Calcified tissue histochemistry: from microstructures to nanoparticles

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It has long been recognized that histochemistry and cytochemistry offer the only ways of gathering information about the biochemical composition of tissues and cells without disrupting their microscopic architecture. A variety of methods have been put forward for studying nuclei acids, proteins, carbohydrates, lipids, enzymes and other components of intact tissues and cells. By now, many of these have only a historical interest. Some do, however, survive in microscopic and ultramicroscopic applications, and have become incorporated in the most refined and precise techniques that are currently available. Histochemical reactions range from the classic procedures carried out on histological sections to yield final stained products recognizable under the light microscope (Figure 1), to those which are applied on ultrathin sections, using heavy metals or other electron-dense compounds to reveal specific components under the electron microscope (Figure 2A); others range from procedures based on the antigen-antibody reaction that are capable of revealing the presence of specific biological molecules (Figure 2B), to the biophysical techniques which permit the qualitative and quantitative analysis of elements (Figure 3); lastly, there are the recently proposed ultra-high resolution methods that allow nanoparticles to be recognized. This brief review, which is based on personal experience and on the data in the literature, will discuss the most important methods now being used.

Calcified tissues consist of specific cells and an organic matrix that occupies more space than cellular elements and whose outstanding feature is that of being the site where calcification takes place. In bone, the most often studied of all calcified tissues, the most typical component of the organic matrix is type I collagen. Other less well represented constituents are non-collagenous proteins, proteoglycans and lipids bearing traces of enzymes and growth factors. Inorganic structures, whose biophysical characteristics approach those of hydroxy-

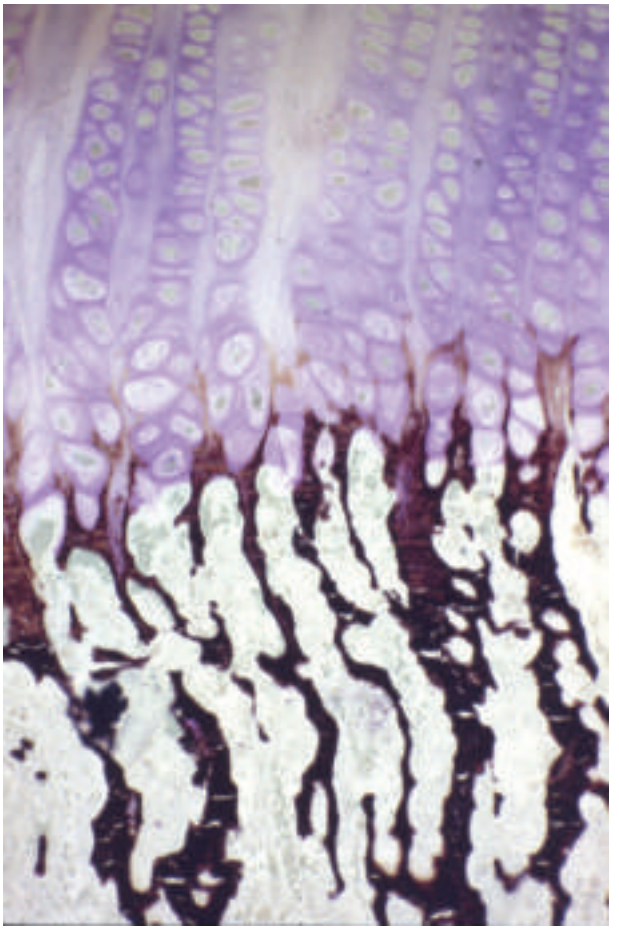

Figure 1. Histological section of the epiphyseal cartilage and metaphyseal trabeculae of the rat; von Kossa staining. The positivity (black stain) of the hypertrophic-degenerated cartilage and of metaphyseal trabeculae documents the presence of calcium phosphate in calcified areas. x 60.

apatite, and for this reason are named *crystals* or *crystallites*, are enmeshed in the matrix and are very closely connected with its organic components. Matrices of other calcified tissues (cartilage, dentin, enamel, cementum, as well as mollusc shells, crustacean exoskeletons, spicules of sea urchin embryos, several unicellular organisms and pathologically calcified tissues) differ in their composition but all basically consist of an organic matrix permeated by inorganic substance (see Bonucci 1992a).

One of the histochemical reactions most often applied to the study of the calcification process explores alkaline phosphatase activity. Long considered responsible for phosphate accumulation in areas of early calcification (Robison 1923; Majno and Rouiller 1951), without ever being assigned a precise functional role (Whyte 1989), alkaline phosphatase is a membrane-bound enzyme typically expressed in osteoblasts and chondrocytes (Doty and Schofield 1976) but present in almost all calcifying areas (de Bernard *et al.,* 1986) and closely correlated with the calcification process itself (Gomez and Boyde 1994). It can be studied in histological sections using Gomori's method (Majno and Rouiller 1951), one of whose modifications, based on the use of cerium (Robinson and Karnovsky 1983; Van Goor *et al.,* 1989; Bonucci *et al.,* 1992; Hulstaert *et al.,* 1992), can be used to localize enzymatic activity under the electron microscope. By applying this method, alkaline phosphatase activity has been demonstrated not only on the osteoblast membrane, but extracellularly too, in areas of calcification (Bonucci *et al.,* 1992) and, most clearly, in matrix vesicles, where the enzyme is the most typical marker (Ali *et al.,* 1970; Matsuzawa and Anderson 1971; McLean *et al.,* 1987). Alkaline phosphatase can be studied using immunohistochemical techniques under the light or the electron microscope (De Bernard *et al.,* 1986; Bruder and Caplan 1990; Masuhara *et al.,* 1992; Morris *et al.,* 1992).

Other often applied histochemical methods aim to study the presence of glycoproteins and acid proteoglycans in histological sections of calcified tissues and their changes as calcification proceeds. Glycoproteins are typically shown by the periodic acid-Schiff (PAS) method, which is based on the production of aldehyde groups by periodate oxidation of vicinal hydroxyl groups and on their staining by Schiff's reagent (Puchtler *et al.,* 1974). The diffuse PAS-positivity of the cartilage matrix and the osteoid border in bone are well-established findings (Cabrini 1961), and glycoproteins are supposed to play a direct role in calcification (de Bernard *et al.,* 1977; Addadi *et al.,* 1989) in all calcifying matrices, from vertebrates to invertebrates (Termine *et al.,* 1981; Butler *et al.,* 1985; Goldberg and Septier 1986; Nanci *et al.,* 1989; Albeck *et al.,* 1996; Marxen *et al.,* 1998; Wilt 1999; Levi-Kalisman *et al.,* 2001). PAS-like methods can be applied in ultrastructural studies of glycoproteins by using, rather than Schiff's reagent, aldehyde-reactive electron-dense compounds such as alkaline bismuth (Ainsworth *et al.,* 1972) or thiocarbohydrazide-silver proteinate (Scherft 1970; Spicer and Schulte 1982). In electron microscopy, acidic phosphotungstic acid can be used to identify glycoproteins along cell membranes (Marinozzi 1967; Barsotti and Marinozzi 1980) and in calcifying areas (Bonucci and Gherardi 1975; Bonucci

2002). There is a rich literature on the histochemistry of acidic proteoglycans in calcified tissues, reflecting the interest these substances have always raised in relation to biological calcification (reviewed by Kobayashi 1971, Buckwalter 1983, Takagi 1990, Shepard 1992). They have long been considered to have a prominent, but still imprecisely known, role in the process: some authors (reviewed by Schubert and Pras 1968; Takagi *et al.,* 1984) see them as promoting calcification, others (Blumenthal *et al.,* 1979) as inhibiting it, and their function may well vary according to whether they are immobilized on a substrate or free in solution (Linde *et al.,* 1989), according to their state of aggregation and the hydrodynamic size of their molecules (Chen *et al.,* 1984), or according to their calcium phosphate content (Schubert and Pras 1968). Under the light microscope, their acidic character is exploited to demonstrate them through the reaction of their acid groups with cationic dyes (Szirmai 1963). In this context, Alcian blue is probably the most often used dye (Quintarelli *et al.,* 1964), but other cationic molecules can be utilized, so giving rise to a metachromatic (Toluidine blue) or basophilic reaction with acid proteoglycans (Cabrini 1961). The link between the strongly positively-charged colloidal iron and acid proteoglycans can be demonstrated under the light microscope through its reaction with potassium ferrocyanide and the formation of Prussian blue and, under the electron microscope, by the intrinsic iron electron density (Figure 2A).

Actually, some of the methods routinely used to show the presence of specific substances in histological sections by light microscopy can be applied to show the same substances in ultrathin sections by electron microscopy. This is true not only of colloidal iron (Curran *et al.,* 1965; Matukas *et al.,* 1967; Takagi *et al.,* 1982), but also of other cationic, electron-dense molecules which react with acid proteoglycans, such as ruthenium red, cationized ferritin, tannic acid-uranyl acetate (Spicer and Schulte 1982), colloidal thorium dioxide (Scherft and Moskalewski 1984), and bismuth nitrate (Smith 1970). These substances have been applied to the study of the calcification process in bone, cartilage, dentin and other hard tissues (Kobayashi 1971). A proportion of the acid proteoglycans may be lost during fixation, so better results are obtained when they are used in tissues that have been fixed with cetylpyridinium chloride-glutaraldehyde (Eisenstein *et al.,* 1971; Chardin *et al.,* 1990; Hirabayashi *et al.,* 1995), ruthenium red (Nuehring *et al.,* 1991), cationic dye-formaldehyde or -glutaraldehyde (Hunziker and Schenk 1987; Takagi 1990; Shepard 1992), or when cryopreservation methods are used (high pressure freezing, freeze substitution, and low temperature embedding; discussed by Hunziker and Schenk 1984). These methods, in fact, stabilize the proteoglycan molecules so that, even if they collapse during fixation by tissue soaking in aldehyde solutions and take on a granular shape (Matukas *et al.,* 1967), they keep their original, extended, filament-like shape when they are stabilized by cations (Shepard 1992) or are cryopreserved (Hunziker and Schenk 1984, 1987; Hunziker and Herrmann 1990).

Acid proteoglycans in calcified tissues have also been studied with immunohistochemical methods

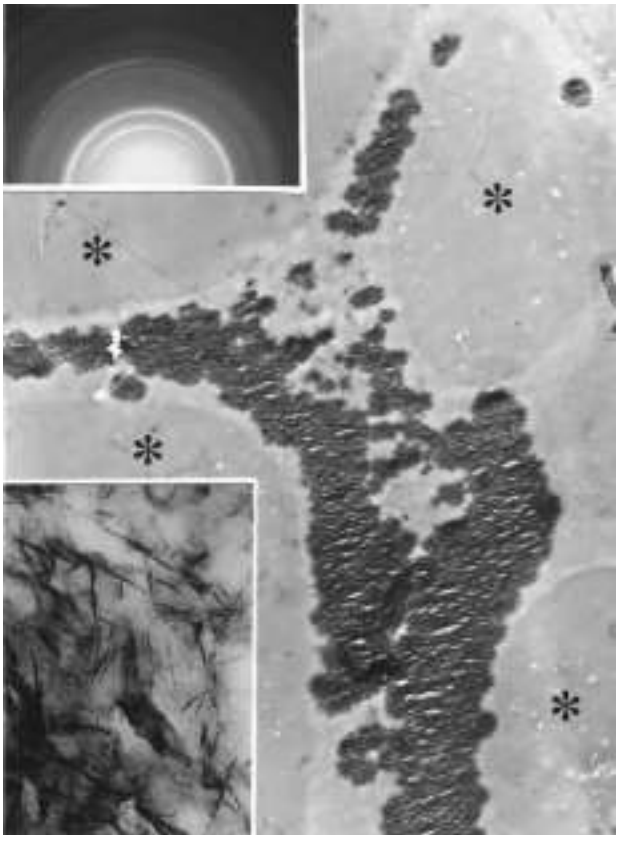

Figure 2. Electron microscopy of an unstained section of epiphyseal cartilage: the inorganic substance appears as an electron-dense material located in intercellular matrix; asterisks show chondrocyte lacunae. x 7,500. Upper inset: electron diffraction of the same calcified area; the diffractogram shows that the inorganic substance is hydroxyapatite-like and that it probably consists of needle-shaped crystalline nanoparticles. Lower inset: The enlargment of the electron microscope picture confirms that the calcified areas contain needle-shaped nanoparticles. x 145,000.

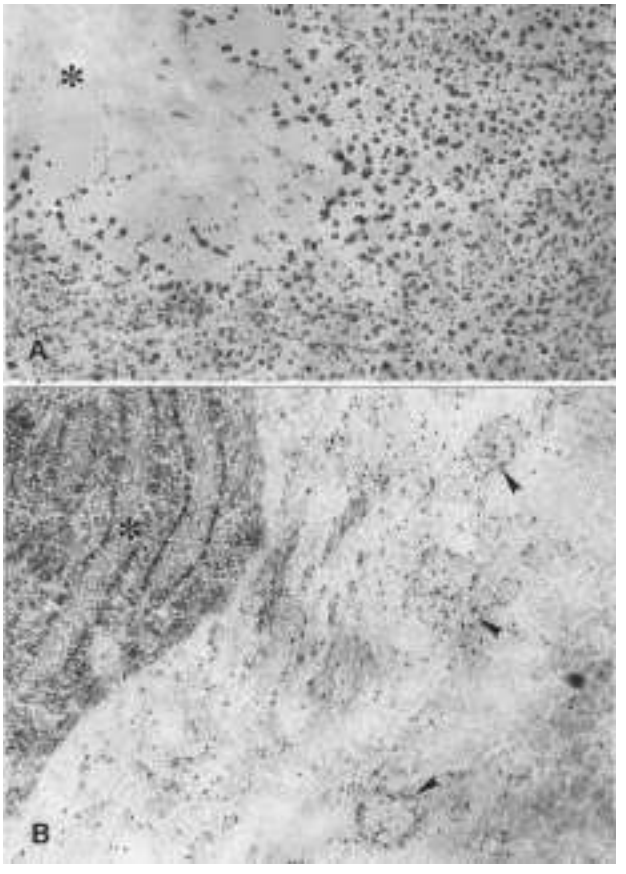

Figure 3. Different ways of showing acid proteoglycans under the electron microscope. A) Matrix of epiphyseal cartilage stained with colloidal iron at pH 2.8; iron 'stains' acid proteoglycans which appear as granular structures because their molecules collapsed during specimen fixation and dehydration. The asterisk shows a chondrocyte lacuna. X 20,000. B) Immunohistochemistry of an area of ossification; CS-56 monoclonal antibody immunospecific for the glycosaminoglycan portion of chondroitin sulfate: immunospecific gold particles are present in the uncalcified osteoid matrix and in that of early areas of calcification (calcification nodules; arrowheads). The asterisk shows part the cytoplasm of an osteoblast. X 25,000.

both under the light and the electron microscope (Figure 2B). A fall in the protein-polysaccharide content of cartilage before or during calcification has been shown with fluorescein-labeled antibodies by Hirschman and Dziewiatkowski (1966). The antibody CS-56 has been used to show chondroitin sulfate distribution in epiphyseal cartilage and to document its absence in the proliferative zone (Nakano *et al.,* 1996). The same antibody has been used to verify the presence of chondroitin sulfate in cartilage calcification nodules under the electron microscope using colloidal gold post-embedding immunocytochemistry (Bonucci and Silvestrini 1992). The monoclonal antibody 2-B-6, which specifically recognizes chondroitin-4-sulfate or dermatan sulfate after digestion with chondroitinase ABC in RHT-fixed, LR-White-embedded tissues, has been used to show their distribution in cartilage (Hagiwara 1992) and in dentin (Septier *et al.,* 1998). Antibodies are also available for the localization of biglycan (Takagi *et al.,* 2000) and keratan sulfate (Daugaard *et al.,* 1991; Nakano *et al.,* 1996). Immunohistochemical studies of Nakamura *et al.,* (2001) have shown the presence of keratan sulfate around matrix vesicles and in calcification nodules of the rat calvarium.

The so-called *non-collagenous* proteins are the components within calcifying matrices to which the interest of investigators is at present primarily directed. They comprise a wide group of mostly phosphorylated (acidic), glycoprotein molecules whose inappropriate, generic denomination possibly derived from a wish to stress that bone calcification was not exclusively due to collagen fibrils, so countering a widespread view of that period, and that specific (non-collagenous) proteins of the matrix may also have a role in the process. Whatever their generic name, proteins of that type have been found in all calcifying tissues and, although their role is still uncertain, many believe they are the main regulators of the calcification process (reviewed by De Bernard 1982; Butler *et al.,* 1985; Fisher and Termine 1985; Boskey 1989; Bonucci 2000). This view is supported not only by their presence in tissues, such as tooth enamel or mollusc shells, which contain no collagen fibrils, but, above all, by their affinity for calcium ions (calcium-binding proteins) and by their close connection with inorganic substance in calcified areas. They can, in fact, only be totally extracted from the calcified matrix after this has been decalcified (Linde *et al.,* 1980; Termine *et al.,* 1980, 1981). They can be studied with immunohistochemical methods (Bianco 1990) and a number of antibodies directed to specific proteins are now available. The immunoreaction can be carried out on histological sections, where it can easily be recognized either by fluorescence, if an antibody linked to a fluorochrome has been used, or by the development of a stained product, if the antibody has been linked to an enzyme such as peroxidase or alkaline phosphatase. Immunoreaction can also be applied to thin sections for electron microscopy, mostly using the protein A-colloidal gold method (McKee and Nanci 1995) or the enzymes reported above, and/or can be carried out by non-morphological methods such as immunoblotting (Nanci *et al.,* 1998; Fukae *et al.,* 2001).

Using these and other types of immunoreaction, a number of proteins thought to have a role in calcification have been detected in calcified matrices (Bronckers *et al.,* 1989; Bianco 1990). The main ones found in bone are osteocalcin, osteonectin, osteopontin (OPN), bone sialoprotein (BSP) and bone acidic glycoprotein-75 (BAG-75). Except for the first, these are all phosphorylated glycoproteins. Their role in calcification is still under discussion; it is interesting, however, that three of them, OPN, BSP and BAG-75 co-localize in *calcification nodules*, i.e., areas of initial calcification which develop in the osteoid tissue (Bianco *et al.,* 1993; Riminucci *et al.,* 1995; Nanci 1999). They are also found in cement lines (Chen *et al.,* 1994).

Similar proteins are found in other calcified tissues. A highly phosphorylated protein, called *phosphophoryn*, has been demonstrated in dentin (Dimuzio and Veis 1978), where its concentration appears to be directly related to the amount of mineral (Rahima *et al.,* 1988); its concentration is, in fact, high at the predentin-dentin interface but gradually falls in passing towards the enamel (Nakamura *et al.,* 1985). No collagen fibrils are present in enamel matrix, which consists of non-collagenous proteins only (for review see Nanci 2003). These are highly acidic proteins tightly bound to enamel crystals. They can be divided into two groups, amelogenins and nonamelogenins; the latter include ameloblastin (also named amelin, or sheathlin), tuftelin, enamelin and a 65 kDa glycoprotein. With enamel maturation, they undergo progressive extracellular proteolysis, which characterizes various stages of enamel development and ultimately leads to their almost complete disappearance, as shown both by extraction methods and immunohistochemistry (Blumen and Merzel 1972; Nanci *et al.,* 1992; Nanci *et al.,* 1994; Smith and Nanci 1996). Mineral-bound glycoproteins are also found in the matrix of mollusc shells (Travis and Gonsalves 1969; Wheeler 1992; Sudo *et al.,* 1997) and other calcified structures of invertebrates (Benson and Wilt 1992; Wilt *et al.,* 2003).

Besides collagen fibers and non-collagenous proteins, other organic components can be found in calcifying matrices. These include lipids, which have long been known to occur in calcifying areas, where they are so strongly bound to the inorganic substance that a fraction of them can only be extracted after decalcification (Shapiro 1970a; Shapiro 1970b). First shown histochemically in calcifying cartilage (Irving 1960), their presence as acidic phospholipids has been confirmed under the electron microscope in cartilage (Bonucci and Silvestrini 1994) and in bone (Takahashi *et al.,* 1991; Nefussi *et al.,* 1992; Bonucci and Silvestrini 1995). They have also been demonstrated in dentin fixed in the presence of malachite green (Goldberg and Septier 1985) or treated with iodoplatinate (Vermelin *et al.,* 1994), and in cartilage and bone fixed with malachite green and treated with the phospholipase A2-gold method (Silvestrini *et al.,* 1996). The results of these investigations, in agreement with those of biochemical studies (Peress *et al.,* 1974; Wuthier 1975; Wuthier and Gore 1977; Wu *et al.,* 2002), have shown that lipids are contained in matrix vesicles. Calcium-acidic phospholipid-phosphate complexes are formed which may initiate the calcification process in cartilage (Boskey *et al.,* 1980) and bone (Boskey and Posner 1976; Boskey *et al.,* 1982), as well as in unicellular organisms (Boyan *et al.,* 1984) and pathological vascular calcification (Dmitrovsky and Boskey 1985). Phospholipids have been shown immunohistochemically in cartilage and bone (Bonucci *et al.,* 1997) and in dentin matrix vesicles (Tsuji *et al.,* 1994) using the MC22-33F monoclonal antibody, which specifically reacts with choline-containing phospholipids (Mark *et al.,* 1992).

Considering that the most distinctive feature of the calcified matrices is that they contain inorganic substance, it is hardly surprising that this has been the topic of a large number of studies. Over a long period these studies have mainly been based on X-ray diffraction, which has shown that the inorganic substance of bone and other calcified tissues is hydroxyapatite. This technique (Finean and Engström 1953; Carlström and Finean 1954), along with electron microscopy (Bocciarelli 1970; Ascenzi *et al.,* 1978; Jackson *et al.,* 1978), suggested that apatite appears in bone as very thin, needle- or platelet-like crystals (reviewed by Elliott 1973). These structures (shown in Figure 2, inset) are, however, poorly crystallized and highly impure and, especially at the earliest stage of formation, show very little crystal arrangement (Bachra 1967), so it has been suggested that they are paracrystalline structures comparable with biopolymers (Wheeler and Lewis 1977; Arnold *et al.,* 2001). Possibly for this reason, they have been called *mineralites* instead of crystallites (Eppell *et al.,* 2001; Tong *et al.,* 2003).

Several highly refined techniques, some of which allow nanoparticles and atoms to be detected, have been used to study the problem of the structure and composition of the earliest inorganic particles and their relationships with organic components. These techniques have the disadvantage of being rather complex and not available in all laboratories, but they yield important results, ranging from the high spatial resolution of element distribution to the evaluation of the local concentration of nanoparticles and single elements, from the assessment of the degree of crystallinity to the appraisal of macromolecular crystals at subnanometer resolution, and from mapping specific molecules to imaging protein surface and membrane components. An excellent example of what these techniques can achieve is the images of individual aggregans macromolecules and their constituent glycosaminoglycan chains obtained by Ng *et al.,* (2003) using atomic force microscopy.

Some of these method are longstanding, others are new. The list is quite long. Mentioning only the most important ones, they comprise wide (Engfeldt *et al.,* 1985) and small (Fratzl *et al.,* 1991) angle X-ray diffraction and synchrotron radiation (Ascenzi *et al.,* 1985); selected area electron diffraction (shown in Figure 2, inset; Landis and Glimcher 1978; Arnold *et al.,* 1999); neutron diffraction (Wenk and Heidelbach 1999; Girardin *et al.,* 2000); electron spin resonance spectrometry (Ostrowski *et al.,* 1972; Roufosse *et al.,* 1976; Ascenzi *et al.,* 1977); energy dispersive X-ray elemental analysis, otherwise known as electron-probe analysis (Lewinson and Silbermann 1990); energy-filtering electron microscopy (Egerton 2003; Leapman 2003); infrared spectroscopy (Paschalis et al. 1996; Pleshko *et al.,* 1991); and atomic force microscopy (Reich *et al.,* 2001; Santos and Castanho 2004), besides other biophysical techniques. Taken together, these methods have produced an impressive corpus of data which, together with those obtained using histochemical (Brighton and Hunt 1976; Appleton and Morris 1979; Morris and Appleton 1980; Lewinson and Silbermann 1990) and autoradiographic (Lacroix 1960; Nagai and Frank 1974) procedures that aim to determine the movements of calcium and phosphate ions within, and their binding to, the organic matrix, have contributed a great deal to knowledge of the physiopathology of bone and other calcified tissues. The fine structure and composition of the earliest inorganic particles are, however, still uncertain and so far even the availability of techniques as sophisticated as these has failed to produce a definitive explanation of the way inorganic substance is actually deposited in the matrix.

Many of the difficulties encountered springs from the close relationship that links inorganic and organic substance. The former masks the latter, which can, in its turn, hinder detection of the former. The merging of these two components often makes it imperative to prepare for morphological studies by using decalcification to unmask the organic matrix. Regrettably, decalcification disrupts the structure of the calcified matrix (reviewed by Callis and Sterchi 1998). Several methods have been proposed to preclude the extraction artefacts that derive from the removal of inorganic substance and the loss of organic components. The method which appears to yield the best results is known as PEDS (post-embedding decalcification and staining). In this case, sections of embedded tissues are floated on a decalcifying solution before they are stained (Bonucci 1967; Bonucci and Reurink 1978). This leaves the organic components intact, because they are protected against the dangerous effects of decalcification by being embedded in the resin, as shown by their ultrastructure, which is so well preserved that the tissue seems not to have been decalcified at all (Bonucci 1992b). Histochemical and immunohistochemical reactions are possible after PEDS, provided that the embedding resin is appropriate (Bonucci and Gherardi 1975; Bonucci *et al.,* 1986, 1988, 1989; Goldberg *et al.,* 1980).

The results of PEDS confirm that the inorganic and organic substance are closely linked in all calcified tissues and suggest that the earliest inorganic particles, rather than being true crystals, are organic-inorganic, crystal-like structures whose formation and shape derive directly from the template role of organic molecules (reviewed by Bonucci 2002). The biochemical, histochemical and immunohistochemical detection of acidic, phosphorylated glycoproteins in many, if not all, calcified matrices, and their very close relationship with the inorganic substance, have brought with them a new attitude to the way crystals are formed, about the mechanism of their maturation, and about the way these proteins could trigger the process. Much remains to be done, especially on the function of single proteins and the relationship between them.

In this connection, the possibility of using transgenic, knock-out or null animal models opens up new, promising perspectives (Boskey 1998).

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