Biological Calcification

Normal and Pathological Processes in the Early Stages

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4 The Nature and Composition of the Inorganic Phase

4.1 Introduction

The nature and composition of the inorganic phase in calcified biological tissues were first studied using X-ray diffraction and other physical techniques. These were mainly applied to the study of compact bone, probably because the sheer compactness of this calcified biological tissue compared with all others makes it easier to prepare specimens for physical examination. The results obtained from compact bone have often been extrapolated to cover other calcified tissues, but it must be stressed that clear-cut differences can be found between different minerals, and even for the same type of mineral in different calcified tissues. This is shown, for example, by comparing calcium phosphate in bone, calcified cartilage and enamel, or calcium carbonate as it occurs in various lower organisms. Boyan et al. (1992) reported that as many as 31 biogenic mineral compounds, most of them including calcium, are distributed across all phyla, and Mann (1988) found that over 40 different minerals can be identified in living organisms. Most of these minerals are calcium salts, but other ions can contribute to the formation of intra- and extracellular inorganic substance. Iron can form magnetite in some bacteria and in the teeth of some mollusks (limpets and chitons), barium can be found as sulfate in the organules of some desmids, strontium contributes to the formation of spines in *acantharians*, and silica is found in unicellular organisms and plants (Mann 2001). It is just the variability of the inorganic substance composition that lead many to prefer the term mineralization instead of calcification; this topic has been discussed in Sect. 2.4.

Of all these mineral compounds, those which form the bulk of, and are found most often in, inorganic substances in the animal kingdom will be considered in the following pages, with special reference to calcium phosphate and calcium carbonate. Silicates present in higher and lower plants and in terrestrial and marine organisms are not discussed; several articles and chapters of books can be consulted on that topic (Arnott and Pautard 1970; Arnott 1976; Leadbeater and Riding 1986; Mann 2001; Perry 2003; Sumerel and Morse 2003; Foo et al. 2004).

4.2 Vertebrates

The normally calcified tissues of vertebrates are those of the skeleton (bone and growth cartilage), of teeth (dentin, cementum, enamel) and, as an aging process, some cartilaginous segments like the rib, trachea and larynx cartilages, and tendons in some animals.

4.2.1 Bone

The pioneering studies of deJong (1926) and Roseberry et al. (1931) were the first to show that the inorganic substance of bone consists of very small particles whose X-ray diffractograms resemble those of polycrystalline samples of natural hydroxyapatite. This is the most likely reason why inorganic particles came to be called "crystals" or "crystallites", even in referring to developing bone salt which, as noted by Arnott and Pautard (1967), is usually said to consist of crystals, without any proof being offered that any portion of the area is specifically crystalline. One of the conclusions of the in vitro studies of Eanes and Meyer (1977) was that the first crystals formed in solution show marked divergences from apatite in their morphology, composition, structure, and solubility, and that only with maturation do they take on apatite-like features. Setting this problem aside for a moment, the term "crystals" will be used from now on to refer to the inorganic particles found in calcifying and calcified areas, independently of their degree of crystallinity and without any strictly crystallographic implications. It must be stated, however, that, as outlined by Eanes and Posner (1970) in their review of the structure and chemistry of inorganic substance in bone, it is generally accepted that in bone the inorganic substance is crystalline – irrespective of its stage of formation – and that it is apatitic in structure and composition, a conclusion supported by a number of X-ray diffraction studies and selected area electron diffraction (Lefèvre et al. 1937; Bale 1940; Carlström 1955; Trautz 1955; Wallgren 1957; Urist and Dowell 1967; Lénárt et al. 1968, 1971, 1979; Landis and Glimcher 1978). The inorganic particles of bone have been considered to be crystals; this is the most likely reason why they have been regarded from a strictly mineralogical standpoint as structures possessing the same atomic configuration as hydroxyapatite, whose unit cell is a rhombic prism with an *a*-axis of 943 nm and a *c*-axis of 688 nm, and with a formula of $Ca_{10}(PO_4)_6(OH)_2$ (often simplified as $Ca_5(PO_4)_3OH$; see Posner 1987).

The inorganic ions which go to form bone crystals are not, however, calcium and phosphate alone, as the formula reported above seems to suggest. Carbonate, which accounts for about 5% of the total weight of bone ash, is found together with magnesium, sodium, potassium, and other ions, even if

their exact position within the crystal unit is not known (Eanes and Posner 1970). The Ca/P molar ratio is itself variable; it ranges between 1.57 and 1.71 (Woodard 1962), and therefore only rarely reaches the theoretical value of 1.67 the bone inorganic substance should have, if the formula given above is correct (Mellors 1964; Glimcher 1990). On the other hand, Zipkin (1970), from an analysis of the literature, reported that the Ca/P values, given as weight ratio, range between 2.09 and 2.25 in adult human bone, and that values from 1.82 to 1.98 are specific for fetal human bone. Variations may depend on the fact that the Ca/P molar ratio increases from a value of 1.35 in the earliest inorganic deposits (osteoid calcification nodules) to 1.60 in the heavily calcified regions (Wergedal and Baylink 1974) or, according to Landis and Glimcher (1978), from 1.60–1.70 to 1.81–1.97.

Several theories have been put forward to explain the non-stoichiometry of bone hydroxyapatite, such as lattice substitutions, excess ion adsorption on the crystal surface, deficiency of calcium ions, and the addition of a second phase (Posner 1969, 1987; Eanes and Posner 1970; Elliott 1973). Apart from its non-stoichiometry, bone apatite differs from natural apatites in leading to the formation of pyrophosphate when heated to 200–600 °C, in containing some tightly-bound water of crystallization, and in being associated with considerable amounts of extraneous ions (Glimcher and Krane 1968). Several different constituent ions may, in fact, be substituted in the crystal lattice of bone apatite – to give three examples, hydroxyl or phosphate ions may be replaced by carbonate ions, hydroxyl ions by chloride or fluoride ions, and calcium ions by magnesium ions (McConnell 1952; Trautz 1955; François and Herman 1961; Posner 1969, 1987; Elliott 1973; Young 1974). Strontium, barium, zinc, iron, lead, aluminum, bromine are common trace elements in biological apatites (Posner 1987; Zylberberg et al. 1992). As a result, the values of the *a* and *c* axes within the crystal unit cell, as well as the *a*/*c* ratio, may change with the type of substituting ions (Smith and Smith 1976).

It can be concluded that bone "hydroxyapatite" is by no means identical with natural hydroxyapatite. The various formulas that have been suggested for the inorganic phase of bone, as well as the inorganic composition of adult human bone, dentin and enamel, have been reviewed by Zipkin (1970). According to Arnold et al. (2001), the primary crystals of developing hard tissues (bone, dentin, enamel) are apatitic but their crystal lattice may contain so many distortions that they should be viewed as belonging to a state intermediate between amorphous and crystalline; in other words, they have a paracrystalline character, comparable with biopolymers. These lattice fluctuations appear to decrease with the loss of organic material in the matrix and with crystal maturation. Wheeler and Lewis (1977) too reported on the paracrystalline nature of bone apatite: they calculated that the paracrystalline mean distance fluctuations are 1.5 and 2.9% for the basal and prism planes, respectively, and that the corresponding paracrystalline sizes are 2.2 and 0.7 nm. They

also observed that heating above 600 °C increases the degree of crystalline regularity. As discussed below, it is known that at this temperature, or above, the structural characteristics of bone crystals are completely subverted (Fig. 4.1).

The structure and composition of crystals can vary. They become more apatite-like with age and maturation (Smith and Smith 1976; Eanes and Meyer 1977), even if the high degree of crystallinity of natural or synthetic apatites is never approached. X-ray diffraction and infrared spectroscopy concur in indicating that the degree of crystallinity increases with bone age (Posner et al. 1965; Burnell et al. 1980). Bonar et al. (1983) used density fractionation to reduce the heterogeneity of bone and select bone fractions of increasing density, hence of increasing degree of calcification and, presumably, tissue age; they confirmed that the degree of crystallinity increases with tissue age, and therefore with mineral age, as well as animal age – a clear indication that changes in bone inorganic substance occur even after calcification is complete

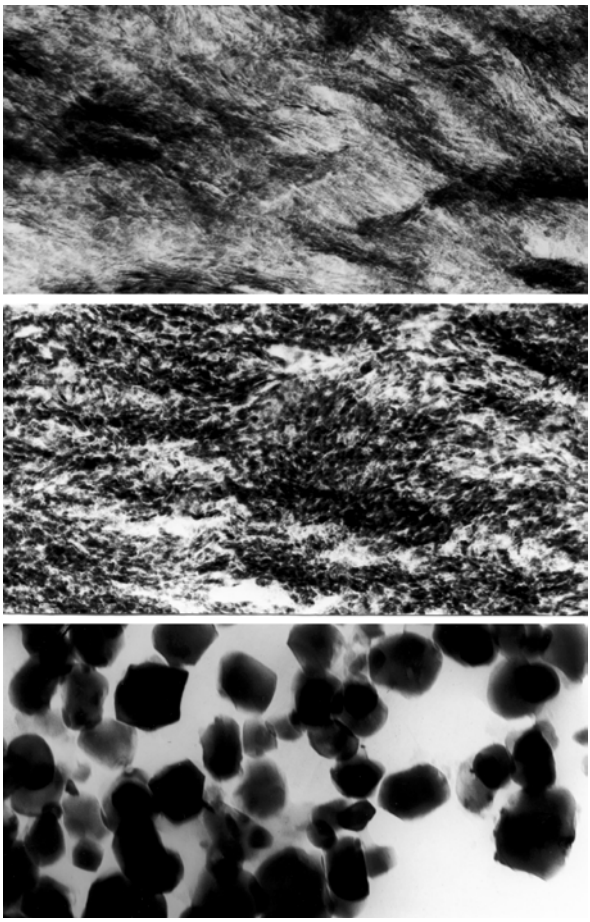

Fig. 4.1. Ultrastructural picture of natural compact ox bone (*above*), and of the same type of bone after heating at 350–550 °C (*middle*) and at over 650 °C (*below*). Untreated, ×60,000

or nearly complete. Wu et al. (2002), by ^{31}P solid state nuclear magnetic resonance spin-spin relaxation studies, found that, in bone, enamel and synthetic hydroxyapatite crystals, a significant fraction of the protonated HPO_4^{2-} has a superficial location, while the unprotonated PO_4^{3-} is concentrated within the apatite lattice. Ascenzi et al. (1977) found that in isolated osteons at different degrees of calcification the crystallinity coefficient – defined as the ratio of the number of radiation-induced paramagnetic defects in the crystal lattice of hydroxyapatite to the total ash content – increases with the degree of matrix calcification, i. e., with mineral age. They reported that human osteons at the initial stage of calcification contained 57% ash when their crystallinity coefficient was 40.6, whereas human fully calcified osteons contained 60% ash when their crystallinity coefficient was 52.1.

For all these reasons, the composition of bone crystals has been the topic of a large number of investigations, which have not, however, led to a definitive solution. It has gradually become clear that although the close similarity between the bone inorganic substance and hydroxyapatite is undeniable, the unit cell dimensions of bone crystals have been given a range of different values (Caglioti 1935; Gruner et al. 1937; Stuehler 1937; Bale 1940; Hendricks and Hill 1942) and their true atomic configuration is still an unresolved issue.

It was realized by several investigators that the main cause of confusion was due to the broad, fuzzy reflections of the X-ray diffraction of bone, caused by the extreme smallness of the crystals and their haphazard orientation (reviewed by Engström 1960). The fact that crystals are mixed with the components of the organic matrix does not seem to account for the low degree of resolution of the diffraction rings, because their appearance is not improved by hydrazine deproteinization (Termine et al. 1973). What can be said is that the lack of order in crystal structure attributable to the presence of carbonate could be partly responsible for lowering the resolution of the X-ray diffraction (Blumenthal et al. 1975). It must be added that X-ray diffractograms of the various isomorphic types of apatite (hydroxyapatite, carbonatoapatite, fluorapatite, oxyapatite, and so on) are so similar (Hirschman et al. 1953) that it is very hard, or impossible, to distinguish between them. Neuman and Neuman (1953) clearly stated that any preparation of basic calcium phosphate with a Ca/P ratio between 1.33 and 2.0 shows the X-ray diffraction of the apatite lattice, and that this should not be considered a compound but a spatial arrangement of atoms common to a number of minerals. The difficulty is increased by the fuzziness of the bone diffraction reflections. It has been reported that sharpness can be increased by heating bone to 500–600 °C, which produces sharp apatite diffractograms (Carlström and Finean 1954), but this procedure turns the inorganic bone substance into a carbonatoapatite (Dallemagne and Brasseur 1942) and induces changes in its molecular arrangement (Fig. 4.1; Bonucci and Graziani 1976; Sakae 1988; Rogers and Daniels 2002). It is striking that the diffuse diffraction lines of developing enamel crystals become sharper as calcification progresses, indicating crystal growth, or even perfection (Nylen et al. 1963).