solutions, they show mainly extravesicular apatite deposition (Boskey et al., 1994). They are considered to arise mostly from maturation and early hypertrophic cells (Anderson, 1995). Hypertrophic chondrocytes, in any case, continue to produce MVs throughout their life-span (Gomez et al, 1996), although not all these new MVs are TNAP-positive (Akisaka & Gay, 1985, Bonucci et al., 1992), in spite of the fact that their calcium-binding sites are invariably stained.

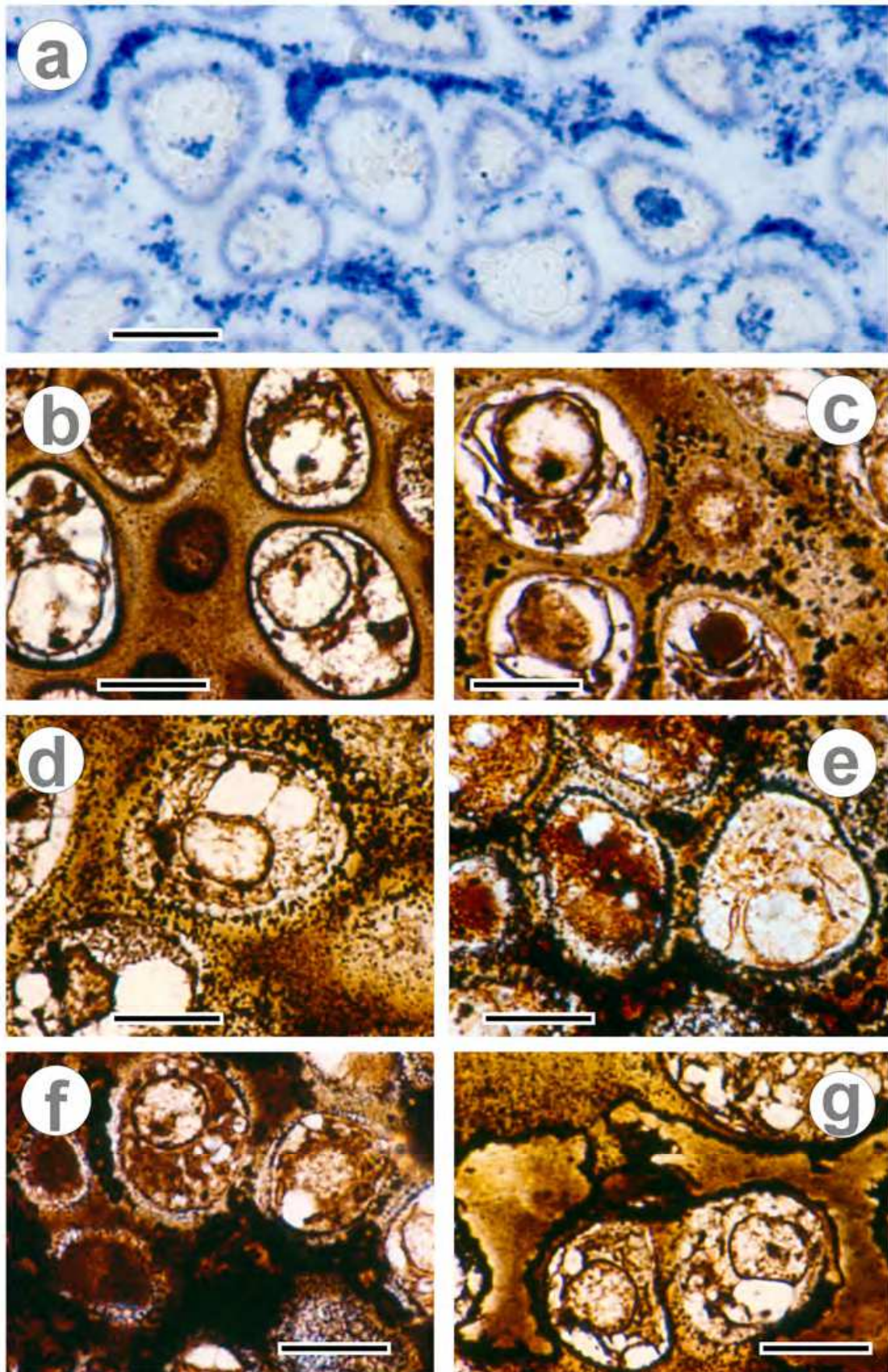


Fig. 4. TNAP (a), Calcium-binding staining (b-g) in chick embryonic cartilage. (Bar = 10 μm)

Staining for calcium-binding sites requires the incubation of slices of fresh cartilage in a solution containing 10-15mM of lanthanum chloride. This method was proposed by Morris and Appleton (1984) for electron microscopy. It was later studied in depth, at optical and electron microscopic level, using backscattered electron imaging and transmission electron microscopy (Gomez et al., 1996). The method is based on the premise that  $\text{La}^{3+}$  and  $\text{Ca}^{2+}$  have the same ionic radius but the  $\text{La}^{3+}$  has a greater charge, so that it is less easily displaced. Lanthanum allows very precise staining of the initial mineralization ( $\text{Ca}^{2+}$ -binding) sites, which then appear electron-dense.

Calcium-binding sites (after using lanthanum ions) are revealed under the light microscope by the ammoniacal silver impregnation method. In the growth plate, the upper hypertrophic chondrocytes show slightly stained dots at their peripheral membrane, and staining is also found focally in the territorial matrix (Figure 3d). In the lowest zone, the last hypertrophic chondrocytes (before apoptosis) show linear silver deposits on the peripheral membrane (Figure 3e), and the mineralizing matrix is completely stained, whereas the calcified deposits remain unstained (Figure 3f). In chick cartilage, the findings are similar in the upper territories (Figure 4 b, c), whereas in the lower zones many chondrocytes show thicker linear deposits, and a large number of dots are seen near the cells (Figure 4d-g). Matrix is stained first in the middle of the upper hypertrophic zone (Figure 4c), and is heavily stained in the lower zones (Figure 4e, f). A peripheral rim can be made out too around the bulk of the unstained calcified deposits (Figure 4g). Interestingly, the figures depicted by the La-incubation method are very similar to those displayed by confocal laser microscopy using fluorescent  $\text{Ca}^{2+}$  probes in sections of fresh chick cartilages and in cell cultures (Wu et al., 1995, 1997b). Chick chondrocytes are believed to maintain a sort of 'breathing' process by releasing calcium-Pi packets into the matrix.

The optical study has limited resolution; when electron microscopy was used, MVs proved to be stained in a rather different way. Figure 5 shows the different types of MVs stained by lanthanum and their approximate location.

MVs marked (a) are found early in the interterritorial matrix of upper zones; lanthanum staining is extravascular and is attached to the membrane of calcified MVs. There is an ultrastructural similarity between these complexes and TNAP-rich MVs in which the reaction product is localized at the periphery. MVs marked (b) are located in the peripheral rim of the upper hypertrophic chondrocytes. They appear as globules homogeneously filled with lanthanum. MVs of the last type (c) are found in large numbers around the lower chondrocytes in connection with their peripheral membranes. They are filled with lanthanum and also show numerous intravesicular densities about 10nm thick. The chondrocytes in this zone often show intracellular La-deposits.

Backscattered electron imaging of these lanthanum sites makes it possible to obtain a map of selected areas by energy dispersive X-ray analysis (Figure 6). The early lanthanum sites found in the matrix showed Sulphur (S), Phosphorus (P) and Lanthanum (La) co-localization (Figure 6a). At the pericellular rim of upper chondrocytes, mapping shows La and P co-localization (Figure 6b), whereas the lower ones show La, P and, surprisingly, Calcium (Ca) peaks (Figure 6c). All the MVs produced by hypertrophic chondrocytes bind lanthanum, and therefore have calcium-binding capabilities; in addition, the lower ones already contain calcium when released, possibly as preformed labile calcium mineral nuclei, as suggested by Wuthier and Lipscomb (2011).

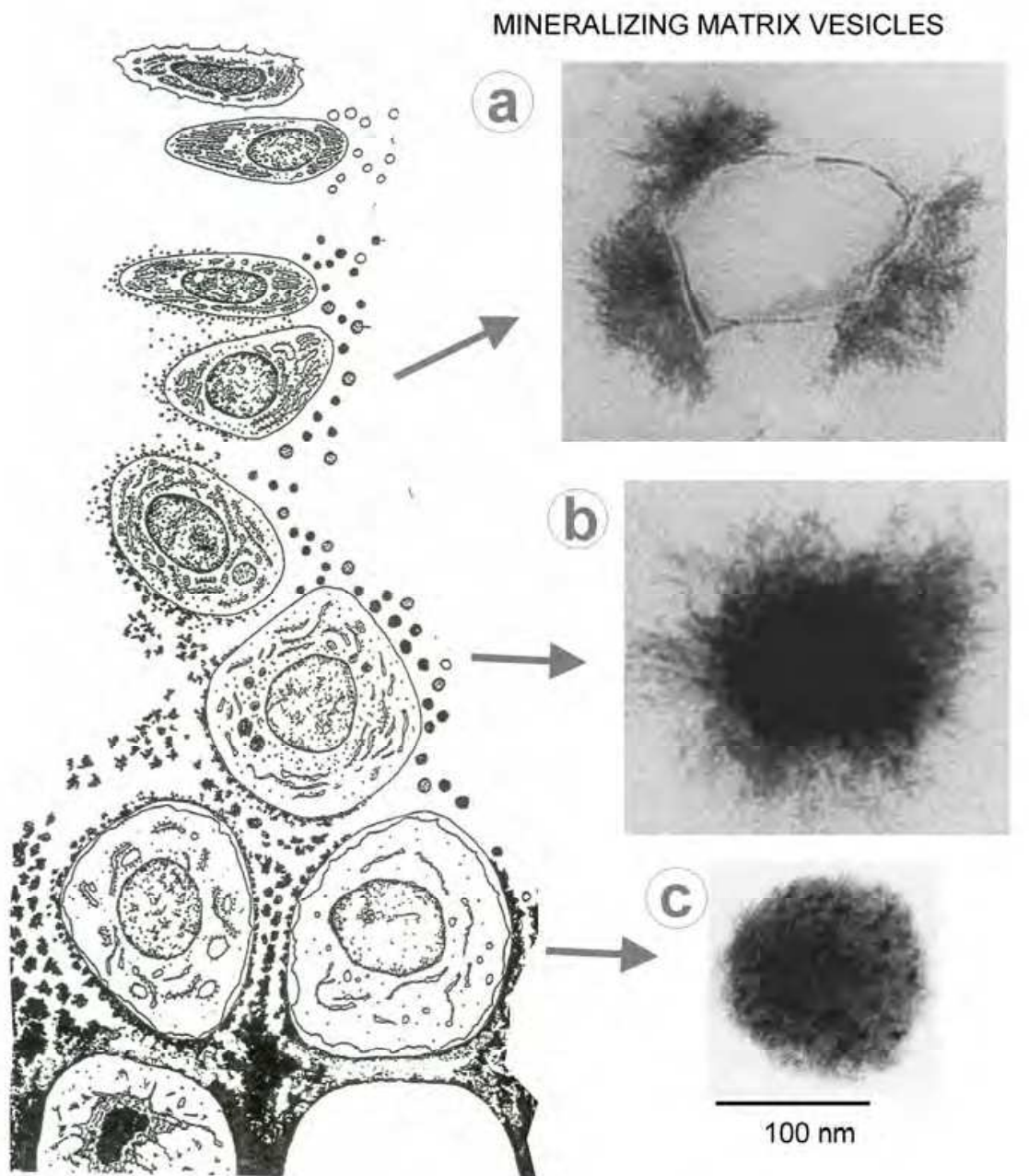


Fig. 5. Scheme of mineralizing MVs as detected by calcium-binding staining using lanthanum ions.

These morphological types of MVs, which are TNAP-rich and calcium-binding, seem to correspond to those studied by isolation methods. In fact, MVs have been separated into different density fractions corresponding to slow or quick mineralization (Warner et al., 1983). Slowly mineralizing MVs require organic phosphate substrates, and mineralization is blocked by the release of alkaline phosphatase. The activity of quickly mineralizing MVs depends on the presence of Annexin V,  $\text{Ca}^{2+}$ -protein phospholipids complexed to form an unstable mineral nucleational complex (revised by Wuthier and Lipscomb, 2011) – so they do not require organic phosphate to accumulate calcium and phosphate *in vitro*, and the removal of TNAP has only a minor effect.



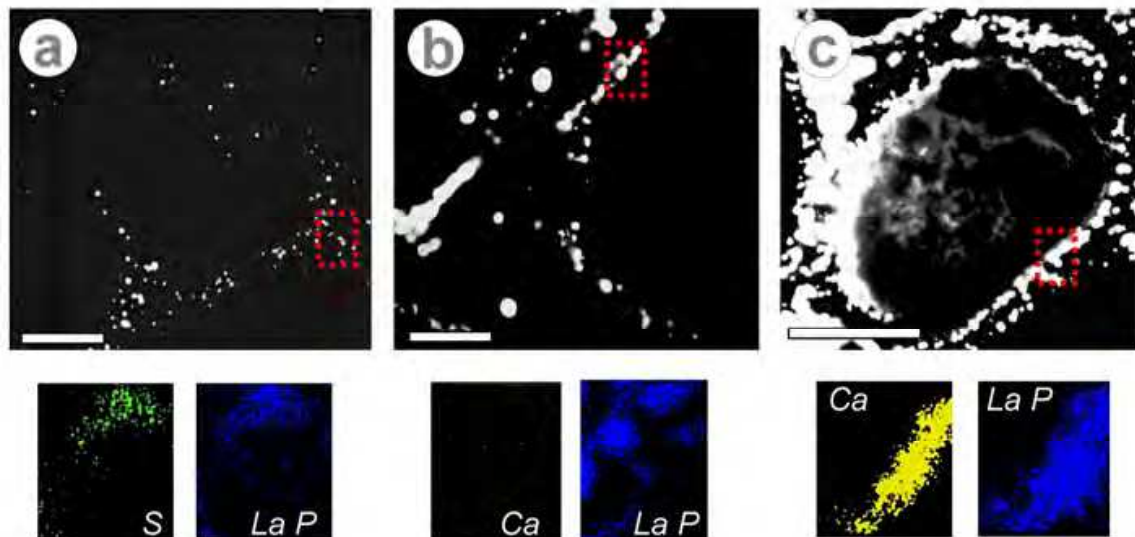


Fig. 6. Backscattered electron imaging in conjunction with elemental X-ray mapping of selected areas (red boxes) from upper matrix in chick cartilage (a); peripheral rim of hypertrophic rat chondrocytes (b); and peripheral rim of hypertrophic chick chondrocytes (c). (Bar = 5  $\mu\text{m}$ )

The production of MVs during the mineralizing sequence is probably the hallmark of the mineralization process, but its mechanism is still a topic for discussion. Current concepts on MVs support two conflicting viewpoints based on mammalian and avian growth cartilage studies. *In vitro* studies of MVs isolated from the normal and rachitic rat cartilage or from normal calves have led to the conclusion that alkaline phosphatase,  $\text{Ca}^{2+}$ -ATPase, is needed for calcification (Hsu and Anderson, 1984, 1995 a, 1995b, 1996; Hsu et al., 1999; Kanabe et al., 1983). By contrast, studies on MVs isolated from chick cartilage have stressed the role of an intravesicular nucleational core (Genge et al., 1988, 1989, 1990, 1991; Kirsch et al., 1997; McLean et al., 1987; Nie et al., 1995; Register et al., 1984, 1986; Sauer & Wuthier, 1988; Wu et al., 1993, 1997a; Wuthier, 1992). Considering that chondrocytes produce various different types of MVs during the sequence, this contradiction may only be apparent, because some mechanisms could be redundant. On the other hand, re-examination of the ultrastructural micrographs accompanying Anderson's studies shows that apatite deposition is found mainly around MVs (though some MVs are mineralized within), so that the doubt arises that the TNAP-mineralizing mechanism proposed by Anderson's group should actually refer exclusively to the early mineralization of the matrix.

### 3. Nature of calcified deposits

The calcified deposits in cartilage are believed to be similar to those in bone, dentin and other calcified tissues: they are considered to consist of calcium phosphate and to correspond to very small crystals which, when viewed under the electron microscope, have a needle- or filament-like shape and measure from about 2 to 5 nm in thickness and from 40 to 160 nm in length. Given that structures with similar characteristic give in bone diffractograms of hydroxyapatite type, the cartilage structures, too, are usually labelled 'hydroxyapatite crystallites'. The force of habit is so strong that this denomination has

been retained by many, although it is seriously misleading. First, as discussed in several reviews (Bonucci, 2007; Boskey, 1998; Veis, 2003), the inorganic deposits in cartilage – in parallel with those in bone, dentin and other hard tissues – contain, besides calcium and phosphate, other ions too, notably carbonate, magnesium, sodium, potassium and zinc. Early mineral deposits are, in fact, complexed with Zinc ions that can be found either as components of the mineral or as elements bound to metallo-enzymes such as alkaline phosphatase (Gomez et al., 1999). Second, the Ca/P molar ratio of the mineral substance is not only lower than that of hydroxyapatite, but is also variable and increases with the age of the deposits. Third, the crystalline organization is questionable, because, in our experience, and in agreement with results previously reported in bone (Landis & Glimcher, 1978), the early deposits of inorganic substance formed in epiphyseal cartilage fail to generate any electron diffraction patterns of the specific calcium phosphate solid phase, which are, in fact, only produced by the more calcified regions, whose reflections, in any case, remain those of poorly crystalline hydroxyapatite. According to Wheeler and Lewis (1977) and Arnold et al. (2001), the structures that form the early calcified deposits in bone are apatitic, but their crystal lattice contains so many distortions that they come to be intermediate between amorphous and crystalline; i.e., they have a paracrystalline character comparable with biopolymers. Fourth, the question is complicated by the possibility that amorphous calcium phosphate precedes the formation of the crystalline phase (Nudelman et al, 2010). This question is further discussed below (Chapter 5.2).

#### 4. The matrix component related to calcification

There can be little doubt that components of the matrix are crucial for the induction and regulation of the calcification process. This is borne out by the findings that *in vitro* some of them induce the formation of hydroxyapatite, that a close association exists *in vivo* between the mineral substance and most of them (so that their total extraction can only take place after decalcification), that some are changed by the calcification process, and that their spontaneous or induced changes may cause abnormal calcification of the matrix. The specific role of each of them in calcification is, however, hard to determine, mainly because of their heterogeneity, their reciprocal interactions and their possible post-translational changes. In addition, serum proteins which permeate the matrix may have an inhibitory role during the earliest phase of the calcification process (Heiss et al, 2003).

##### 4.1 Collagen

The results of investigations on the calcification of bone, dentin, tendons and other collagen-rich tissues have led to the conclusion that the collagen fibrils of the matrix play a leading role in the deposition of inorganic substance. In areas of initial calcification, in fact, this substance shows a close relationship with the periodic banding of collagen, due to its location within the 'holes' zone that results from the rearrangement of collagen molecules into fibrils. The development of inorganic bands exactly corresponding to the period of the collagen fibrils or, more exactly, to their holes, has led to the conclusion that calcification occurs through a process of heterogeneous nucleation catalysed by a particular atomic organization of these fibril 'holes' (Glimcher & Krane, 1968).

This theory, on which several reviews can be consulted (Bonucci, 1992, 2007; Höhling et al., 1995; Veis, 2003), can hardly be operative in the case of cartilage calcification. The typical

pattern of electron-dense bands that coincide with the collagen periodic binding found in the areas of early calcification in bone has never been found in cartilage, where the early inorganic aggregates correspond to roundish calcification nodules. This agrees with the fact that cartilage contains type II collagen, whose thin fibrils, formed by homotrimers of  $\alpha 1(\text{II})$  chains, have a poorly recognizable period and display no identifiable 'hole' zones.

This does not rule out the possibility that cartilage collagen participates in the calcification process. Calcification nodules contain chondrocalcin (reviewed by Poole et al., 1989), a calcium-binding protein associated with calcification and later identified as the C-propeptide of type II collagen (van der Rest et al., 1986). The exact role of this protein is not known; it is of interest that it is found at the beginning of the calcification process, whereas it is absent from completely calcified nodules. Calcification might also be mediated by FACIT (fibril-associated collagen with interrupted triple helices) collagens, which are characterized by the interposition of non-triple-helical domains between two or three triple-helical domains, so acquiring the possibility of association and formation of cross-links with collagen type II and with other molecules that contribute to stabilizing the matrix (Olsen, 1989). Collagen type X appears to possess the best credentials in this respect among these numerous collagens: it is specifically expressed, in fact, by hypertrophic chondrocytes (Linsenmayer et al., 1988) and its synthesis precedes matrix calcification (Iyama et al., 1991), so that it might well play a role in the process. It is covalently cross-linked to type II collagen and both bind to matrix vesicles; it binds calcium in a dose-dependent manner. Isolated matrix vesicles deprived of the associated type II and type X collagens show a marked fall in Ca uptake, which can be restored by collagen reconstitution (Kirsch & Wuthier, 1994). Type X collagen transgenic animals undergo disruption of the matrix around hypertrophic chondrocytes, anomalous proteoglycan distribution, and abnormal vertebral development (Jacenko et al., 2001). The function of type X collagen, however, remains uncertain.

#### 4.2 Proteoglycans

The wide spaces outlined by type II collagen fibrils contain abundant non-collagenous components, the most representative of which are acid proteoglycans. These mainly consist of aggrecan, whose molecules aggregate by binding to hyaluronic acid (hyaluronan) which, in its turn, is bound to a globular link protein, so that macromolecular aggregates are formed. The composition of the aggrecan glycosaminoglycan chains varies, although there is a prevalence of chondroitin sulphate and, to a lesser degree, of keratan sulphate. An additional factor is that the protein core itself can vary, giving rise to different members of the aggrecan family (versican, neurocan, brevican). Perlecan and syndecans are cartilage proteoglycans that contain high concentrations of heparan sulphate. Decorin and biglycan have only been found in the resting cartilage.

A family of four oligomeric extracellular matrix proteins, the first and third of which are mainly expressed in cartilage, have been described as matrilins (Deák et al., 1999). They share a structure consisting of von Willebrand factor A domains, epidermal growth factor-like domains and a coiled coil alpha-helical module; post-translation proteolytic processing may cause extensive heterogeneity of their tissue forms. Matrilins contribute to the regulation of matrix assembly by binding to collagen fibrils, to other noncollagenous proteins and to aggrecan (reviewed by Klatt et al., 2011).

Acid proteoglycans have long been associated with calcification, since the early suggestion of Sobel (1955) that a complex of chondroitin sulphate and collagen in a critical conformation constitutes the 'local factor' that is responsible for calcium deposition. This hypothesis found wide support, on the basis of the observation that plenty of acid proteoglycans are found in the cartilage matrix, that they can bind high concentrations of calcium and that this can be released locally by degradation of their molecules, so creating an environment suitable for the precipitation of hydroxyapatite. By contrast, it has been shown that acid proteoglycans in solution inhibit the precipitation of calcium and phosphate (Dziewiatkowski & Majznerski, 1985) and that the breakdown of their molecules fails to trigger precipitation (Blumenthal et al., 1979). Without further considering this controversial question (see reviews by Bonucci, 2007; Roughley, 2006; Schaefer & Schaefer, 2009; Shepard, 1992), the available results suggest that the function of acid proteoglycans chiefly depends on their being aggregates or monomers, and on their hydrodynamic size.

In this connection, a number of data show that matrix proteoglycans undergo modifications pertinent to the calcification process. This has been shown by immunohistochemistry (Hirschmann & Dziewiatkowski, 1966) and confirmed by energy dispersive X-ray elemental analysis showing that matrix sulphur levels fall with calcification (from 3.5% in the uncalcified matrix to 0.3% in the fully calcified matrix; Althoff et al., 1982; Boyde & Shapiro, 1980). Lohmander and Hjerpe (1975) also found, by centrifugation of finely ground material in acetone/bromoform density gradients followed by density gradient ultracentrifugation, that the cartilage matrix loses about half its proteoglycan content with the onset of calcification, and that the proteoglycans of the calcified matrix differ in composition and size from those of uncalcified cartilage. These results were in line with electron microscope data showing that during calcification a marked decrease occurs in the size of the granules that correspond to collapsed acid proteoglycans (Buckwalter et al., 1987; Matukas & Krikos, 1968; Takagi et al., 1983, 1984). Loss of proteoglycans with calcification was also reported by de Bernard et al. (1977), Mitchell et al. (1982), Vittur et al. (1979). On the other hand, Barckhaus et al. (1981) did not find any significant sulphur loss in frozen, freeze-dried cartilage studied by electron microscope microprobe analysis, and Scherft and Moskalewski (1984), on the basis of the number of matrix granules and the affinity of the matrix for colloidal thorium dioxide, concluded that degradation of proteoglycans is not a first, indispensable step in cartilage mineralization.

These controversial results can be settled on the basis of the autoradiographic observation, already made by Campo and Dziewiatkowski in 1963, that, although the protein of the proteoglycans is somehow removed before calcification of the cartilage, a portion of the chondroitin sulphate is retained and becomes part of the calcified matrix. Rather than being lost, the cartilage proteoglycans could be depolymerized and modified, to be finally entombed in the calcified matrix (Campo & Romano, 1986). These topics concur, and will be further considered, with those concerning crystal ghosts (Chapter 5.2).

The degradation of proteoglycan molecules might be due to the effects of enzymes (Boskey, 1992). Proteoglycan-degrading enzymes are produced by cartilage cells and their concentration is higher in the lower hypertrophic and calcification zones than in the other cartilage zones (Ehrlich et al., 1985). Most of these enzymes, such as acid phosphatase and aryl sulphatase, have a lysosomal origin (Meikle, 1975; Thyberg et al., 1975); only the former would be found in the extracellular matrix of the cartilage (Meikle, 1976). Matrix vesicles are

also selectively enriched in enzymes which degrade proteoglycans (Dean et al., 1992), although their most typical enzyme is alkaline phosphatase.

### 4.3 Alkaline phosphatase (TNAP)

Since the earliest suggestion of Robison (1923), that the formation of calcium phosphate might be dependent on the hydrolysis of phosphate esters by alkaline phosphatase, plenty of studies have been centred on this enzyme (reviewed by Orimo, 2010; Wuthier and Lipscomb, 2011) whose importance has been heightened by its already discussed relationship with MVs (Chapter 2). Its function in the calcification process, however, remains controversial, although it is certainly fundamental in all processes of biological calcification. This topic is further discussed below (Chapter 5.1).

### 4.4 Glycoproteins and phospholipids

The cartilage matrix positively reacts when treated with the periodic acid-Schiff method, a histochemical method that shows molecules having vicinal glycol groups, i.e., glycoproteins. Some of these molecules are phosphorylated and expressed by both chondrocytes and osteoblasts, as in the case of osteopontin and bone sialoprotein. The localization of these glycoproteins, and their function in calcification, remain rather elusive (reviewed by Gentili & Cancedda, 2009).

Phospholipids too are components of the cartilage matrix and are probably involved in the calcification process, as shown by the accumulation of  $^{32}\text{P}$ -orthophosphate at the calcification front and by the observation that a fraction of them can only be extracted after decalcification (Eisenberg et al., 1970). Their incorporation in the calcified matrix has been confirmed by immunohistochemistry using MC22-33F, an antibody that recognizes phosphatidylcholine, sphingomyelin and dimethylphosphatidylethanolamine, and that gives a strong reaction at the periphery of the calcification nodules (Bonucci et al., 1997). Further confirmation was obtained by combining malachite green fixation with the complex phospholipase A<sub>2</sub>-gold; again the reaction was stronger at the periphery than at the centre of the calcification nodules (Silvestrini et al., 1996). These results, together with the long-standing knowledge that bone and other calcifying tissues contain calcium-phospholipid-phosphate complexes (Boskey & Posner, 1976) and that in calcifying cartilage the interaction of Ca and P ions with phosphatidylserine can give rise to phospholipid-Ca- Pi complexes (Boyan et al., 1989), make the phospholipids good candidates as molecules capable of inducing or controlling the calcification process.

## 5. Matrix calcification

The calcification of the cartilage has long been considered a straightforward process comprising just one single phase- the precipitation of calcium phosphate in specific areas of the matrix. In this area of research, things turn out to be much more complicated, and the process actually goes forward through at least three phases: the intervention of specific calcifying structures, the development of crystal-like, organic-inorganic particles, and their gradual transformation into inorganic structures that finally mature into hydroxyapatite crystallites.

### 5.1 First phase: Structures that undergo calcification

A number of electron microscope studies have shown that in epiphyseal cartilage the early mineral aggregates are connected to MVs, which are often partly or totally filled by crystals, or may be found in contact with peripheral crystal aggregates. The sheer abundance of MVs suggests that there are many crystal centres from which mineralization spreads out into the matrix. In reality, most of the early matrix mineralization begins around MVs (Figure 7), i.e., is an extravascular process, which is probably favoured by the existence of a mineralization centre (none other than the intravesicular mineral), but is regulated by a second mineralizing mechanism. This is suggested by the observation that there are pathological conditions - hypophosphatasia, in particular - in which the mineralization process occurs within and around MVs, but fails to spread into the surrounding matrix (Anderson et al., 1997). For mineralization to spread beyond MVs, therefore, a second, facilitating mechanism is required.

The possible role of collagen fibrils, acid proteoglycans, glycoproteins, and phospholipids in calcification has been discussed above. These structures are components of the uncalcified matrix; some type of modification therefore seems necessary for them to be able to take part in the calcification process. Removal of inhibitors might be one, molecular changes of various types might also be involved. Unfortunately, these processes are poorly known (reviewed by Boskey, 1992). The role of TNAP in calcification must obviously be emphasized: as already mentioned, any serious lack of TNAP (as in hypophosphatasia) prevents the calcification process from spreading beyond MVs into the matrix. It is present in all tissues that calcify, and is located precisely in the areas that will calcify, so much so that its histochemical reaction product gives an ultrastructural picture similar to that of the mineral substance around MVs. The role of TNAP, however, is still in doubt,

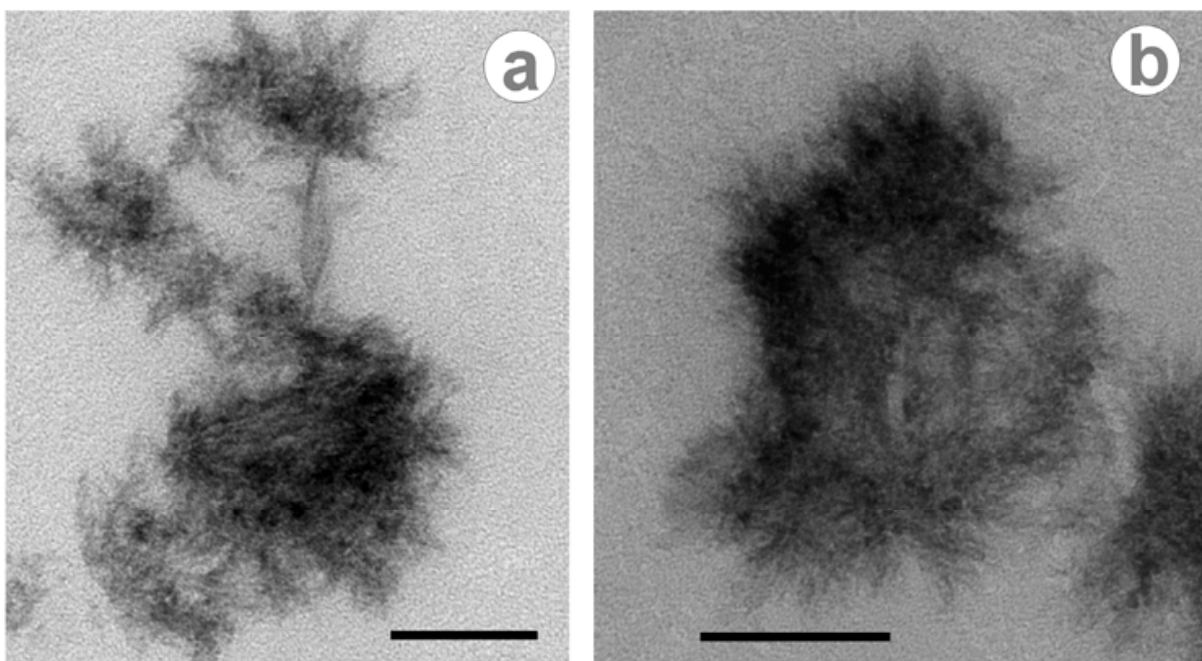


Fig. 7. Early matrix mineralization sites around calcified MVs as shown by calcium-binding staining using lanthanum ions. (Bar = 100 nm)

and as many as six different functions have been attributed to the enzyme (Wuthier & Register, 1985), the two most often put forward being an increase in the local concentration of phosphates, which would permit hydroxyapatite formation, and the removal of inorganic pyrophosphate (PPi), which is an inhibitor of calcification. Another function is often neglected: the TNAP of MVs from growth cartilage is a calcium-binding glycoprotein (de Bernard et al., 1985), a property that might permit crystal formation through the mechanism depicted below for crystal ghosts (Chapter 5.2). In this context, it is interesting that a zinc-containing glycoprotein can be demonstrated in the matrix of calcified deposits (Gomez et al., 1999). Molecular studies have shown that TNAP contains a specific metal binding domain (different from the zinc-active site). A synchrotron radiation X-ray fluorescence study confirmed that the metal in the metal-binding site is a calcium ion (Mornet et al., 2001).

## 5.2 Second phase: Development of organic-inorganic, crystal-like structures

The structures that develop within and around matrix vesicles during the early calcification phase are usually called 'crystallites', although, as reported above (Chapter 3), they appear to be intermediate between amorphous and crystalline, i.e., they have a paracrystalline status comparable with biopolymers. Because of their prevalent origin from matrix vesicles, most of them are collected in roundish aggregates called 'calcification nodules'. Electron microscope studies have contributed much to the knowledge of these structures. As already mentioned in Chapter 3, the cartilage 'crystallites' appear under the electron microscope as filament- and needle-like structures, which have intrinsic electron-density; as a result, they do not need to be stained to become visible on the microscope screen. This advantage, which is directly attributable to their inorganic content, is counterbalanced by the fact that their electron density completely masks the organic structures they are associated with. This masking effect, which prevents recognition of the organic components of the calcified matrix and their relationship with the inorganic substance, can only be eliminated by decalcification. This procedure, however, leads to the removal not only of the inorganic material, but also of a number of organic molecules, so that the decalcified areas appear as almost empty zones crossed by collagen fibrils (reviewed by Bonucci, 2007). This disadvantage can be overcome by using special decalcification techniques such as the PEDS method and the cationic dye stabilization method.

### 5.2.1 'Crystal ghosts'

The acronym PEDS stands for Post-Embedding Decalcification and Staining, a method that, unlike the usual decalcification by immersion of whole specimens in the decalcifying solution, followed by dehydration and embedding, decalcifies the tissue after its embedding in a resin, that is, by floating ultrathin sections on the surface of the decalcifying solution (Bonucci & Reurink, 1978). The extreme thinness of the sections (usually less than 1  $\mu\text{m}$ ) allows their decalcification to become complete in only a few minutes. At the same time, because the tissue is embedded in the resin, its organic components are blocked and stabilized, and therefore protected from solubilization and extraction. The preservation of the organic structures is confirmed by the ultrastructure of the cells and of the uncalcified matrix: this is conspicuously altered by the usual methods of decalcification, while after the PEDS method it is indistinguishable from the ultrastructure these structures show in the undecalcified sections. Strangely enough, the calcification nodules appear electron-dense and contain filament- and needle-like structures similar to untreated 'crystallites'. This effect, which at first glance may

appear to be due to the lack of decalcification and to the persistence of the so-called 'crystallites', must actually be put down to the fact that organic structures previously masked by the mineral substance have become unmasked by decalcification and have then been stained. The unexpected finding is that these structures have practically the same shape and size as the 'crystallites' and must consequently be considered as their 'organic ghosts' (Figure 8). This is why they were first called 'crystal ghosts'. As a consequence, the early 'crystallites' found in the calcification nodules must be considered organic-inorganic hybrids, each consisting of an organic filament with attached calcium and phosphate ions.

Confirmation of these findings has come from studies that have adopted the second decalcification method mentioned above, i.e., the cationic dye stabilization method (reviewed by Bonucci, 2002). This is based on the notion that acid polyanions react with basic substances like cationic dyes, so that the latter can be used to reveal the former in a tissue (according to the process often referred to as 'basophilia'). The same staining substances stabilize the acid molecules, so that these become insoluble and resistant to decalcification, and do not collapse into granules during dehydration. The ultrastructural study of cartilage treated with cationic dyes, after its decalcification by immersion in a decalcifying solution, and later embedding in a resin, shows that the calcification nodules appear as aggregates of organic, filament-like structures which bear a close resemblance to 'crystallites' on one hand and to crystal ghosts on the other (Figure 8a). The importance of these findings is not limited to their confirmation of the existence of crystal ghosts, but includes their demonstration that crystal ghosts cannot depend, as suggested by Dong and Warshawsky (1995), on the penetration by staining heavy metals of the voids left in the resin by the dissolution of the crystallites, because the decalcification procedure is always carried out before embedding.

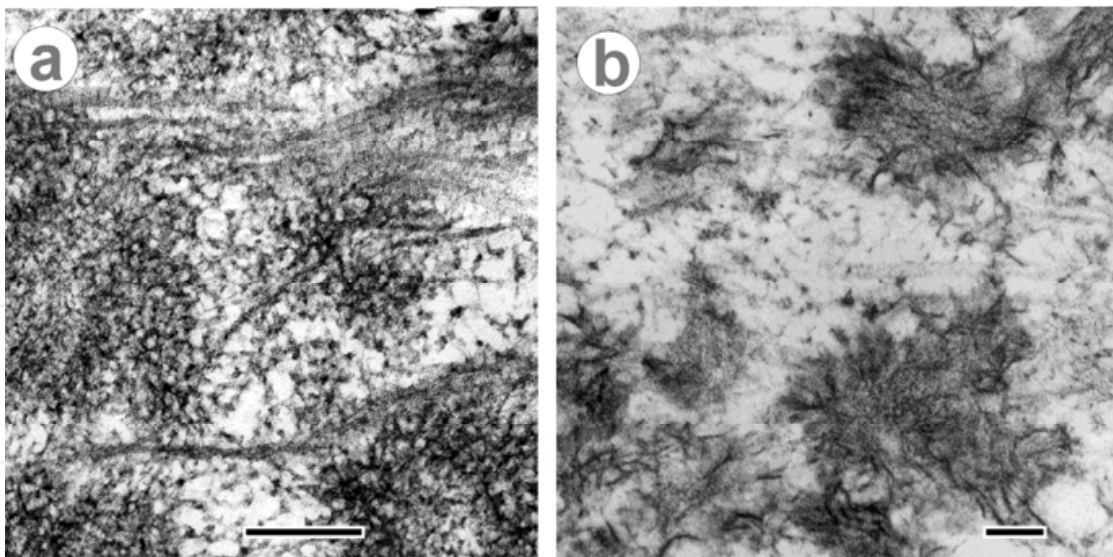


Fig. 8. (a) Crystal ghosts in rat growth plate after acridine orange dye stabilization and EDTA decalcification; (b) Crystal ghosts in embryonic chick cartilage after PEDS. (Bar = 200nm)

### 5.2.2 The nature of crystal ghosts

The results reported in the previous chapter show that the early 'crystallites' of the calcifying cartilage are organic-inorganic hybrids and that the crystal ghosts represent their organic component. On this basis, it becomes mandatory to establish their nature and composition.



As reported in Chapter 3.4.2, the acid proteoglycans are the most abundant components of the cartilage matrix and are responsible for its long-recognized basophilia and metachromasia. It is true that calcification slightly reduces staining properties, but they do, on the whole, persist, which shows that the calcification nodules, too, contain acid proteoglycans. Because the crystal ghosts are the most abundant components of the calcification nodules, the logical step forward is to suppose that they are proteoglycan molecules.

This hypothesis is strongly supported by electron microscope histochemistry. The crystal ghosts, in fact, react with, and are stained by, ruthenium red, ruthenium hexammine trichloride, terbium chloride, colloidal iron, or bismuth nitrate (Bonucci et al., 1989; Bonucci, 2002). All these histochemical reactions can occur at pH as low as 1.8, showing that the organic substrates have strong acidic groups, such as the sulphate groups of acid proteoglycans. The possibility that the crystal ghosts of the calcified cartilage are acid proteoglycans is confirmed by the observation that the histochemical reactions are inhibited by methylation, which blocks sulphate and carboxy groups, and are not restored by saponification, which only re-establishes the carboxy groups (Bonucci et al., 1988). Further confirmation is given by the immunoreaction of crystal ghosts with the antibody CS-56, which is specific for the glycosaminoglycan portion of chondroitin sulphate (Bonucci & Silvestrini, 1992). It must be added that crystal ghosts also react with acidic phosphotungstic acid, which is a glycoprotein stain.

### 5.2.3 The function of crystal ghosts

The relationship between crystal ghosts and inorganic substance in the 'crystallites' is so close that they cannot be distinguished from one another under the electron microscope, even if the former are stained by heavy metals (uranyl acetate, lead citrate). The hybrids that they form appear as unique structures; only after decalcification can their organic component be made out. It is obvious that this close organic-inorganic relationship necessarily brings with it strong implications.

Actually, as reported in Chapter 4.2, acid proteoglycans have long been considered to be responsible for the calcification process in cartilage. The identification of crystal ghosts as molecules of acid proteoglycans strongly supports this possibility. The acid groups of these molecules can bind high concentrations of calcium and give rise, with the possible initial formation of amorphous calcium phosphate, to the organic-inorganic structures that are commonly called 'crystallites'. The proteoglycan molecules could function as templates, and the 'crystallite' shape and size could simply reflect the filamentous shape and size of their organic framework. In this connection, the suggestions must be taken into consideration that prenucleation clusters of calcium carbonate may be stabilized by organic molecules (Gebauer et al, 2008) and that the template-directed aggregation of these clusters give rise to the formation of amorphous calcium carbonate nanoparticles which assemble at the template and develop into crystalline domains (Pouget et al, 2009).

Crystal ghosts have been described not only in the calcifying cartilage, but in similar terms in other hard tissues during the early stage of calcification (reviewed by Bonucci, 2007). The acid proteoglycans are ubiquitous in these tissues and it might be thought that they behave as crystal ghosts in all of them. It must be noted, though, that other acidic molecules could play exactly the same role; a number of polyanions, some characterized by the repetitive sequences of aspartic acid, have been found in all calcified tissues and each of them could

theoretically initiate and regulate the calcification process (Gotliv et al., 2003; Rahman & Oomori, 2010; Takeuchi et al., 2005; Weiner & Addadi, 1991). Moreover, it cannot be disregarded that, as reported above (Chapter 3.5.1), TNAP, too, is a glycoprotein with calcium-binding properties. It can be demonstrated histochemically in all areas of the matrix undergoing calcification, whereas it is left unstained, even if still present, in already calcified areas. It may be speculated, therefore, that TNAP can bind mineral ions, as crystal ghosts do, so becoming deactivated and embedded in the calcified matrix.

#### 5.2.4 Are there pre-crystal ghosts?

If crystal ghosts have the role suggested above, then the question arises whether they are preformed in the matrix and exactly what kind of mechanism induces their activation as mineral-binding structures. It may be hypothesized that the matrix proteoglycans, and the other proteins mentioned above, must in some way be modified to acquire calcium-binding properties; the great variety of proteolytic enzymes located in the cartilage might well carry out this function. The reaction of lanthanum with components of the matrix seems to strengthen this hypothesis (Figure 9).

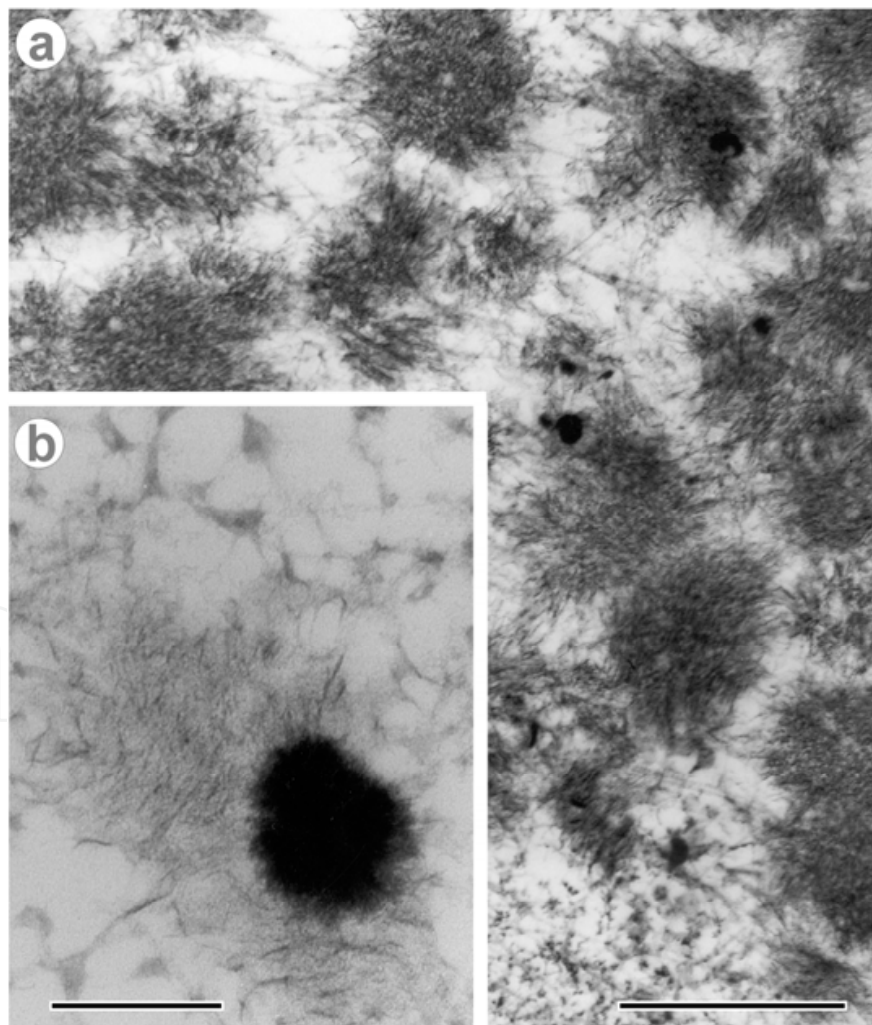


Fig. 9. (a) Pre-crystal ghosts in rat growth plate (bar = 1  $\mu\text{m}$ ); (b) and in chick embryonic cartilage, (bar = 200 nm)

Staining the growth cartilage with lanthanum chloride unexpectedly shows that the still uncalcified matrix of the upper chondrocyte zones contains roundish aggregates of very fine filaments which closely resemble crystal ghosts and, on the whole are similar to calcification nodules (Gomez et al., 1996). They correspond to focal concentrations of proteoglycans with a high La-binding capacity, or, in a broader perspective, strong calcium-binding properties. These findings are in agreement with the hypothesis that the acid proteoglycans of the cartilage matrix, which are inhibitors in their native state, can become inducers of the calcification process after their molecules have in some way become modified. The lanthanum-stained structures might be pre-crystal ghosts, that is, organic molecules that are ready to bind calcium ions and so trigger 'crystallite' formation.

### 5.3 Third phase: Changes in crystallites as mineralization progresses

The progression of the calcification process implies that the early calcification nodules acquire new 'crystallites' at their periphery, so increasing in size and gradually coalescing with each other till the whole matrix is calcified. As a consequence, the developing calcification nodules become fully calcified in their central area while they are still calcifying at their periphery, where 'crystallites' continue to be formed. Another process occurs at the same time: the PEDS method shows that, as the calcification nodules enlarge, the crystal ghosts disappear from their central, fully calcified zones, where all that can be recognized is an amorphous material; the crystal ghosts remain visible at the periphery of the nodules, where the process of calcification is still under way (Figure 8b). The completion of the calcification process takes place, therefore, in parallel with a loss of the organic components that constitute the crystal ghosts. In agreement with these observations, the loss of organic material during calcification has been found biochemically not only in cartilage (Lohmander and Hjerpe, 1975; Vittur et al., 1979), but in other calcified tissues, too, especially in bone (Pugliarello et al., 1970) and enamel (reviewed by Bartlett & Simmer, 1999; Simmer & Hu, 2002). These results suggest that, as calcification progresses, the organic components of the 'crystallites', recognizable as crystal ghosts, are gradually lost, probably through the lytic effects of proteases. The fall in amounts of organic material as the calcification process is completed can be seen as converging with other two processes mentioned in the preceding chapters: first, the Ca/P molar ratio increases and approaches that of hydroxyapatite; second, the electron diffractograms, which are of amorphous type if obtained from the early, small, incompletely developed calcification nodules, become of poorly crystalline type if obtained from the central zone of the biggest nodules or from the already diffusely calcified matrix. The loss of crystal ghosts therefore seems to be a pre-requisite for the inorganic component of 'crystallites' to acquire a definitive hydroxyapatite organization.

## 6. Conclusion

The results reported above allow a few conclusions to be drawn. The calcification of the cartilage matrix seems to occur through two mechanisms, one related to the development of matrix vesicles, another involving TNAP and other matrix components. The first is characterized by the formation of early mineral aggregates within MVs, mostly through their annexin calcium-binding properties; the second consists in the formation of organic-inorganic hybrids and implies the activation of alkaline phosphatase. These hybrids,

incorrectly called 'crystallites', undergo a process of transformation which includes the gradual loss of their organic component, the increase of their Ca/P molar ratio, and the transformation of their electron diffractograms from amorphous to crystalline. It seems that the acidic molecules that function as templates allow the linkage and organization of inorganic ions along planes that approximate those of hydroxyapatite, and that their enzymatic breakdown permits the ions to move all over the definitive hydroxyapatite reticulum. The organic components of the 'crystallites' are pre-formed in the matrix and must in some way be activated, probably by metalloproteases derived from lysosomes and MVs. These changes are most directly pertinent to acid proteoglycans which, as shown by the lanthanum reaction, acquire a focal capacity to bind calcium and phosphate ions.

Although most of these concepts must still be regarded as speculative, they appear to offer a rational explanation for all the main aspects of the calcification mechanism in cartilage - an explanation whose basic features are probably applicable to all calcifying tissues.

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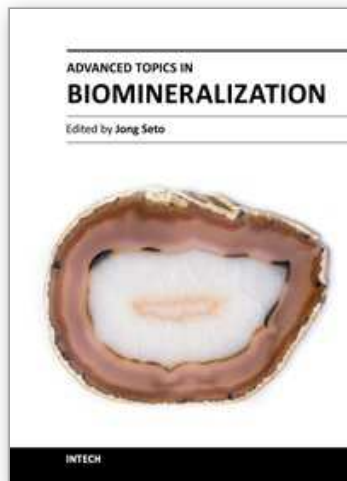


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