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Chapter 3

STRUCTURALLY INCORPORATED WATER IN BONE APATITE: A CAUTIONARY TALE*

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ABSTRACT

The calcium phosphate mineral hydroxylapatite [OHAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] exhibits remarkable properties from both a medical-biological and a mineralogical viewpoint. These physical and chemical properties were quantitatively evaluated over many decades of research. Even more time has been required to determine (or, at least, to infer) the physical-chemical mechanisms that control those properties, such as crystal shape, hardness, and solubility in synthetic materials, and the ability to change composition over time and to strongly adhere to collagen in the biological milieu. This chapter summarizes and analyzes the slow acceptance, particularly from the medical and dental communities, of the notion that molecular H_2O resides at the few weight-percent level both in synthetic carbonated OHAp and in bone mineral. The purpose of this review is to document the very convoluted pathway leading to the still-incomplete acceptance of structurally incorporated water in OHAp and to analyze why this recognition took so long. Some comparisons are made with related controversies over the chemical form in which carbonate occurs in bone and tooth and over the state of hydroxylation of biological apatites. This retrospective literature analysis, going back to the mid-1800s, provides an example of why one should not disregard reproducible but unexpected results simply because of the absence of a readily apparent model to account for them. The fact of structural water in apatite initially was overlooked and subsequently avoided. It currently has worked its way to the level of simply undervalued. The culmination of this story lies

* This chapter provides information on the development of knowledge specifically about biological apatite that has been referenced in Chapter 1 by Sergey Dorozhkin but could not be evaluated in much detail there. Hence these two chapters are designed to completely cover the complex, convoluted, controversial, comprehensive and labyrinthine ways in which acquisition of information and knowledge on one of the most important minerals on Earth has progressed. The Editor.

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in the future, because there are always ramifications to structural-chemical variations in a mineral. It is time to begin to explore the effects of crystallographically incorporated water on the properties of bioapatite.

1. INTRODUCTION TO CARBONATED BIOLOGICAL AND SYNTHETIC APATITE

As reported in numerous mineralogy sources [1, 2], the mineral name ‘apatite’ is derived from the Greek for ‘to deceive.’ The textbook explanation for the nature of the deception is that samples of natural, geologic apatite can be mistaken for other minerals of similar appearance. Review of the chemical, mineralogic, and medical literature of the past one hundred or more years, however, suggests that the real deceptiveness of apatite comes from its ability to accept into its structure about half the elements in the periodic table [3,4], thereby causing substantial changes in its chemical and physical properties. The simplified mineral formula for the apatite group is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH},\text{F},\text{Cl})_2$, representing its three most common natural species, i.e., hydroxylapatite (OHAp), fluorapatite (FAP), and chlorapatite (ClAp). The species most similar to that which occurs biologically in bone and tooth is hydroxylapatite. That said, there is a large and medically very important difference between biological apatite (bioapatite) and hydroxylapatite. Although many of these differences are now understood, in numerous cases after years of controversy, the model of bone apatite is still not complete.

Although in a sense it is only *details* that remain to be worked out, these very specific aspects of bone apatite could be of considerable importance to the understanding of, for instance, what changes occur in bone mineral during the ageing process and how such changes might be prevented or slowed, and what parameters of the mineral and the process of its fabrication might be tweaked for the purpose of producing better biomaterials.

The present chapter begins with an historical overview of the many steps to our current understanding of the presence of structurally incorporated water in bone apatite and in its synthetic carbonated apatite analogs. The conclusion of this documentation is that the notion of structural water in bioapatite still has not pervaded the major literature in the medical, dental, and related fields. A retrospective analysis follows, concerning how this important topic has remained as more of a backwater discussion than a raging controversy for well over one hundred years.

The associated research activities and minor debates summarized below illustrate the importance of models (as well as their limitations), demonstrate temporal changes in the notion of what constitutes interdisciplinary research, and suggest future pathways to enhanced understanding of bone and tooth tissue.

2. SETTING THE STAGE: ONE CONTROVERSY AFTER ANOTHER

Apatites follow the compositional template $\text{X}_4\text{Y}_6(\text{PO}_4)_6\text{Z}_2$, which in hydroxylapatite, OHAp, becomes $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$. Among calcium phosphate phases, apatites are

recognized by their anion-filled channels oriented along the *c*-axis of the structure (see Figure 1). Those six of the ten calcium ions that define the boundary of the channels are labeled Ca(2), and the other four calcium ions within the structure are labeled as Ca(1).

One of the earliest revelations about *bioapatite* was that, in addition to phosphate, it also contains carbonate. Even within the past 60 years, however, there has been debate about whether the carbonate in the bulk bone material represents the coexistence of the calcium carbonate mineral calcite (CaCO_3) together with apatite or whether the carbonate is a constituent of the apatite phase [5-7]. It was ultimately established that carbonate is an actual substituent within the crystalline structure of the apatite [8], but that fact did not fully permeate the medical literature until many years later (see debate in [9,10]).

Subsequently, multiple research groups worked to show that bone apatite incorporates about 5-8 wt% carbonate (CO_3)²⁻ and that the carbonate dominantly substitutes for phosphate (PO_4)³⁻ tetrahedra in the crystal lattice, a substitution labeled as type B [11-13]¹. From the viewpoint of the modeling of crystal behavior, one should note that this is actually an unlikely substitution. The phosphate and carbonate atomic clusters have greatly different sizes and shapes (3-D tetrahedral vs. 2-D triangular, respectively), as well as different ionic charges.

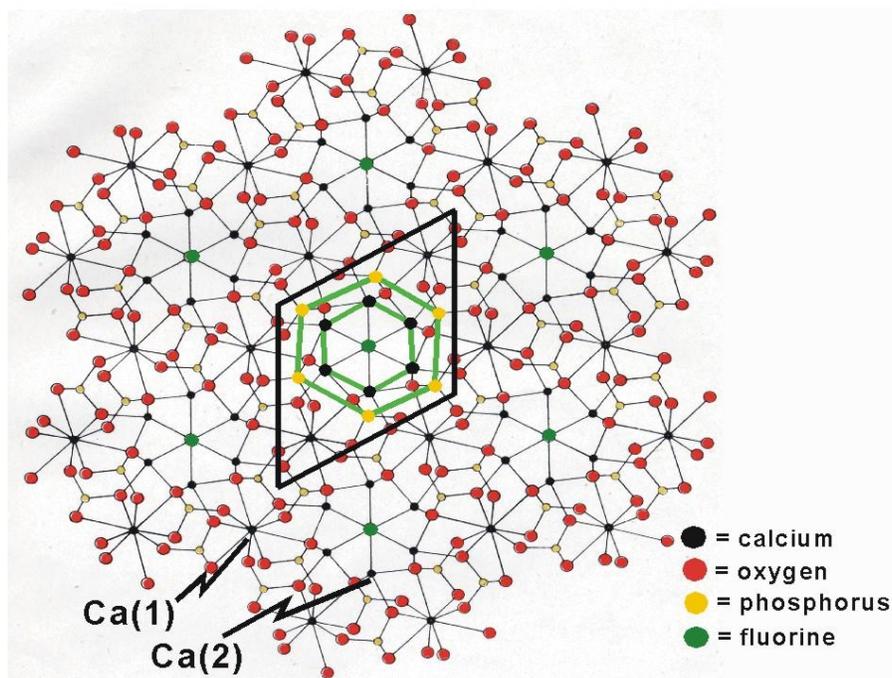


Figure 1. Atomic structure of the fluoride analog to hydroxylapatite, i.e., fluorapatite, $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$, in which one unit cell is outlined with a black rhombus. The site for the channel anion (central green, filled circle), e.g., OH^- in OHAp, is outlined in a small hexagon. The black, filled circles at the apices of the inner hexagon represent the channel-defining Ca(2) cations. Ca(1) cations do not share any bonds with the channel anions. Figure from [40] (their figure 5), with some additions. (Reproduced with kind permission of the Mineralogical Society from a paper by Beever and McIntyre (1946)).

¹ For more detailed information on carbonate substitution see Chapter 2. The Editor.

A much less common incorporation mechanism in bone mineral is $(\text{CO}_3)^{2-}$ substituting for OH^- in the channel sites of apatite, referred to as type-A substitution, which also necessitates additional charge balancing [11]. One therefore can understand some reluctance in the initial acceptance that large amounts of $(\text{CO}_3)^{2-}$ substitute into apatite, even though carbonate does not even appear in the OHAp formula. Intriguingly, there was an even longer period of uncertainty about the exact concentration of the hydroxyl ion (OH^-) in bone mineral, despite hydroxyl's explicit appearance in the formula of OHAp. Bone apatite's deficiency in hydroxyl compared to pure OHAp was first realized more than 35 years ago [14,15] and experimentally explored in synthetic calcium phosphate phases shortly later [16]. In spite of these data, a long-extended controversy later ensued concerning exactly how much/little hydroxyl ion (OH^-) exists in bone mineral and what controls its concentration [17-19]. The most recent analyses indicate that bone mineral contains only about 15-20% of the hydroxyl content in pure OHAp [20,21], i.e., about an 80-85% hydroxyl deficiency. To a large degree, the deficiency in hydroxyl can be explained by the need for charge balance as CO_3^{2-} ions substitute for PO_4^{3-} , as represented by the formula $\text{Ca}_{10-x}[(\text{PO}_4)_{6-x}(\text{CO}_3)_x](\text{OH})_{2-x}$ [12,13,17,19,22,23]. As a result of these chemical-structural adaptations, bone mineral typically does not have a Ca:P ratio of 1.67. It also is hydroxyl-deficient, as recorded in a hydroxyl (OH^-) ion content of less than 2 per crystallographic stoichiometric unit cell. There is, however, yet another complexity in comparing bioapatite with pure OHAp. For over 120 years, chemical, geological, and medical researchers sometimes have argued, but more often simply dithered, about whether any of the molecular water repeatedly shown to be associated with both synthetic and bone-derived carbonated apatite is actually part of the latter's crystallographic lattice or just adsorbed on the mineral's surface. In summary, analyses and discussions that have spanned many decades of time, multiple scientific fields, and several generations of ever-improving analytical instrumentation somehow have never gained community-wide recognition of the fact that bone mineral contains as essential hydrogen-bearing components not only the hydroxyl ion (OH^-), but also molecular water (H_2O), in amounts as great as a few weight percent.

3. THE STORY THAT WOULD NOT END: WHAT ABOUT THE WATER?

'Modern' scientific investigation of bone mineral began about 150 years ago with the recognition that the composition of the solid phase in bone is dominated by calcium and phosphate [24] but that it also contains carbonate [25-27]. It was about 100 years later, however, before most researchers in the field could be said to agree that the solid, i.e., mineral, portion of bone was a *single crystalline* compound and that the compound could be broadly labeled as apatitic [28] (compare debate between [10] and [9]). This chapter is organized so that one may either read the complete decade-by-decade summary below or simply read the highlights of the research up through 1969 in Table 1 and then re-rejoin the summaries of modern post-1970 research concerning water in apatite.

Interest in the water content of whole bone and the perception that some portion of the water was contained in the bone crystals themselves began in the 1800s (e.g., [25-27]; also see the excellent summary, in German, by Gabriel [5]).

Table 1. Brief Overview of the History from 1842 to 1969 of the Analysis and Interpretation of Bone Mineral with Special Reference to its Water Content

Reference	Paper type*	Some specific contribution of data, interpretation, or explicit formula for bone mineral
Compiled by Hendricks and Hill 1942		Some published formulas for the inorganic component of bone. Berzelius (1845): $\text{Ca}_5\text{H}_2(\text{PO}_4)_4$; Gassman (1937): $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$; Gruner et al (1937): $(\text{Ca,C})_{8-9}[(\text{P,C})\text{O}_4]_6\text{Ca}(\text{OH})_2$; Klement (1938): $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$
<i>1840s: Books present new bone analyses and summarize older bone analyses carried out over several previous decades.</i>		
von Bibra 1842 Book <i>Chemical Investigations on the Bones and Teeth of Humans and Vertebrates</i> (in German)	E/A	Remarkable work providing detailed chemistry of whole bones and of bone and tooth mineral of many animals and humans. Reported values for CaO, P_2O_5 , CaCO_3 , Fe, F, K, and other trace elements. Sketches of bone cross-sections as seen under microscope.
Gmelin 1849 Book <i>Handbook of Chemistry, Vol. III, Metals</i>	R	Analyses compiled on <i>bone ash</i> . Berzelius is cited as stating that this "salt" is $8\text{CaO}, 3\text{PO}_5$
<i>1870s: Analysis and interpretation of bone has become more common, and the approaches and results are more sophisticated and well documented.</i>		
Maly and Donath 1873 <i>Contributions to the chemistry of bones</i> (in German)	E/A	Investigation of solubility of bone mineral and other Ca phosphates in various solvents. Bone mineral has same solubility as synthetically precipitated Ca phosphates. Bone powder is extremely hygroscopic.
Wibel 1874 (in German) <i>The constitution of bone phosphates, especially the existence and formation of a basic compound; $(\text{Ca}_3\text{P}_2\text{O}_8 + x\text{CaO})$</i>	R	Summary of the Ph.D. thesis and related work by Aeby. Strongly discredited Aeby's analytical approach and, thereby, his proposed formula for bone mineral as $[6\text{Ca}_3\text{P}_2\text{O}_8 + 2\text{H}_2\text{O} + 2\text{CaO} + \text{CO}_2] + 3\text{H}_2\text{O}$, which Wibel described as calcium phosphate + carbonic acid + basic water + water of crystallization (author's translation of p. 114).
Aeby 1875 (in German) <i>On the chemistry of bones</i>	R	Results of several approaches to investigating content of water in whole bone and bone mineral. Proposed more than 1 wt% H_2O as water of crystallization in bone mineral. Focused on bones "naturally calcined" by fossilization, which contain about 12 wt% H_2O (including water released below 100°C).
Watts 1875 Book <i>A Dictionary of Chemistry and of the Allied Branches of Other Sciences</i>	R	Report on the composition of geological francolite [carbonate fluorapatite] with 1.5 wt% H_2O . Summarized Aeby's analyses on fossil bone as showing that both CO_2 and water of crystallization can be expelled by heating to 450°C .
<i>1890s: Analyses of both bone and other calcium phosphate phases.</i>		
Gabriel 1894 (in German) <i>Chemical investigations on the mineral components of bones and teeth</i>	R, E/A	Summary and evaluation of bone mineral analyses of previous 50 years, plus Gabriel's own work. Gabriel chemically extracted organic components from bone before doing controlled heating experiments, which revealed about 2 wt.% crystallographically incorporated H_2O in bones and teeth.
<i>1930s: Still much confusion about the nature of bone mineral. Mounting evidence for H_2O in bioapatite.</i>		
Schleede et al 1932 (in German) <i>On the understanding of calcium phosphates and apatites</i>	E/A	Weight loss from 200 to 900°C of carefully prepared (at 100°C) OHAp was 2.5 wt%. Water was almost continuously released over this interval. Water believed to be strongly bound in the crystal. Channels large enough for both OH^- ions and H_2O molecules.

Table 1. (Continued)

Reference	Paper type*	Some specific contribution of data, interpretation, or explicit formula for bone mineral
Henricks et al 1932 The crystal structures of some natural and synthetic apatite-like substances	E/A	Report of "structural reasons for supposing ...that the phosphate compound of bone is hydrated" (p. 369). Bone mineral = $\text{Ca}_{10}(\text{CO}_3)(\text{H}_2\text{O})(\text{PO}_4)_6$, p. 368.
Huggins 1937 The composition of bone and the function of the bone cell	R	Excellent summary of still-ongoing debate about WHAT bone mineral is. X-ray diffraction is making a huge contribution to this discussion. Huggins did not think bone mineral is a single compound of definable composition, but he believed water is present and held firmly in bone mineral.
McConnell 1938 The problem of the carbonate-apatites: A carbonate oxy-apatite (dahllite)	R	Report that geological, carbonated fluorapatites released approx. 1-2 wt% H_2O during heating at 110-300°C. McConnell believed that it is important to evaluate H_2O in the structural context of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH},\text{F})_2$ and that both OH^- and H_2O can be accommodated in the apatite channels.
<i>1940s: Increasing research to link the chemistry (incl. H_2O) together with the actual structure of apatite.</i>		
Eisenberger et al 1940 The basic calcium phosphates and related systems	R	Summary of the contributions of both chemists and mineralogists. Recognized the remarkable flexibility of apatite structure in accepting chemical substitutions.
Henricks et al 1942 The inorganic constitution of bone	E/A	Current (cf. 1932) support for a bone mineral formula of $[\text{Ca}_{8.50}\text{Mg}_{0.25}\text{Na}_{0.19}][(\text{PO}_4)_{5.07}(\text{CO}_3)_{1.24}](\text{H}_2\text{O})_{2.0}$. Provided structural diagrams showing how Ca^{2+} and 2F^- can be substituted by $2\text{H}_2\text{O}$.
<i>1950s: Increasing contributions from dental researchers; hydration shell recognized to enclose apatite; more structural modeling of where H_2O resides.</i>		
McConnell 1952 Problem of the carbonate apatites	E/A	Report that even when all anion sites in FAp are filled with F^- , channels still contain additional OH^- and H_2O . Proposed that H_2O substitutes for $\text{Ca}(2)$ ions, and $(\text{OH})_4$ substitutes for (PO_4) .
Neuman et al 1952 Surface chemistry of bone	E/A	Report that "...considerable amounts of water are intimately associated with the crystals of hydroxylapatite and of bone" (p. 4239). Hydration shells surround apatite crystallites, being strongly held by their surfaces.
Burnett and Zenewitz 1958 Studies of the composition of teeth: VII The moisture content of calcified tooth tissues	E/A	"In spite of the many studies of the composition of human calcified tissues, their moisture content has neither been investigated extensively nor established conclusively" (p. 581). Measured moisture release in tooth materials up to 100 and 197°C. Not all such water could be restored during (even lengthy) rehydration.
Reference	Paper type*	Some specific contribution of data, interpretation, or explicit formula for bone mineral
Neuman and Neuman 1958 Book <i>The Chemical Dynamics of Bone Mineral</i>	R	Excellent summary and enlargement upon previous research on bone mineral, with insights into questions still to be answered and experiments to be done. "...composition of most apatite preparations does not agree with the composition predicted by the assumed structure...lose significant quantities of water in prolonged heating" (p. 41).
<i>1960s: Additional and more sophisticated XRD analysis of enamel; NMR and IR analyses come into their own.</i>		
Little and Casciani 1966 The nature of water in sound human enamel	E/A	Application of NMR to define 3 types of water in enamel, released 1) up to 100° C, 2) 100-900°C, 3) 900-1300°C. #2 and 3 types are both "caged" but "freely tumbling" water and, thus, "unbound."

Reference	Paper type*	Some specific contribution of data, interpretation, or explicit formula for bone mineral
Brophy and Nash 1968 Compositional, infrared, and X-ray analysis of fossil bone	E/A	Heating experiments at 1250 or 1400°C that released up to 6 wt% H ₂ O in fossil bone samples, i.e., carbonated (~3 wt% CO ₂), fluoride-rich (~3 wt% F) apatite.
Simpson, 1968 Substitutions in apatite: Potassium-bearing apatite	E/A	Report that apatites synthesized at <100°C typically are Ca-deficient and water-rich. Can replace up to half of OH ⁻ with H ₂ O. Recorded 4.5-6.5 wt% H ₂ O. H ₂ O incorporation accounts for increase in a-dimension of unit cell.
LeGeros et al 1969 Two types of carbonate substitution in the apatite structure	E/A	Meticulous XRD and IR analyses of synthetic carbonated OHAp. Can only substitute CO ₃ for OH (in channels) in the absence of H ₂ O. "Entrapped water" in the channels could explain small increase in a-dimension of unit cell.
Paulik et al 1969 Investigation of the composition and crystal structure of bone salt by derivatography and IR spectrophotometry	E/A	Detailed TGA and IR analyses on crystallites from natural bone that had been defatted and deproteinated. Called bone mineral a "water- and carbonate ion-bearing so-called 'hydroxylapatite of non-stoichiometric composition'" (p. 421). IR monitoring of loss of much structural water by about 600°C.
Elliott 1969 Recent progress in the chemistry, crystal chemistry and structure of the apatites	R	Report of three conferences in 1967-68 focused on apatites. Recorded need to understand apatites more generally in order to understand bioapatites better. Progress being made in growth of single crystals. Record of water in non-stoichiometric hydroxylapatites. Review of NMR data on different types of water in calcified tissues.

*Paper type: R = review paper; E/A = experimental and/or analytical paper.

Although the intimate relation between water and mineral already was recognized in the 1800s, the complex internal architecture of the whole bone and the extremely small sizes of its mineral and organic components clearly were not understood. This lack of detailed knowledge did not prevent several of the earliest researchers on bone from reporting, with obvious surprise, the extremely strong hygroscopic affinity of bone, especially of pulverized, clean bone material [27, 29].

4. A SHORT HISTORY OF WATER IN INORGANIC AND BIOLOGICAL APATITES

4.1. Most of What We Needed to Know Was Revealed by the Mid-1890s

One of the major breakthroughs in the understanding of the complexity of bone mineral was made by German chemist Carl Aeby, whose several related publications in the early to mid-1870s also started a controversy concerning the appropriate means and samples by which to analyze the mineral component of bone [30]. Until Aeby's work, many of the published so-called bone analyses actually were made on bone ash, which is the residue after heating bone to at least several hundred degrees Celsius. Aeby [27] analyzed fresh bone, however, finely pulverizing it and applying a number of tests to establish its water content. He recognized not only the strongly hygroscopic nature of bone powder but also that the attraction of water increased as the mineral concentration in the bone increased. In addition, there was a rise in

temperature as the bone powder took on water. Aeby viewed the latter response as evidence for a chemical interaction between the bone mineral and water. He also observed that the bone powder could not be fully desiccated at temperatures of even 130°C. Among his other insights, Aeby [27] also realized the advantage of being able to work with bone mineral that was free of organic matter. To his credit, he declined using bone ash, which he viewed as compromised by intense heating. Instead, he looked to bone material that was naturally devoid of organics, namely fossil material (on which he had written his doctoral dissertation). Aeby [27] acknowledged that one could not claim a quantitative understanding of bone mineral by analyzing fossil bone, which he referred to as metamorphosed. He did believe, however, that a better qualitative understanding of fresh bone mineral could be derived from such work.

One of his fossil bone samples released approximately 12 wt% water during heating to about 500°C. Aeby's [31] recognition that fresh bone samples had ~10 wt% of what he called "hygroscopic moisture" led him to understand that it was about 2 wt% rather than 12 wt% water that was of mineralogic interest. [It should be noted that these water values were specified to two places to the right of the decimal.] Aeby was fully convinced that actual 'crystal water', i.e., water of crystallization, was chemically bonded with the calcium phosphate mineral in bone. He [31] reported the composition of the mineral in bone to be $[6 \text{ Ca}_3\text{P}_2\text{O}_8 + 2 \text{ H}_2\text{O} + 2 \text{ CaO} + \text{CO}_2] + 3 \text{ H}_2\text{O}$ (as formally expressed by [30]), thereby distinguishing two types of water.

Aeby was just one of several German chemists in the late 1800s who were analyzing bone material. Interestingly, it was a fellow German analyst of fresh and fossil bone material, F. Wibel, who most strongly took Aeby to task for the latter's conclusions about the chemical nature of bone material. In doing so, Wibel [30] both succinctly summarized Aeby's work and brought it to the attention of the English-speaking scientific community, where both men's work was summarized in such publications as *The Journal of the Chemical Society* [of London] in its section on Abstracts of Chemical Papers Published in Other Journals. Wibel [30] concentrated on critiquing two aspects of Aeby's analytical results: (1) the 10:3 molar ratio of CaO:P₂O₅ (i.e., 10:6 atomic Ca:P ratio) rather than the 3:1 ratio reported decades earlier by the well respected Swedish chemist, J.J. Berzelius, who published the relationship $3 \text{ CaO} + \text{P}_2\text{O}_5$ as the composition of bone ash (apparently equated with the composition of fresh bone mineral by many), and (2) the proposal of water and carbonate as explicit chemical components of bone mineral. Wibel questioned Aeby's strong reliance on heat-treatment (similar to modern-day thermogravimetric analysis) of *fossil* material, whose mineral component clearly could have changed in composition from that of fresh bone.

Dr. S. Gabriel, a later German chemist wrote an extensive publication 'Chemical investigations into the mineral component of bones and teeth' ([5], in German), which is regarded by some as the first 'modern' scientific article on that subject. The early analyses, which focused on CaO, MgO, P₂O₅, and CO₂, were done almost exclusively on ashed bone, as indicated above. Gabriel, just as Aeby twenty years earlier, realized the importance of isolating the bone mineral without heating the sample to vaporize the organic matter. Heating had always made it difficult to determine the carbonate concentration in the mineral, but, Gabriel further pronounced, it would make it impossible to evaluate the presence of "chemically bound water" (p. 274), which hardly anyone had experimentally undertaken to analyze.

Gabriel adopted the use of a 200°C mixture of glycerin and potassium hydroxide to strip the organic components from the bone, which then was further cleaned in boiling water. The residual bone showed no sign of darkening when heated, indicating its lack of residual hydrocarbons, but it was extremely hygroscopic. To remove the adsorbed water, Gabriel heated the material to 130°C and held it at temperature long enough to stabilize its weight. Additional weight loss was monitored as the sample was heated further to about 350°C. When the latter heating step was carried out in a small glass tube, the released volatiles could be condensed and shown to be water. Gabriel indicated that such water “must be regarded as chemically bound” (p. 276) to the apatite. Upon stepwise heating, the evolution of water alone was followed by that of carbon dioxide.

Gabriel was pleased that the notion of chemically bound water in fresh (unfossilized) bone had raised considerable interest, but he also was intrigued by Aeby’s [27] report of water in elephant ivory. Using the same bone and tooth samples of his own choosing, Gabriel [5] compared the amount of chemically bound water between (1) the residue of his glycerin-treated bone and the typical ash that is a residue of bone heating and (2) between a bone sample and an enamel sample. In all four cases, the amount of bound water was found to be about 2.25 wt%. Subsequent analyses showed more variability in water content among bones from different animal species.

A recurring concern was that even the most trustworthy analyses of actual bone mineral typically summed to somewhat less than 99 wt%. The question was whether this deficit was a specific, but unrecognized component. The long-sought answer to the deficit problem was eventually found by Gabriel [5] when he determined through heating to very high temperature that the bone mineral also had a considerable hydroxyl (OH⁻) component. He referred to the latter as ‘constitutional water’ to distinguish it from the ‘crystal water’ (water of crystallization) referred to above as chemically bound water. Gabriel believed his water analyses to be less accurate and reproducible than his determinations for other components in bone mineral, but estimated that the chemically bonded water amounted to about 1 mole of water of crystallization per mole of bone mineral. Thus, by 1894, it was, in principle, known that the mineral in bone (as well as in dentin and enamel) is a crystalline, hydrated, hydroxylated calcium phosphate phase.

Another important aspect of this time period is that information pertinent to bone mineral began to appear in the literature of other scientific fields. For instance, Gabriel [5] implied the usefulness of studying geological materials when he correctly concluded that natural phosphates, phosphorite rocks, and geological apatite were compositionally very similar to bone mineral. Watts [32] already had summarized earlier literature on the 1.5 wt% H₂O measured in a geological form of carbonated fluorapatite called francolite. Förster [33] followed with an in-depth chemical study of the calcium-phosphate system in order to better understand a process used in steelmaking to eliminate unwanted phosphorus from molten iron ore.

Researchers thus had defined remarkably well what bone mineral is – at least chemically – by the mid-1890s. Yet, over 100 years later, no review article on bone mineral (e.g., [13, 19, 34, 35]) listed molecular H₂O as an intrinsic component of bioapatite. As active, respected members of the community of bone and tooth researchers, the above authors were reflecting the apparent general rejection of the concept of water as an intrinsic component in bioapatite or the apparent widespread judgment that any such water was of little consequence.

4.2. Moving through the 1900s

The application of X-ray diffraction (XRD) to minerals in the early 1900s opened up the way for structural analysis of members of the apatite group. By the mid-1920s, the crystallographer de Jong [36] had used XRD on bone to irrefutably demonstrate that its mineral component was a type of apatite. Náray-Szabó [37] and Mehmel [38, 39] in the early 1930s had characterized fluorapatite crystallographically. Their work was followed 15 years later by Beevers and McIntyre's [40] small changes to fluorapatite's unit cell parameters and the exact positioning of phosphate in the structure, as reproduced in Figure 1 of this chapter. Meanwhile, the German chemists continued to play an active role in exploring the various phases in the CaO-P₂O₅-H₂O system, their solubilities in a variety of organic and inorganic acids, and the conditions under which they are stable, as well as in comparing them with the now-recognized mineral hydroxylapatite [41-43]. In their careful experiments, supported by XRD analysis of the solids, Schleede et al. [42] carried out thermogravimetric analyses (TGA) on hydroxylapatite synthesized at 100°C. During subsequent heating from 200 to 900°C, a total of approximately 2.5 wt% water was released almost continuously. Based on the very high release temperatures, the authors concluded that this water was strongly bound within the apatite. Schleede and colleagues also demonstrated that their carefully synthesized OHAp was thermally stable up to 1500°C. Inorganic chemist Klement [44] subsequently published his very meticulous analyses on bone mineral from a wide range of vertebrates, focusing on the components Ca, PO₄, and CO₃, especially their molar ratios. There was no discussion of water in the mineral, but repeated reference to hydroxylapatite's stoichiometric formula of Ca₁₀(PO₄)₆(OH)₂.

Apatite research was also being carried out on several fronts in the United States at this time. Soil and fertilizer chemists Hendricks et al. [45] were analyzing and comparing the properties of geological and synthetic apatite-related substances with a view toward understanding the most useful phosphate sources for agricultural fertilizer. They proposed that bone mineral was a member of a solid solution series of OHAp that has the formula Ca₁₀CO₃(PO₄)₆·xH₂O, in which x most likely is 1. Medical researcher C. Huggins [46] wrote a review article addressing the composition of bone, in which he accepted the hydrated formula proposed by Hendricks et al. [45], in part due to the obvious retention of water by apatite to temperatures of hundreds of degrees Celsius. Interestingly, though, Huggins was not sure that the solid component of bone consisted of only a single phase, and he therefore referred to the 'calcium phosphate complex' (p. 124).

In the 1930s, American mineralogist Duncan McConnell also began writing what would become an extensive list of articles on apatite, published in a wide range of scientific journals. His work, although shown later to be flawed in several significant ways, played an important role in changing the view of bone apatite and related carbonated apatites from that of merely *chemical* entities. In his papers, McConnell brought the most meticulous XRD analyses together with the highest quality chemical analyses (e.g., [1]). In doing so, McConnell forced several scientific communities to consider the structural as well as chemical implications of the crystallographic incorporation of carbonate into the apatite phase. Many of his samples at this time were geologic in nature, but his later work explicitly involved bone mineral and tooth enamel.

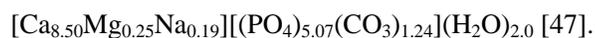
Even in his 1938 paper, McConnell already had identified some of the complications in the analysis for water in apatite that would plague researchers for decades to come. (1) Even

excellent analysts who made TGA measurements on samples apparently of very similar material could derive water concentrations that differed by 100% or more from each other's. (2) It matters greatly which temperature intervals are used to distinguish between [as McConnell, 1938, defined them] a) hygroscopic water [released below 110°C], b) *OH-ions* released as H₂O [above 300°C], and c) *water* released that "is very firmly held by superficial forces but probably is not present as OH-groups in the crystal" (between 110 and 300°C, p. 301). (3) The choice of 300°C is quite arbitrary to distinguish the origin of different aliquots of released water. In any event, the amounts of water released at 110-300°C from his carbonated apatites ranged from 1.20 to 2.10 wt% [1], marking water as a clearly significant component.

Moreover, McConnell [1] sought to determine not only where in the structure the CO₃²⁻ ions resided (a contentious and complex issue in its own right), but also where the H₂O molecules resided. For the latter, he focused on the channel sites, but unfortunately did not clearly distinguish between those TGA-derived H₂O contents that are actual water molecules in the structure and those that represent OH-ions. For the latter determination, McConnell should have selected a much higher heating temperature than 300°C for his lower bound.

In several of his papers, McConnell clearly stated that he did not believe apatite could be stable if there were large numbers of vacancies in the channels. This notion is both intriguing crystallographically (i.e., how *do* channels remain defined if they lack the contents to support them?) and very significant to the development of models for how water *could* be incorporated in apatite. For one of his carbonated apatite samples, McConnell [1] was concerned that "it does not contain sufficient water to fill the fluorine positions...[and even if all detected water] were assumed to enter the structure as hydroxyl ions there would still be a significant deficiency [less than complete fill of all the channel sites]..." (p. 301). Both the ideal formula Ca₁₀(PO₄)₆(OH)₂ and structure of OHAp had become parts of a model to which to adhere. From this time forward, others would try to assign sufficient anions and water molecules to completely fill the two channel sites per formula unit.

By the 1940s, Hendricks had investigated the chemical complexities of bone mineral further, arriving at a chemical formula that indicated more solid solution and a higher state of hydration than his previous formula, i.e.,



Meanwhile, chemical engineers Eisenberger et al. [48] had further investigated the basic Ca-phosphates, as distinguished from acidic (HPO₄)²⁻ and (H₂PO₄)⁻ phases, with a view toward both theoretical and practical applications. They especially emphasized the crystallographic conclusion of Schleede et al. [42] that the structural channels in OHAp were of sufficient size to accommodate *both* their full complement of hydroxyl ions and water molecules. The stage appeared to be set for more detailed crystallographic investigation of the structural site of water molecules.

The 1950s brought more detailed structural models for the accommodation of water molecules in apatite as well as a more sophisticated view of the 'hygroscopic water' that had been recognized since the 1800s. Thus, McConnell [8] presented crystallographic diagrams of apatite (somewhat analogous to Figure 1 in this chapter) in which H₂O molecules were shown partially replacing Ca ions, especially those in the Ca(2) sites that define the channels in the

apatite structure. The latter vacancies (absences of Ca^{2+}) were necessitated by a charge imbalance created when $(\text{CO}_3)^{2-}$ ions replaced $(\text{PO}_4)^{3-}$ ions in carbonated apatite. In addition, he proposed that some $(\text{PO}_4)^{3-}$ ions were substituted by $[(\text{OH})_4]^{4-}$ ions. These specific sitings and O-H speciations have not withstood the test of time. More importantly, however, they were proposed in response to actual analyses that showed the presence of water in apatite.

Medical researchers W.F. Neuman and colleagues [49] meanwhile had done several meticulously controlled experiments on synthetic hydroxylapatite (unfortunately, not further described or characterized) and on powdered, de-fatted bone. Among other analyses, they did high-speed centrifugation studies on synthetic OHAp in an aqueous slurry and on similar OHAp in which the aqueous phase had been isotopically spiked. The spiking allowed them to determine that the water held by the crystals does not contain the solutes of the bulk solution. Despite similar grain sizes, the particles of fresh bone became hydrated to only 40% of the level acquired by the synthetic OHAp. Based on their data, Neuman et al. [49] proposed that bone crystallites are encased in a hydration shell, much like that on natural, geological clay particles (typically less than 1 micrometer in diameter). These ideas about the surface chemistry of bone mineral were further developed and formalized in Neuman and Neuman's (1958) fine book *The Chemical Dynamics of Bone Mineral* [50].

By the end of the decade, however, dental researches Burnett and Zenewitz [51] still felt compelled to report: "In spite of the many studies of the composition of human calcified tissues, their moisture content has neither been investigated extensively nor established conclusively" (p. 581). Fortunately, the challenge of investigating water in bioapatite prompted the dental community to focus its research on enamel, which is a moderately close analog to bone. Enamel's larger and better crystalline mineral phase, however, made it more amenable than bone crystallites to chemical and structural analysis [51].

The 1960s brought a huge increase in our knowledge about the nature of bone and enamel apatite, through experiments in synthesizing apatites under controlled conditions and through the application of different and more sophisticated analytical techniques. Several important papers were published by independent dental research groups. Marguerite Little and colleagues from the Eastman Dental Center in Rochester [52, 53] specifically addressed the issue of water in enamel. Through a combination of TGA and nuclear magnetic resonance (NMR) spectroscopy, Little and Casciani [53] identified three types of water based on their analyses: (1) Water released up to 100°C , which they called unbound, freely tumbling H_2O ; (2) water released at $100\text{-}900^\circ\text{C}$, 0.8 moles H_2O based on TGA, interpreted from NMR spectra as caged but not really 'bound' in that it still was freely tumbling; and (3) water released at $900\text{-}1300^\circ\text{C}$, which they identified as 2 moles of H_2O structurally sited where others had claimed that OH resides. They also labeled the latter as caged, but freely tumbling H_2O . Little et al. [53] ultimately "speculated that enamel apatite and synthetic apatites may not be a hydroxy- but a hydro-apatite" (p. 565). It is unfortunate they did not recognize that the H_2O released above 900°C actually represented dehydroxylation of OHAp in which hydroxyl ions were released from the apatite channels in the form of H_2O . They did, however, identify 0.8 moles of actual molecular H_2O that was structurally incorporated in enamel apatite. Between the two studies referenced above came a short publication by Myers [54] on water trapped in enamel. In comparing proton NMR analyses on bioapatite samples dried at 110°C , Myers found a strong, relatively narrow peak for H_2O in enamel, even after heating it to 200°C , but only weak and very broad water peaks for dentin and bone.

Heating enamel to 500°C did eliminate the peak. Myers concluded that the persistence of such a narrow water peak to temperatures well above those that remove adsorbed moisture demonstrated this water to be “ ‘trapped’ rather than bound in the structure” (p. 713). A larger-scale NMR study on enamel and dentine by Myrberg [55] for the most part confirmed the findings of Little and Casciani and of Myers [53, 54]. The above NMR papers are based on wide-line ^1H NMR spectroscopy.

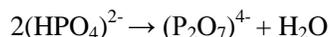
Biophysicists Carlström et al. [56], whose work had stimulated Myers [54] to carry out his investigation, believed that their optical microscopic analyses of the (changes in) birefringence of tooth enamel under controlled conditions indicated approximately 3.3 wt% ‘firmly bound’ H_2O in enamel, a result that they said confirmed Little et al.’s [52] work. Dental researcher Raquel LeGeros and colleagues in 1969 published one of the first of their many important contributions to our knowledge of enamel and bone mineral [11]. This particular paper focused on new determinations for two, specific structural sites that can be filled by carbonate ions as they substitute into the OHAp structure, now known as type A and type B. The paper is noteworthy for two additional contributions. The authors combined XRD and infrared (IR) spectroscopic information on carefully synthesized carbonated hydroxylapatite samples that had been heated to specific temperatures, thereby correlating the application of three analytical techniques. In addition, they hypothesized, that in apatites precipitated from solution, a slight increase (over geological, almost pure OHAp) in the a -axis length of the unit cell could be due to trapped water in the structure. This hypothesis became an accepted criterion in future years for inferring the presence of water in OHAp. They also observed that the (type-A) substitution of CO_3 for OH (in the channels) only took place if the synthesis was done in the absence of water.

The geologists of the 1960s were not to be outdone in the realm of new developments. Brophy and Nash [57] also applied XRD and IR analysis to heated samples, in this case fossil bone. They recorded about 5 wt% H_2O . Their work was an interesting extension of Aeby’s heat-treatment analyses of fossil bone in the mid-1870s. Dale Simpson wrote a series of papers in the 1960s about carbonated apatites and alkali-bearing apatites that he synthesized at approximately 100°C and subsequently heated to 900°C. He reported [58] that these low-temperature apatites synthesized in aqueous solutions were both Ca-deficient and water-rich. Based on a ‘modified Penfield method’ of analysis (see [59]), his potassium-bearing apatites yielded 4.5-6.5 wt% H_2O , the structural incorporation of which he believed was the cause of the enlarged a -axis value in their unit cell. In other sodium- and potassium-bearing carbonated apatites, Simpson [60] found that a total of 11 wt% H_2O was continuously evolved from room temperature to 900°C. Some of this water clearly must have been adsorbed, but a large proportion of it would appear to have been structurally incorporated. Simpson seems to have adopted McConnell’s [61] model of bone mineral, in that he proposed the water in his samples to occur as H_3O^+ ions substituting for Ca^{2+} and $(\text{H}_4\text{O}_4)^{4-}$ ions substituting for $(\text{PO}_4)^{3-}$ groups. In characterizing the rather rare geological occurrences of hydroxylapatite, McConnell [62] stated: “On the basis of the premise that all structural positions of the apatite are filled (i.e., there are no ‘holes’) the structural formula becomes $[\text{Ca}_{10}[\text{F}_{0.2}(\text{OH})_{1.5}(\text{H}_2\text{O})_{0.3}][(\text{PO}_4)_{5.7}(\text{H}_4\text{O}_4)_{0.3}]$ ” (p. 425), in this case placing molecular H_2O in the channel sites after calculating full occupancy of calcium in its two sites.

Analytical chemists Paulik and colleagues [63] applied IR analysis to deproteinized natural bone in conjunction with TGA and differential thermal analyses (DTA). From their results, they concluded that “bone salt may be considered as a water- and carbonate ion-

bearing non-stoichiometric hydroxylapatite” (p. 418), or more specifically, “a transitional substance between pure hydroxylapatite, carbonateapatite [sic!], and octocalcium [sic!] phosphate” (p. 421). Their analyses, done on materials that had been superficially dehydrated in methanol, yielded 5.4 wt% of what they labeled as structural water. Paulik et al. [63], however, seemed as confused as several chemists before them concerning the final release of 1.8 wt% H₂O at temperatures over 1000°C, still referring to this reaction as the dehydration of apatite rather than as the destabilization of OHAp as it fully dehydroxylates to form a mixture of Ca₃(PO₄)₂ and Ca(OH)₂ (the latter probably formed initially as CaO, which hydrates to Ca(OH)₂ very readily in air [18]).

James Elliott, a crystallographer who would focus his attention on natural and synthetic apatites for the next 40 years, summarized the decade’s progress in the synthesis and structural characterization of chlor-, fluor-, and hydroxyl-apatites of sufficient size for high-precision x-ray-diffraction analysis [64]. He acknowledged that the carbonated forms of apatite were more of a challenge to grow and thus to fully characterize, but he was hopeful that more modern analytical techniques such as nuclear magnetic resonance and neutron-scattering diffraction would provide additional information even in the absence of large single crystals. The issue of water in (carbonated) hydroxylapatites arose in several different contexts. First, there is Elliott’s [64] recognition that a number of chemical analytical techniques do not distinguish among the possible speciations of hydrogen within the apatite structure, such as OH⁻, molecular H₂O, (HPO₄)²⁻, or H₃O⁺. Secondly, there is the confounding factor that the heating of samples in order to determine the concentration of lesser components in apatite, a technique that had been used since the mid-1800s, actually could cause reactions that changed the species that were retrieved. For instance, there was the well-accepted method of quantifying the amount of (HPO₄)²⁻ in bone mineral or synthetic carbonated apatite by measuring the amount of pyrophosphate derived by heating the sample to 600°C:



Heating apatite therefore could evolve water that did not previously exist as that species. Such water might be misinterpreted as the high-temperature release of crystallographically incorporated water. Elliott [64] summarized several papers in which proton magnetic resonance had been used, especially on enamel, to determine whether water was crystallographically controlled in the apatite. As indicated in the paragraphs above, the spectra from this technique did not lend themselves to unique interpretation. Thus, the chemical affinity (including bonding) of water for the apatite and the state of mobility of water in bulk samples of enamel were not totally clear. Elliott [64] pre-saged a number of types of analytical studies that would prove important for the characterization of apatite minerals, including Rietveld analysis in x-ray diffraction. However, he was perhaps too sanguine in his final comment: “We can therefore expect in the near future a rapid increase in knowledge, and therefore understanding of these compounds” (p. 305).

The 1970s brought more detailed modeling of how and where water molecules were sited in the apatite structure, but not from the mineralogists. McConnell [65] instead wrote a rather defensive paper in which he reiterated that many apatites seem to contain appreciable structural water and that various observed anomalies/variations in the a-axis length of the unit cell could be explained by H₂O occupancy in the lattice. Although McConnell’s model of

incorporation of $(\text{H}_4\text{O}_4)^{4-}$ groups in carbonated apatites never had been fully substantiated, many researchers had attributed their water analyses in apatite to this explicit species. McConnell [65], overlooking this lack of documentation, was quite indignant at Elliott's [64] reminder of the lack of direct evidence for that species. Elliott and his colleagues would go on to study biological and synthetic apatites in great detail, particularly applying the vibrational spectroscopic methods that McConnell [65] indicated "should be disregarded as meaningless" (p. 1665). The times were changing, however, and the vibrational spectroscopic methods of IR and, later, Raman would become of increasing application and value in the characterization and structural-chemical understanding of biological and synthetic carbonated apatites.

In 1970, the chemists Joris and Amberg [66,67] published a pair of papers in which they investigated the mechanism behind the non-stoichiometry of uncarbonated hydroxylapatites. They documented that Ca-deficiency in the apatites was accompanied by a decrease in the concentration of hydroxyl ions in the channels. Among other observations, the increase in value of the unit-cell *a* parameter in Ca-deficient apatites suggested to Joris and Amberg [66, 67] that H_2O was in the channel sites, most likely residing in the vacancies left by OH-ions whose absence was demanded by charge balance. They provided a quantitative and diagrammatically illustrated model of how the H_2O molecules could be accommodated in the channel sites of hydroxylapatite [67]. This paper, however, does not appear to be well-cited in the modern literature.

The dental researchers once again used modern techniques of the day to document water in bioapatite. In his presentation at the 1969 international Enamel II conference (published in 1971), Casciani [68] presented his interpretation of extensive NMR analyses of enamel, dentine, and bone. He acknowledged not only general statements that he felt confident to make, but also uncertainties about several details. For instance, he firmly stated that crystallographically incorporated water exists in all types of calcified tissues and that the less crystalline of these tissues (dentin and bone) contain more water than the more crystalline (enamel). His estimate still remained at approximately 6 wt% water within enamel, and his description of how water was retained and bound in the apatite structure did not seem to have progressed beyond the interpretations of Little and Casciani [53], as described above. Moreover, Casciani [68] re-introduced confusion into the understanding of the nature of enamel itself, by suggesting that some of this 'trapped' but 'unbound' water was contained in other non-apatite phases that he thought were constituents of the 'hard parts' of mineralized tissue. Among these other known hydrated phases that he called upon were octacalcium phosphate $[\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}]$ and calcium hydrogen phosphate dihydrate, brushite $[\text{Ca}(\text{HPO}_4) \cdot 2\text{H}_2\text{O}]$. Dibdin [69] followed with additional NMR analyses of enamel, in which he tried to clarify some of the complications in interpreting NMR line width in terms of the mobility of H_2O and the spectral distinction between H_2O and OH. He showed evidence for both of those hydrogen-species in enamel, and he warned about the need to remove and exclude adsorbed water before analysis of the samples. As in the previous decade, the NMR papers referenced above from the 1970s are based on proton NMR of powdered samples (no MAS, magic-angle spinning) in which interpretations of water mobility are based on peak widths.

The published work of LeGeros in the 1960's and their own experimental syntheses and analyses of carbonated apatites led French researchers Vignoles [70] and Bonel et al. [71] (as

summarized in [72]) to propose the following charge-balancing mechanism in carbonated apatites synthesized in Na-bearing solutions:



They not only recognized vacancies in both the Ca- and OH-sites, but they also listed H₂O as an explicit component, whose concentration was controlled by the number of vacancies in the apatite structure, most specifically by vacancies in the channels.

LeGeros et al.'s [73] frequently cited 1978 paper appears to have marked a turning point in the acknowledgment of, as the title indicates, the different "types of 'H₂O' in human enamel and in precipitated apatites." The authors used IR spectroscopy in conjunction with heating techniques to monitor both the release of water at specific temperatures from and the accompanying changes in synthetic carbonated apatites. They concluded that adsorbed water could be distinguished by its release below 200°C based on the reversibility in its re-absorption. Lattice water was released at 200-400°C, and this water could not be reversibly re-incorporated. The proof offered for water's structural incorporation was that the a-axis length (at room temperature) was smaller in the apatites after the high-temperature release of water. LeGeros et al. [73], however, once again brought up the drawback to heating a sample in order to quantify its structural water content; they demonstrated in their IR spectra that original (HPO₄)²⁻ thermally decomposed into (P₂O₇)⁴⁻ with the evolution of H₂O. Likewise, Termine et al. [74] had recognized the release of several weight percent H₂O at 600°C. They also observed, however, that the (HPO₄)²⁻ proportion of bone mineral "phosphate" is substantial (15-20%) in the youngest of animals, but decreases with age. This age-dependence in hydrogen phosphate content of bone could produce additional water artifacts in the TGA data. The application of new analytical techniques in the late 1960s and throughout the 1970s, coupled with a clearer understanding of both the usefulness and pitfalls of heat-treatment techniques (e.g., TGA), would appear to have set the stage for final clarification of the structural-chemical relations of water in the apatite lattice. That great breakthrough did not materialize imminently, however, as related below.

In the 1980s, D.W. Holcomb and colleagues documented some useful modifications in their application of TGA. Holcomb and Young [75] carried out heating studies on tooth enamel, with the additional precaution of making their accompanying IR analyses in a dry nitrogen atmosphere to avoid spectral interference from adsorbed water. They agreed with LeGeros et al. [73] concerning the presence of lattice water and how its thermal release affects the XRD a-axis parameter. In a remarkably data-rich published study, Young and Holcomb [76] used multiple analytical techniques to characterize (uncarbonated and carbonated) hydroxylapatite synthesized by six different accepted methods of the day. They detected numerous differences in IR spectral features, XRD parameters, ease of deuterium exchange with the hydrogen species, etc. among samples that, in principle, were very similar. There was much evidence for structural water, especially in samples precipitated at 1 atm. from aqueous solutions, therefore at temperatures below 100°C. The authors saw a possible correlation between the amount of structural water in the latter samples and the ease with which diffusion occurred (monitored as deuterizability). Interestingly, Young and Holcomb [76] did not find a correlation in their IR data between the abundance of hydroxyl vacancies and the amount of incorporated water in their samples. The wealth of data in this paper likely could be further mined by modern analysts.

By the later 1980s, researchers were using temperature-programmed analysis of apatites in conjunction with IR and XRD monitoring of changes in the solid sample. Mortier et al. [77] summarized their understanding of how the broader community viewed the calcium-phosphate phases. “Compounds with compositions intermediate between HAp and OCP are conventionally called calcium-deficient apatites (DAp)...Some authors...have proposed that they be described by the following formula $\text{Ca}_{10-z}(\text{HPO}_4)_z(\text{PO}_4)_{6-x}(\text{OH})_{2-z} \cdot n\text{H}_2\text{O} \dots$ ” (p. 266). This statement suggested some degree of acceptance of water as an intrinsic component of non-stoichiometric apatite by the rapidly growing and diversifying biomaterials community.

The 1990s brought re-consideration of the accepted two mechanisms for substitution of carbonate in apatite and re-invigoration of studies into the variability of hydroxyl concentration in apatite, both of which topics are crystallochemically intertwined with the mechanism of H_2O incorporation. Analytical chemists De Maeyer et al. [78] published an insightful paper on Na- and carbonate-containing apatites that were produced through hydrolysis of the more acidic calcium hydrogen phosphate phase monetite (CaHPO_4). The authors did explicit chemical analyses for Ca, P, Na, and CO_3 . Unfortunately, OH concentration was calculated, based on the assumption of charge balance, and H_2O subsequently was calculated by difference from an assumed 100 wt% total. Their IR spectra still showed water in samples dried under vacuum at 25°C . The carbonate concentrations of the samples ranged from approximately 8 to 21 wt%, i.e., from the level in bone upward. In this perhaps earliest of studies in which water concentration was evaluated as a function of carbonate concentration, water was *calculated* as approximately 5 wt% across the entire range of samples. Later work suggests this finding of constant water concentration to be very significant. Despite the lack of correlation between the concentration of water and that of the carbonate substituent believed to induce vacancies in the apatite, De Maeyer et al. [78] inferred that this “structural water” resides in vacancies. Christian Rey and colleagues [17], who have made numerous contributions over several decades to the understanding of nanocrystalline carbonated apatite, applied magic-angle-spinning (MAS) proton NMR to natural bone. They concluded that the concentration of OH ions in bone apatite crystallites was sufficiently low to be undetectable by that technique. One would infer thereby a high density of vacancies in the channels. As part of this study, the authors indeed detected H_2O in their samples, but they regarded this water as simply a constituent of collagen or an adsorbent on the apatite crystallites.

4.3. Heated Apatites But Unheated Discussion: 2000-2011

As most readers of this book and chapter will realize, in the past decade there has been an explosion in the literature on carbonated apatite within the biomedical (e.g., medical, dental, biological engineering), chemical, environmental, and biomaterials sciences. The chemical-structural adaptability of the mineral apatite has allowed it to be tailored in a multitude of ways for a huge range of applications. Thus, based on their specific goals and needs, individual research groups have explored the relationships among the chemistry, crystallographic structure, and properties of apatite.

In this time period in the progression of apatite research, one can ask whether there are still researchers who do not agree that there is structurally incorporated water in non-stoichiometric (carbonated) apatite and what their evidence is. Likewise, one can query those

who do provide explicit evidence of the presence of structural water regarding the nature of their sample preparation and analytical techniques. The follow-on questions to the latter group concern where in the crystal structure the H₂O resides and what the mechanism is that controls its incorporation. Lastly arises the question that brought about the research for and writing of this chapter – why is water still not regarded in the broader medical-dental research community as an *essential* and relatively abundant constituent of the mineral in bone, dentin, and enamel? Why is it that modern review articles that carefully explain the incorporation of carbonate into apatite and carbonate's relation to the vacancies in hydroxyl sites do not emphasize H₂O in a like manner?

Not all the recent literature that addresses hydrogen species in bone mineral and its synthetic equivalents discusses the issue of water. Among the studies not explicitly focused on water, those that address OH-ions in bone apatite typically have also uncovered H₂O in their analyses. The present author freely admits, however, that even she ignored the issue of water in her own paper dealing with OH in non-carbonated apatite [18]. Cho et al. [20], and Kolmas and Kolodziejwski [21], who applied NMR spectroscopy to bone and dental apatites, respectively, were concerned primarily with quantifying the concentration of OH-ions in bioapatites. Those two studies revealed consistent results, e.g., that bone and dentin apatite (long recognized to be very similar in properties) contain only about 20% of the concentration of OH-ions that is found in pure hydroxylapatite. Both groups also detected considerable H₂O in their NMR spectra, yet both dismissed this water as adsorbed on the crystallites or contained within the collagen.

It would appear that TGA-based studies of apatite would produce irrefutable evidence for or against the structural incorporation of water. All thermal experiments on non-stoichiometric apatites release water. Surely the temperature of release would distinguish adsorbed water from structural water. Several published papers, however, fail to make this distinction, and the reader is left wondering at how high of a release temperature the authors still would label the water as 'adsorbed'. Thus, Tas [79] followed an accepted biomaterials preparation procedure in heating to 900°C the OHAp precipitated at 37°C from synthetic body fluids. The author reported that the broad IR band for 'adsorbed water' on the initial material 'diminished' after the sample had been heated to 900°C, leaving one to wonder about the distribution of water released throughout that large temperature interval. Unexplained is why the IR spectrum of the 900°C sample in their Figure 2d (in [79]) still records the presence of some, although much less, water. Likewise, in a paper on the thermal decomposition of carbonated apatites aqueously precipitated at 90°C, Lafon et al. [80] carried out TGA studies up to 1400°C in air, N₂, or CO₂. They reported "20-600°C: departure of adsorbed water and synthesis residuals" (p. 1131). Their paper does not distinguish the amount of non-adsorbed water. Similarly, Taylor et al. [81] had reported for inelastic neutron scattering analyses of synthetic hydroxylapatite that "after heating SRM 2910 to 850°C the bands...are better resolved following the removal of the underlying water bands after heating...The absence of translational modes that could be assigned to water would imply that the water present in both samples is surface and not lattice bound water..." (p. 3143). Antonakos et al. [82] straightforwardly, but inappropriately, stated that "...sample #6 was heat-treated at 530°C to derive the adsorbed water..." (p. 3044).

In contrast, several studies based on a combination of TGA results on tooth material and synthetic apatites in conjunction with analyses of the associated solids were carried out with due attention to the temperatures of volatile release. Reyes-Gasga et al. [83] heated dentine

and enamel, in addition to synthetic apatites, while noting the specific steps in the TGA traces and simultaneously monitoring changes in resistivity in the solids. Their results correlated well with the interpretation that adsorbed water was released from room temperature to 200°C, lattice water and organics (in the dentine and enamel) were removed from 200 to 600°C, and carbonate decomposed and was released as CO₂ at temperatures above 600°C [83]. An earlier related study by Shi et al. [84] compared the thermal behavior of tooth enamel and natural geologic, well crystalline fluorapatite from approximately room temperature to almost 500°C. Through IR spectroscopy, they observed essentially no water in geologic fluorapatite but were able to monitor dehydration in enamel. Although these authors did not regard the existence of lattice water with so much certainty as the previous group, they did conclude that at temperatures below about 325°C the adsorbed water and some portion of the lattice water were released from enamel. These papers are further evidence of the dental research community's ongoing research into water in enamel and dentin apatite.

There are also groups that recognize that bioapatite crystallites and their synthetic analogs possess a significant amount of water, but they attribute it to a "structured hydrated layer" (e.g., [19,85-87]), which harkens back to the early work of Neuman et al. [49] and Neuman and Neuman [50]. The above researchers have shown that this reservoir of water is very reactive and also contains a huge ion reservoir that is important to the bulk composition and, perhaps, the maturation of the bone crystallites. Particularly important to the current discussion, as summarized by C. Rey and colleagues [19], is the development of enhanced interpretation of band positions in IR and NMR spectra by which one can distinguish water in the external, structured hydrate layer from water that is structurally (internally) incorporated in apatite.

Many papers since 2001 have acknowledged the presence of structural water in bone apatite, including several papers that were primarily focused on understanding the nature, location, and stabilization of water in several different reservoirs throughout bone. Various applications of nuclear magnetic resonance spectroscopy, particularly in conjunction with Rietveld analysis of XRD data, have proven very powerful in the quantitative analysis of non-stoichiometric apatites. Such attempts have not been without problems and inconsistencies, however. The above-mentioned studies by Cho et al. [20], and Kolmas and Kolodziejcki [21] also were based on NMR analysis, but both sets of authors concluded that the spectral evidence for H₂O actually indicated it was adsorbed. The greater availability of and sophistication of analytical instruments in the past decade clearly have enabled the acquisition of much more data. The selection of the optimal analytical method for water analysis and the interpretation of the acquired data, however, remain a challenge. In the remaining summary, the present author does not always distinguish among the different NMR techniques that have been applied to apatite samples, but typically refers to them all simply as NMR. The reader should take note, however, that the specific technique and the specific isotope observed will affect the information that is derived.

In this author's opinion, the major breakthrough 'in the modern literature' for accepting structural water in non-stoichiometric apatite came in a paper by Ivanova et al. [88]. The latter studied the crystallography, especially the siting of CO₃ ions, in calcium-deficient carbonated apatites through collecting XRD data on sample powders simultaneously with their heating to 750°C. The combined TGA volatile-release data and XRD data on the associated solids had the additional benefit of clarifying "the localization and amount of lattice water that enhanced the reliability of the structural model" (p. 342). Ivanova et al. [88]

measured a 1.1 wt% loss of structural water by TGA, and inferred 1.4 wt% water in the structure based on their Rietveld refinement of XRD data. They recorded as adsorbed all water released up to 120°C and noted that all structural water had been released by 400-500°C. In their apatite formula, they assigned 0.3 moles of H₂O to the PO₄ site and 0.45 moles of H₂O to the channel site. The latter was shared with 1.65 moles of OH, for a total occupancy of 2.0 for the channel sites, i.e., full occupancy.

Other NMR papers directly addressed bone, rather than its synthetic apatite equivalents. Wehrli and Fernández [89] wrote a paper entitled ‘Nuclear magnetic resonance studies of bone water’. Although the authors acknowledged water associated with the crystallites, they focused on the diffusion of intercrystalline water through pores and micro-channel systems in the bone. Kaflak-Hachulska et al. [90] investigated human bone by applying proton NMR under ultra-high-speed magic-angle spinning (MAS), carrying out two-dimensional ¹H-³¹P heteronuclear correlation studies and analysis of ³¹P rotational sidebands in conjunction with their cross-polarization kinetics. Among their results was a lack of detection of OH or (HPO₄)²⁻ ions in bone mineral. The paper also suggested that some structural H₂O still existed in a synthetic OHAp even after it had been heated to 1073°C, which is difficult to envision. A major contribution of this well-explained article is the insight it provides into the complexity and model-dependence of the interpretation of such NMR spectra².

Two sets of published studies, first-authored by two different researchers named Wilson, mark major advancements in the understanding of water in apatite. The group of Erin Wilson, Michael Morris, and colleagues ([91, 92]) applied NMR analysis to bone specifically for the purpose of understanding the different types of water in this complex material. Their 2004 paper identified an ordered arrangement of H₂O molecules apparently located in the grooves/channels between collagen molecules. They also identified an ‘ordered surface water layer’ on the bone crystallites, reminiscent of the surface layer described by Neuman et al. [49] and Rey et al. [87]. Their paper in 2006 specifically identified three different types of water in bone and their apparent structural roles. In addition to recognizing surface (adsorbed) water, Wilson and colleagues identified structural H₂O using ¹H MAS NMR on bone and synthetic type-B carbonated apatite. They monitored differences in hydrogen-bonding environments of the H-bearing species and how the hydrogen-bonding changed as temperature was increased up to 225°C. Wilson et al. [92] found their results to be consistent with those published by Ivanova et al. [88].

Collaborators Rory Wilson and James Elliott published three papers ([72, 93, 94]) in which they applied Rietveld refinements to XRD data on synthetic apatites with different compositions and precipitated through different reactions. For each synthetic, non-stoichiometric apatite product, they provided data on the unit cell contents, which included molar H₂O occupancies of 1.05 (for Na-bearing OHAp with 12.5 wt% CO₃), 1.97 (for Ca-deficient OHAp samples prepared from CaHPO₄), and 2.29 (for Na-free OHAp with 11.2 wt% CO₃). The 2004 study applied Rietveld refinement to both X-ray and neutron diffraction data [72]. After chemical analyses of the samples, which totaled to 93-95 wt%, the OH⁻ ion concentration was calculated based on charge balance, and the H₂O content of the unit cell was calculated to account for the residual mass balance. By this method, 2.11 wt% H₂O (approximately 1 mole H₂O) was derived. Somewhat disconcertingly, the reader is told not to accept as reliable the IR-based OH-concentrations that are obviously lower than the

² See also Chapter 7 of this volume. The Editor.

calculated values. Wilson et al. [93] analyzed synthetic uncarbonated but Ca-deficient apatites by XRD, IR, and Raman spectroscopy followed by Rietveld refinement of the XRD data. Again, assumptions were made to derive specific OH⁻ and H₂O concentrations, which are not spectroscopically quantifiable in this situation. The samples were analyzed by TGA to 900°C, and the raw amounts of evolved water were modified to estimate 'true' H₂O occupancy based on assumptions about thermally induced reactions among original (HPO₄)²⁻ groups, which generate H₂O. The authors' overall assessment is that Ca(2) vacancies surrounding the apatite channels and some proportion of OH-vacancies within the channels may be filled with H₂O molecules. This leads to the proposed 1.97 molecules of H₂O per unit cell. In their 2006 paper on Na-free carbonated apatites that were synthesized through hydrolysis of monetite, Wilson et al. [94] interpreted some resonances in their proton NMR spectra differently than in earlier literature. They attributed the resonances to H₂O that is structurally incorporated in the channels. The results of the TGA experiments and measured density of the samples led the authors to believe that H₂O is part of the unit cell. The proposed cell contents in this case are Ca_{8.241}(PO₄)_{4.344}(CO₃)_{1.656}(OH)_{0.139}·2.29H₂O.

More recently, Harris Mason and Brian Phillips have harnessed the efforts of several collaborative groups of geoscientists to apply NMR spectroscopy to study how H and Na are incorporated into apatites that exhibit combined type-A and -B carbonate substitution [95], the characterization of fluor-chlorapatite with a low OH-concentration [96], and the role of structural water in carbonated fluorapatite [97]. Although some information on H₂O is provided in each of those three articles, the latter is obviously most pertinent to the present discussions. In addition to NMR analysis, Mason et al. [97] applied IR spectroscopy, electron microprobe analysis (for chemical composition), and TGA to well-crystalline examples of natural, carbonated fluorapatites, like those that occur abundantly in large-scale phosphorite (phosphate rock) deposits. The authors observed a peak at 6.4 ppm that contained a set of spinning sidebands in the proton MAS NMR spectrum, consistent with the presence of rigid molecular water bound within the structure of the apatite. After the sample had been heated to 600°C (within a weight-loss step in the TGA pattern), the feature at 6.4 ppm was weaker, which indicated partial loss of water. NMR analysis of the sample after heating to 800°C indicated atomic re-ordering and more complex internal chemical reactions in the apatite. This very informative paper presents an internally consistent model for how and where the H₂O resides. The authors identified abundant water, i.e., 0.84 molecules per unit cell, which is definitely structural in nature and inferred to reside in the channels. Based on additional NMR spectra, the authors proposed that the water molecules are closely associated with and may stabilize structural vacancies.

In an effort to overcome some of the non-unique interpretations of NMR spectra of apatites, our own collaborative group synthesized carbonated hydroxyl- and fluor-apatites in D₂O, which were analyzed by solid-state ²H NMR [98]. Deuteration during mineral formation permitted incontrovertible discrimination between structurally incorporated and adsorbed water. Each spectrum of apatite, containing widely different concentrations of carbonate, showed a single line using the quadrupole echo pulse sequence. Samples dried under several different conditions showed ²H resonance, which was not detected once a sample had been heated to 500°C. These experiments confirmed the presence of lattice water in the carbonated apatite and its release by 500°C.

Thus, several independent research groups, representing a wide range of scientific, medical, and engineering fields, have provided what appears to be irrefutable evidence of the

structural incorporation of significant amounts of water (on the order of 1 to several wt%) in vacancy-bearing hydroxylapatite and carbonated hydroxylapatite of the type found in bones and teeth. All of these groups indicate the c-axis channels of apatite as the major site for the H₂O molecules. Those channels have been recognized for decades as one of many opportune crystallographic features, including the structural flexibility of the Ca and PO₄ sites, that permit variability in the composition of apatite.

An additional crystallographic ramification of carbonate substitution in apatite appears to support the accommodation of water molecules in the structural channels. As explained in the introduction, substitution of carbonate for phosphate in OHAp induces vacancies not only in OH⁻ sites at the center of the channels, but also in Ca²⁺ sites. Numerous studies document or at least suggest that the Ca(2) sites, which are the calcium sites immediately around the channel (see Figure 1), are preferentially left vacant during substitutions that require additional charge-balancing [8,19,93,99].

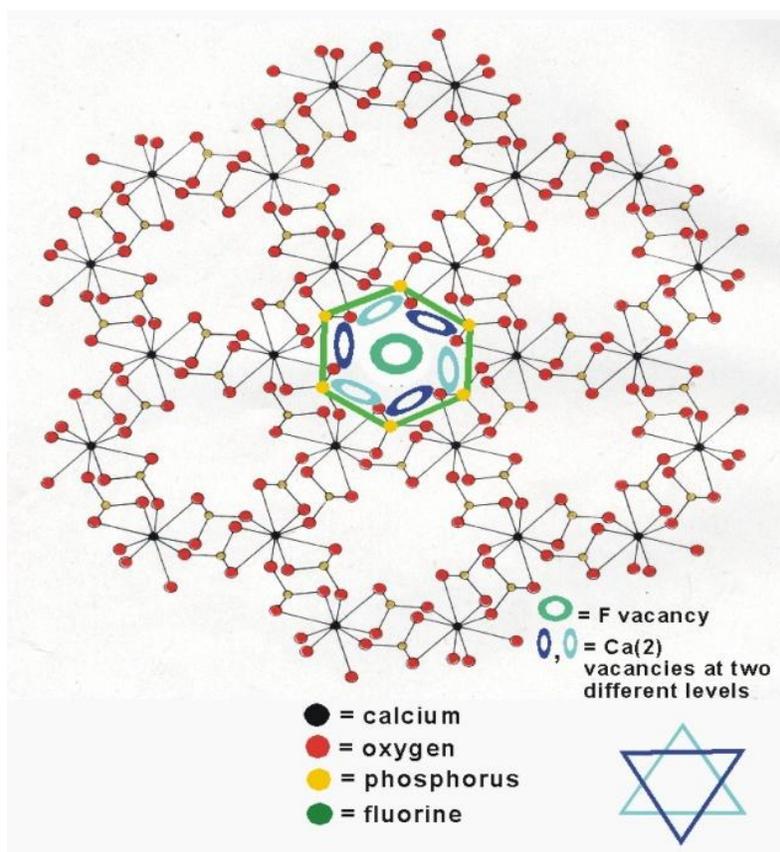


Figure 2. Apatite structure after removal of all the channel-filling anions, as well as the Ca(2) cations. The central green hexagon is the same as the outermost hexagon shown in Figure 1. A huge volume of the crystal is opened up as two types of vacancy sites have formed, i.e., absence of the central anion (shown by central thick green oval) and Ca(2) sites (shown by blue ovals) at two levels along the c-axis (distinguished by light and dark blue color). The enlarged hexagonal channels are lined by phosphate ions with negatively charged oxygens. Figure from [40] (their figure 4), with some additions. (Reproduced with kind permission of the Mineralogical Society from a paper by Beevers and McIntyre (1946)).

Creation of vacancies in all the OH sites and all the Ca(2) sites would produce remarkably large open channels in the apatite, as recognized for fluorapatite by Beevers and McIntyre [40], whose crystallographic representation is reproduced above as Figure 2 [40]. Consider that a typical bone contains carbonated OHAp with approximately 6.6 wt% CO₃. If all the carbonate in such a bioapatite demonstrated B-type substitution, its formula would be Ca₉□[(PO₄)₅(CO₃)](OH)□, where □ represents a vacancy. Hypothetically, if the Ca(2) calcium-site vacancies were three times more abundant than the Ca(1)-site vacancies, then the more specific formula would be Ca(1)_{3.75}□_{0.25}Ca(2)_{5.25}□_{0.75}[(PO₄)₅(CO₃)](OH)□. Half the OH ions and over 12% of the Ca(2) ions would be missing, thereby making accessible for water incorporation about one-fifth of the sites left vacant in the open volume shown in Figure 2. Both the above models of vacancy-modified c-axis channels and the similarity in size between OH⁻ ions and H₂O molecules [100] suggest a number of ways in which H₂O molecules plausibly can be incorporated structurally in non-stoichiometric apatites.

Most proposed models for water incorporation in fact do suggest a very close relationship between the H₂O molecules and the vacancies in the OH-sites ([101] and [102], as summarized in [94]; [70] and [71], as summarized in [72]; [88,92,94,97,103,104]). The present author actually became involved in the water-in-apatite discussion while investigating the relationship between carbonate concentration and hydroxyl concentration in carbonated OHAp. If the incorporation of water is directly tied to the availability of vacancies in which to site H₂O molecules, as proposed by the authors above, then one would expect to find a robust positive correlation between the concentration of carbonate and the concentration of water in carbonated apatites. Although our initial TGA results on a suite of carbonated hydroxyl- and fluor-apatites suggested such a correlation [105], more recent unpublished analyses reveal essentially constant concentrations of water released in the interval from 200 to 500°C from hydroxylapatites of a wide range of carbonate concentrations. These data and our NMR work on deuterated carbonated apatites suggest that most of the H₂O molecules reside between the channel anions [98].

5. INTERPRETING WHY CRYSTALLOGRAPHIC INCORPORATION OF WATER WAS NOT ACCEPTED

5.1. The Issues

The literature on water in bioapatite demonstrates, as does the literature on the controversies surrounding carbonate and hydroxyl ions in bioapatite, that there are multiple reasons for the long intervals of time during which researchers did not share a community-wide view of the details of the structure and chemistry of bioapatite. In the present case, however, the pertinent question concerns why there is a hesitation to formalize the acceptance of what appears to be a well-proven contribution to our knowledge of bioapatite.

Part of the answer may come from a closer look at how well, i.e., to what level of detail, we have documented lattice water in bioapatite. The earliest evidence of lattice water (in the late 1800s) was derived by methods that we no longer acknowledge as ‘cutting edge’. The evidence was only chemical data, which could not be referenced to or tested against any structural model, in that the apatite structure had not yet been determined. Then, several well-

received papers presented detailed crystallographic models of fluorapatite and hydroxylapatite, providing structural constraints for future considerations of water as a component. Multiple documentations of water in carbonated hydroxylapatite subsequently were published, but the specific results did not agree. For instance, although heating experiments such as TGA reproducibly showed the evolution of water at elevated temperatures (thus, not adsorbed water), many different weight-percent values were published. Until quite recently there was not an accepted temperature interval in which to assign the released water as lattice vs. adsorbed water, although many researchers did follow the temperature intervals proposed by LeGeros et al. [73].

The sense of uncertainty of the details about water in apatite continued, however. For instance, certain knowledgeable experimentalists and analysts brought attention to the physical and chemical differences among the synthetic apatites that were under analysis [76,106-108]. The differences among the synthetic products clearly could account for much of the variation in water released during TGA experiments, but this realization only served to show how little was known about the parameters that actually control the amount of lattice water in apatite. Lastly, it became clear that the amount of water evolved from a heated non-stoichiometric apatite sample need not match the amount of lattice water that actually existed in the original sample. Unfortunately, the process of heating could induce internal reactions in the apatite that could either consume or produce molecular water. As documented above, numerous authors had recognized that acidic species such as $(\text{HPO}_4)^{2-}$ and $(\text{HCO}_3)^-$ reacted when heated so as to produce water, e.g., $2(\text{HPO}_4)^{2-} \rightarrow \text{H}_2\text{O} + (\text{P}_2\text{O}_7)^{4-}$. Such reactions would create molecular H_2O that was not originally in the apatite. In contrast, it was also known that carbonated and other non-stoichiometric apatites that inherently were depleted in OH^- would become well-crystalline, fully stoichiometric OHAp after heating to 900°C [14,15,18,23,75,79,84,109]. It was possible that the internally available lattice water in the original apatite (perhaps together with CO_3^{2-}) reacted with the latter to fully hydroxylate it during heating. If so, the TGA-evolved amount of water would be less than that in the original solid. Such variability and uncertainty do not make for a robust model of water's crystallographic incorporation.

Although thermogravimetry continued to be applied to carbonated apatite samples, NMR gradually took on increasing importance as a technique that could specify the structural positions of the isotopic species under analysis. It therefore seemed as though we might finally know exactly where the water resided in the lattice even if there were difficulties in quantifying the exact amount of water. However, the interpretation of the acquired NMR spectra was not as straightforward or objective as desired. It is clear from the literature cited above that most of the interpretations of NMR spectra with respect to lattice water are strongly model dependent. As summarized in Yoder et al. [98], several recent studies of synthetic or biological carbonated apatite have disagreed concerning lattice water even though the NMR techniques applied were quite similar. Multiple hydrogen-bearing species might exist in the same apatite sample (e.g., H_2O , OH^- , HPO_4^{2-} , HCO_3^-) and thereby produce multiple peaks.

Moreover, different degrees of hydrogen bonding between these species and the rest of the structure could shift the recorded peak positions, making species identification less certain. With respect to just the one species H_2O , different 'types' of water (e.g., adsorbed, lattice-incorporated, trapped in fluid inclusions) also could complicate the differentiation among various hydrogen species. NMR, as well as IR and Raman, spectroscopy can be

carried out in a dry gas environment, which at least can eliminate interferences from adsorbed water (see Figure 3). This approach has not been used in most cases, however. One therefore does not gain a sense that multiple types of studies are closing in on one, irrefutable model that accounts for exactly how much water resides in which specific crystallographic site(s) as controlled by some set of definable conditions.

It might appear that a better pathway to resolving the water question would be to focus on actual biological material. It would seem that the variability in the apatite among different samples of bone might be appreciably less than that among carbonated apatites synthesized by means of a dozen or more methodologies. Bone (also dentin) has its own problems, however. If bulk bone samples are measured by TGA or by NMR, there is interference from the abundant collagen and other organic components, which (1) give their own responses to each technique and (2) provide another medium in which non-mineral water can be stored.

It is possible to chemically dissolve the organic components from bone, leaving only the inorganic mineral. However, such aggressive chemical treatment of bone introduces the possibility that the mineral also was chemically changed. Dental enamel, on the other hand, appears to provide the ideal bioapatite for study.

Enamel has a total of only about 4 wt% combined intercrystalline water and organics [110,111], and its crystallites are on the order of 10 times wider and 1000 times longer than those of bone [34,111-113]. Enamel apatite is better crystalline as well as atomically better ordered than bone apatite.

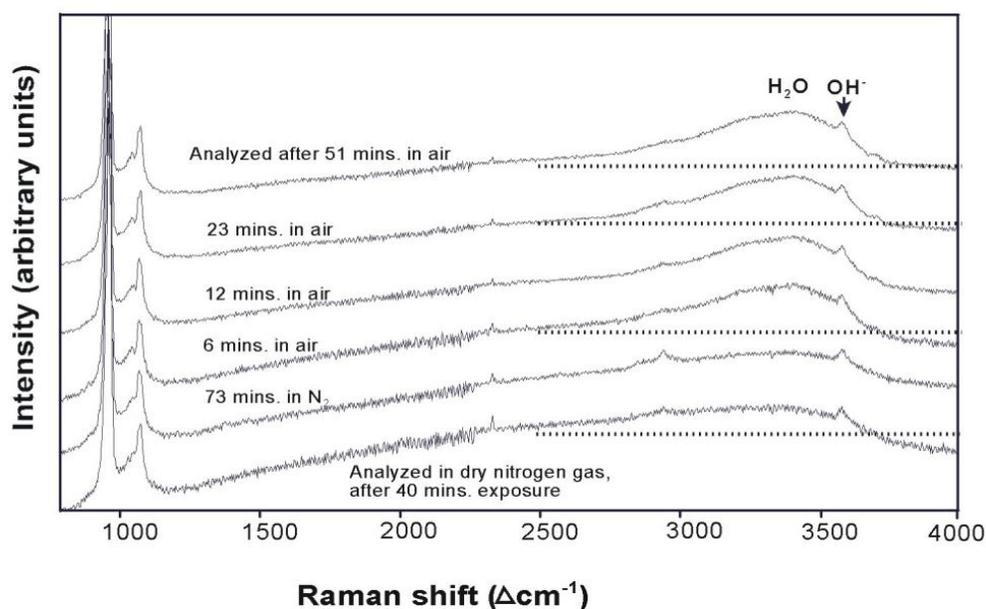


Figure 3. Raman spectra of a single aliquot of carbonated OHAp containing 6.35 wt% CO_3 , synthesized aqueously at 37°C . Bottom to top sequence of spectra shows water adsorption as the apatite powder that had resided in a flow of dry nitrogen gas is exposed to room air. Analogously, viewed from the top downward, the spectra show that even after adsorbed H_2O is removed, the carbonated apatite still contains appreciable structural H_2O , as seen in the broad feature from about 2900 to $3600 \text{ } \Delta\text{cm}^{-1}$ that persists even under dry gas. The O-H stretch for the hydroxyl ion at about $3570 \text{ } \Delta\text{cm}^{-1}$ remains throughout the experiments. All spectra are normalized to the intensity of the P-O stretch at $\sim 960 \text{ } \Delta\text{cm}^{-1}$. Sample kindly provided by Mitchell Sternlieb and Claude Yoder.

It is therefore no surprise that dental researchers over the decades, in many cases in conjunction with colleagues in the natural sciences, have made repeated analyses for water in apatite using increasingly sophisticated techniques. Somewhat counterintuitively, the notion of 2-3 wt% H₂O as an essential component in enamel apatite has not fully pervaded the dental research community.

5.2. Stagnation in Acceptance

The chemists in the 1890s were not particularly excited about the finding of lattice water in ‘bone mineral’. They already had accepted the fact that this calcium phosphate solid was a hydrate, and it was the missing hydroxyl that ultimately interested them. These researchers were not constrained by a preconceived stoichiometry for apatite. Likewise, it has been well accepted for a long time among modern researchers that the non-crystalline (stoichiometrically less constrained) phase amorphous calcium phosphate can contain large amounts of water [114]. Chemists in the 1930s also knew about the lattice water in calcium phosphate materials known as apatitic. It was not considered startling or interesting, in that other hydrated calcium phosphate phases had been identified [7].

By the 1950s, the interesting new revelation about water and bone mineral was the recognition of an ordered hydration shell that extended over the large surface area of such small particles. Interest persists in the nature of and reactivity of that shell, e.g., as an ion reservoir (e.g., [19,115]).

Many researchers who subsequently encountered what we now would call lattice water in apatite considered it more of a nuisance than a component of significance in the mineral. Water (especially adsorbed, but also crystallographically incorporated) created an elevated background in both IR and Raman spectra, which made it difficult to make quantitative measurements for hydroxyl content, in that the narrow O-H stretch band for OH in hydroxylapatite is situated on the high-wave number shoulder of the broad O-H stretch band for water (see Figure 3). Similarly, the signal for water makes it difficult to define the position of the (HPO₄)²⁻ resonance in the NMR spectrum [93]. As noted above, many researchers were more than willing to heat their samples to be rid of this interfering water, not always documenting the specific temperature at which they deemed the adsorbed water to have been removed. In the production of some ceramic apatitic biomaterials, the original synthetic precipitates or bone materials are heated routinely to 700°C or more [116-118].

Ivanova et al. [88] published an apatite study in which they explained the significance of the incorporation of water in the unit cell toward optimizing the Rietveld refinement of XRD data. Numerous subsequent papers have followed their lead. There is still some sense of reluctance, however, in the inclusion of water, in that its quantification remains elusive.

It appears that publications about water in apatite typically received subsequent citations, but that they did not generate a great deal of debate. Such publications were not followed by commentaries that posed pointed questions about the amounts of water that were being recorded or that demanded an internally self-consistent model of how H₂O was accommodated in the apatite structure. Presumably, medical researchers were not impressed by or concerned with a few weight percent water in inorganic mineral within a bone system that obviously has large biological reservoirs of water. The question of H₂O incorporation in bioapatite apparently has not generated either heated discussion or conflicting models (as did

the notion of the incorporation of carbonate within apatite or the presence/absence of hydroxyl in bone apatite). Thus, no resolution was ever reached, because there had never been a battle. The notion of lattice water in carbonated apatite never became THE new idea that had risen through the fray in order to be incorporated into the community's now updated and expanded view of bioapatite.

5.3. Lessons for the Future

Some of the 'take-home messages' from this comparison and analysis of the literature are the importance (1) in reading the (even decades) older literature, especially focusing on papers by those who were considered to be insightful in the field, (2) in not automatically disregarding results or isolated observations simply because they do not fit the current model, and (3) in not assuming if some result is only now becoming recognized that it must not be very important, i.e., we did just fine up until now even without this information. In fact, the current information on water in apatite could help to explain discrepancies about synthetic apatite that have been reported for many years in the literature, for instance, a lack of reproducibility in experimental solubility results.

In a sense, the bone and tooth research communities have always had an identity crisis regarding the inorganic mineral component of interest. Is it hydroxylapatite or not? Well, yes, but not really... Applications of calcium *sulfate* to bone healing and in bone cements, of obvious interest to the bone, tooth, and biomaterials communities [119-122], suggest an alternative path that calcium phosphate research might have traveled. Intriguingly, calcium sulfate exists in two relatively common (geological) mineral forms, one anhydrous (CaSO_4) and the other hydrated ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$). It is also easy to make the hemihydrate phase ($\text{CaSO}_4 \cdot 0.5\text{H}_2\text{O}$) that is employed in the above papers. One wonders how all these decades of bone mineral research would have played out if, very early on, two calcium phosphate phases had been accepted, one a highly crystalline unhydrated (but hydroxylated) phase OHAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and the other a more complex hydrated phase [65] such as $\text{Ca}_{10-x}\text{Na}_z(\text{PO}_4)_6 \cdot x(\text{HPO}_4, \text{CO}_3)_x (\text{OH})_{2-(x-z/2)} \cdot n\text{H}_2\text{O}$.

CONCLUSION

Scientists want to identify those ways of thinking and types of analytical techniques or approaches that will lead to progress and research success. To this end, they can benefit from the analysis of historical successes in which early hypotheses were proven to be correct and then used as springboards to new hypotheses, which, in turn, revealed important knowledge, created beneficial new techniques, and produced widely applicable new biomaterials, etc.

The above is the idealized way in which science purportedly operates: Those ideas that are shown to be wrong are rejected; repetition of this selection process culls the wrong ideas and eventually leads to documentable truths. It is disconcerting to realize, however, that there are many counter-examples to the above scenario, in which a truth or fact is discovered or a correct observation made, but this truth is either rejected or simply overlooked. Such

negative-seeming stories can be equally instructive, however, if analysis of them helps scientists to avoid such mistakes in the future.

The present chapter addresses such a counter-example, the story of water in the mineral apatite. The presentation begins as an historical account, accompanied by modern interpretation of the research results. As the account nears its end, however, it becomes clear that this discussion is not only historical. The understanding of the crystallographically incorporated water in apatite and, perhaps more importantly, the determination of its biological and geological ramifications is an on-going saga. The second part of the chapter suggests possible reasons for the circuitous route to increased acceptance of water's crystallographically controlled presence in apatite, including bone mineral. Somewhat counter-intuitively, it appears that the pertinent medical communities' decades-long resistance to accepting water as an essential component in bone mineral reflects more of an avoidance than a rejection of the idea.

What is exceptional about the investigation of lattice water in apatite aside from its extremely long history? One feature of note is the absence of exciting or embittered battles in the literature that could have highlighted the need to make some major choice between competing models. The accepted formula for bone mineral, a variation on that of hydroxylapatite, did not reveal H₂O as an explicit component or even suggest where H₂O might substitute for some accepted component. Models, like mineral formulas, should provide guidance in our research, but should not obstruct new ways of thinking.

It would appear that the community is still waiting to know if and how the incorporation of a few weight percent of water in bioapatite crystallites should affect current views of inorganic bone mineral and how it responds in a dynamic, watery, organic-rich, biological system. Interested researchers in many fields should take note.

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REFERENCES

- [1] D. McConnell, *American Mineralogist* 23, 1 (1938).
- [2] C. Klein and B. Dutrow, *Mineral Science*, John Wiley and Sons, Inc., New York (2007).
- [3] J. M. Hughes and J. Rakovan, in *Phosphates -- Geochemical, Geobiological, and Materials Importance*, 48 (Eds.: M. J. Kohn, J. Rakovan, and J. M. Hughes), Mineralogical Society of America, Chantilly, Virginia, pp. 1-12 (2002).

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- [4] Y. Pan and M. E. Fleet, in *Phosphates -- Geochemical, Geobiological, and Materials Importance*, 48 (Eds.: M. J. Kohn, J. Rakovan, and J. M. Hughes), Mineralogical Society of America, Chantilly, Virginia, pp. 13-49 (2002).
- [5] S. Gabriel, *Zeitschrift für Physiologische Chemie* 18, 257 (1894).
- [6] D. McConnell, *American Journal of Science* 36, series 5, 296 (1938).
- [7] J. C. Elliott, *Structure and chemistry of the apatites and other calcium orthophosphates*, Elsevier, New York (1994).
- [8] D. McConnell, *Bulletin de la Societe Francaise de Mineralogie et Cristallographie* 7-9, 428 (1952).
- [9] D. McConnell, *Science* 134, 213 (1961).
- [10] A. S. Posner, *Science* 134, 213 (1961).
- [11] R. Z. LeGeros, O. R. Trautz, E. Klein, and J. P. LeGeros, *Experientia* 25, 5 (1969).
- [12] G. Penel, G. Leroy, C. Rey, and E. Bres, *Calcified Tissue International* 63, 475 (1998).
- [13] J. C. Elliott, in *Phosphates -- Geochemical, Geobiological, and Materials Importance*, 48 (Eds.: M. J. Kohn, J. Rakovan, and J. M. Hughes), Mineralogical Society of America, Chantilly, Virginia, pp. 427-453 (2002).
- [14] R. M. Biltz and E. D. Pellegrino, *Calcified Tissue International* 36, 259 (1971).
- [15] J. D. Termine and D. R. Lundy, *Calcified Tissue International* 13, 73 (1973).
- [16] J. L. Meyer, *Calcified Tissue International* 27, 153 (1979).
- [17] C. Rey, J. L. Miquel, L. Facchini, A. P. Legrand, and M. J. Glimcher, *Bone* 16, 583 (1995).
- [18] J. D. Pasteris, B. Wopenka, J. J. Freeman, K. Rogers, E. Valsami-Jones, J. A. M. van der Houwen, and M. J. Silva, *Biomaterials* 25, 229 (2004).
- [19] C. Rey, C. Combes, C. Drouet, and M. J. Glimcher, *Osteoporosis International* 20, 1013 (2009).
- [20] G. Cho, Y. Wu, and J. L. Ackerman, *Science* 300, 1123 (2003).
- [21] J. Kolmas and W. Kolodziejewski, *Chemical Communications* 2007, 4390 (2007).
- [22] R. Legros, N. Balmain, and G. Bonel, *Journal of Chemical Research (S)* 1986, 8 (1986).
- [23] Z. Z. Zyman, D. V. Rokhmistrov, V. I. Glushko, and I. G. Ivanov, *Journal of Materials Science:Materials in Medicine* 20, 1389 (2009).
- [24] E. von Bibra, *Chemische Untersuchungen über die Knochen und Zähne des Menschen und der Wirbeltiere*, Kunstverlag, Schweinfurt, Germany (1844).
- [25] C. Aeby, *Journal für Praktische Chemie* 5, 308 (1872).
- [26] C. Aeby, *Journal für Praktische Chemie* 6, 169 (1873).
- [27] C. Aeby, *Journal für Praktische Chemie* 10, 408 (1875).
- [28] W. E. Brown and L. C. Chow, *Annual Review of Materials Science* 6, 213 (1976).
- [29] R. Maly and J. Donath, *Journal für Praktische Chemie* 7, 413 (1873).
- [30] F. Wibel, *Journal für Praktische Chemie* 9, 113 (1874).
- [31] C. Aeby, *Berichte der Deutschen Chemischen Gesellschaft* 7, 555 (1874).
- [32] H. Watts, *A Dictionary of Chemistry and the Allied Branches of Other Sciences*, Longmans, Green, and Co., London (1875).
- [33] O. Förster, *Zeitschrift für Angewandte Chemie* 5, 13 (1892).
- [34] M. J. Glimcher, in *Medical Mineralogy and Geochemistry* (Eds.: N. Sahai and M. A. A. Schoonen), Mineralogical Society of America, Washington, D.C., pp. 223-282 (2006).
- [35] A. Boskey, *Elements* 3, 385 (2007).

-
- [36] W. F. de Jong, *Recueil des Travaux Chimiques des Pays-Bas* 45, 445 (1926).
- [37] S. Náráy-Szabó, *Zeitschrift für Kristallographie* 75, 387 (1930).
- [38] M. Mehmél, *Zeitschrift für Kristallographie* 75, 323 (1930).
- [39] M. Mehmél, *Zeitschrift für Physikalische Chemie [B]* 15, 223 (1931).
- [40] C. A. Beevers and D. B. McIntyre, *Mineralogical Magazine* 27, 254 (1946).
- [41] G. Trömel and H. Möller, *Zeitschrift für Anorganische und allgemeine Chemie* 206, 227 (1932).
- [42] A. Schleede, W. Schmidt, and H. Kindt, *Zeitschrift für Elektrochemie und angewandte physikalische Chemie* 38, 633 (1932).
- [43] R. Klement, *Zeitschrift für Anorganische und allgemeine Chemie* 237, 161 (1938).
- [44] R. Klement, *Die Naturwissenschaften* 26, 145 (1938).
- [45] S. B. Hendricks, M. E. Jefferson, and V. M. Mosley, *Zeitschrift für Kristallographie, Kristallgeometrie, Kristallphysik, Kristallchemie* 81, 352 (1932).
- [46] C. Huggins, *Physiological Reviews* 17, 119 (1937).
- [47] S. B. Hendricks and W. L. Hill, *Science* 96, 255 (1942).
- [48] S. Eisenberger, A. Lehrman, and W. D. Turner, *Chemical Reviews* 26, 257 (1940).
- [49] W. F. Neuman, T. Y. Toribara, and B. J. Mulryan, *Journal of the American Chemical Society* 75, 4239 (1953).
- [50] W. F. Neuman and M. W. Neuman, *The chemical dynamics of bone mineral*, University of Chicago Press, Chicago (1958).
- [51] G. W. Burnett and J. Zenewitz, *Journal of Dental Research* 37, 581 (1958).
- [52] M. F. Little, E. S. Cueto, and J. Rowley, *Archives of Oral Biology* 7, 173 (1962).
- [53] M. F. Little and F. S. Casciani, *Archives of Oral Biology* 11, 565 (1966).
- [54] H. M. Myers, *Nature* 206, 713 (1965).
- [55] N. Myrberg, *Transactions of the Royal Schools of Dentistry, Stockholm and Umea* series 2, no. 14, 1 (1968).
- [56] D. Carlström, J. E. Glas, and B. Angmar, *Journal of Ultrastructural Research* 8, 24 (1963).
- [57] G. P. Brophy and J. T. Nash, *American Mineralogist* 53, 445 (1968).
- [58] D. R. Simpson, *American Mineralogist* 53, 432 (1968).
- [59] E. F. Cruft, C. O. Ingamells, and J. Muysson, *Geochimica et Cosmochimica Acta* 29, 581 (1965).
- [60] D. R. Simpson, *American Mineralogist* 49, 363 (1964).
- [61] D. McConnell, *Clinical Orthopaedics* 23, 253 (1962).
- [62] D. McConnell, *Archives of Oral Biology* 10, 421 (1965).
- [63] F. Paulik, K. Eröss, J. Paulik, T. Farkas, and T. Vizkelety, *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* 350, 418 (1969).
- [64] J. C. Elliott, *Calcified Tissue International* 3, 293 (1969).
- [65] D. McConnell, *American Mineralogist* 55, 1659 (1970).
- [66] S. J. Joris and C. H. Amberg, *Journal of Physical Chemistry* 75, 3167 (1971).
- [67] S. J. Joris and C. H. Amberg, *Journal of Physical Chemistry* 75, 3172 (1971).
- [68] F. S. Casciani, in *Tooth Enamel II: Its Composition, Properties, and Fundamental Structure* (Eds.: R. W. Fearnhead and M. V. Stack), The Williams and Wilkins Company, Baltimore, pp. 14-23 (1971).
- [69] G. H. Dibdin, *Archives of Oral Biology* 17, 433 (1972).

-
- [70] M. Vignoles, Contribution à l'étude de l'influence des ions alcalins sur la carbonation dans les sites de type B des apatites phosphocalcique, Ph.D. thesis, Institut National Polytechnique de Toulouse, France, 1973.
- [71] G. Bonel, J. C. Labarthe, and M. Vignoles, in *Physico-Chemie et Cristallographie des Apatites d'Interêt Biologique* (Ed.: no. 2. Colloques Internationaux du Centre National de la Recherche Scientifique), CNRS, Paris, pp. 117-125 (1973).
- [72] R. M. Wilson, J. C. Elliott, S. E. P. Dowker, and R. I. Smith, *Biomaterials* 25, 2205 (2004).
- [73] R. Z. LeGeros, G. Bonel, and R. Legros, *Calcified Tissue International* 26, 111 (1978).
- [74] J. D. Termine, E. D. Eanes, D. J. Greenfield, M. U. Nysten, and R. A. Harper, *Calcified Tissue International* 12, 73 (1973).
- [75] D. W. Holcomb and R. A. Young, *Calcified Tissue International* 31, 189 (1980).
- [76] R. A. Young and D. W. Holcomb, *Calcified Tissue International* 34, S17-S32 (1982).
- [77] A. Mortier, J. Lemaitre, and P. G. Rouxhet, *Thermochimica Acta* 143, 265 (1989).
- [78] E. A. P. De Maeyer, R. M. H. Verbeeck, and D. E. Naessens, *Inorganic Chemistry* 32, 5709 (1993).
- [79] A. C. Tas, *Biomaterials* 21, 1429 (2000).
- [80] J. P. Lafon, E. Champion, D. Bernache-Assollant, R. Gibert, and A. M. Danna, *Journal of Thermal Analysis and Calorimetry* 72, 1127 (2003).
- [81] M. G. Taylor, K. Simkiss, S. F. Parker, and P. C. H. Mitchell, *Physical Chemistry Chemical Physics* 1, 3141 (1999).
- [82] A. Antonakos, E. Liarokapis, and T. Leventouri, *Biomaterials* 28, 3043 (2007).
- [83] J. Reyes-Gasga, R. García-García, M. J. Arellano-Jiménez, E. Sanchez-Pastenes, G. E. Tiznado-Orozco, I. M. Gil-Chavarria, and G. Gómez-Gasga, *Journal of Physics D: Applied Physics* 41, 1 (2011).
- [84] J. Shi, A. Klocke, M. Zhang, and U. Bismayer, *American Mineralogist* 88, 1866 (2003).
- [85] S. Cazalbou, C. Combes, D. Eichert, and C. Rey, *Journal of Materials Chemistry* 14, 2148 (2004).
- [86] C. Rey, C. Combes, C. Drouet, A. Lebugle, H. Sfihi, and A. Barroug, *Materialwissenschaft und Werkstofftech* 38, 996 (2007).
- [87] C. Rey, C. Combes, C. Drouet, H. Sfihi, and A. Barroug, *Materials Science and Engineering C* 27, 198 (2007).
- [88] T. I. Ivanova, O. V. Frank-Kamenetskaya, A. B. Kol'tsov, and V. L. Ugolkov, *Journal of Solid State Chemistry* 160, 340 (2001).
- [89] F. W. Wehrli and M. A. Fernández-Seara, *Annals of Biomedical Engineering* 33, 79 (2005).
- [90] A. Kafalak-Hachulska, A. Samoson, and W. Kolodziejcki, *Calcified Tissue International* 73, 476 (2003).
- [91] E. E. Wilson, A. Awonusi, M. D. Morris, D. H. Kohn, M. M. J. Tecklenburg, and L. W. Beck, *Journal of Bone and Mineral Research* 20, 625 (2004).
- [92] E. E. Wilson, A. Awonusi, M. D. Morris, D. H. Kohn, and M. M. J. Tecklenburg, *Biophysical Journal* 90, 3722 (2006).
- [93] R. M. Wilson, J. C. Elliott, S. E. P. Dowker, and L. M. Rodriguez-Lorenzo, *Biomaterials* 26, 1317 (2005).
- [94] R. M. Wilson, S. E. P. Dowker, and J. C. Elliott, *Biomaterials* 27, 4682 (2006).
- [95] H. E. Mason, A. Kozlowski, and B. L. Phillips, *Chemistry of Materials* 20, 294 (2008).

-
- [96] F. M. McCubbin, H. E. Mason, H. Park, B. L. Phillips, J. B. Parise, H. Nekvasil, and D. H. Lindsley, *American Mineralogist* 93, 210 (2008).
- [97] H. E. Mason, F. M. McCubbin, A. Smirnov, and B. L. Phillips, *American Mineralogist* 94, 507 (2009).
- [98] C. H. Yoder, J. D. Pasteris, K. N. Worcester, and D. V. Schermerhorn, *Calcified Tissue International* 90, 60 (2012).
- [99] J. Jeanjean, S. McGrellis, J. C. Rouchaud, M. Fedoroff, A. Rondeau, S. Perocheau, and A. Dubis, *Journal of Solid State Chemistry* 126, 195 (1996).
- [100] G. Graziano, *Chemical Physics Letters* 396, 226 (2004).
- [101] J. C. Labarthe, Contribution à l'étude de la structure et des propriétés des apatites carbonatées de type B phospho-calcique, Ph.D. thesis, Université Paul Sabatier, Toulouse, 1972.
- [102] J. C. Labarthe, G. Bonel, and G. Montel, *Annales de Chimie (Paris)* 8, 289 (1973).
- [103] W. Kolodziejcki, *Topics in Current Chemistry* 246, 235 (2005).
- [104] A. Kafalak and W. Kolodziejcki, *Journal of Molecular Structure* 990, 263 (2011).
- [105] J. D. Pasteris, C. H. Yoder, and B. Wopenka, *Geological Society of America Abstracts with Programs* 42, 220 (2010).
- [106] D. McConnell, *Archives of Oral Biology* 3, 28 (1960).
- [107] A. Ebrahimpour, M. Johnsson, C. F. Richardson, and G. H. Nancollas, *Journal of Colloid and Interface Science* 159, 158 (1993).
- [108] S. Koutsopoulos, *Journal of Biomedical Materials Research* 62, 600 (2002).
- [109] K. D. Rogers and P. Daniels, *Biomaterials* 23, 2577 (2002).
- [110] K. Rogers and P. Zioupos, *Journal of Materials Science Letters* 18, 651 (1999).
- [111] H. C. W. Skinner, *Mineralogical Magazine* 69, 621 (2005).
- [112] R. Z. LeGeros and J. P. LeGeros, in *Phosphate Minerals* (Eds.: J. O. Nriagu and P. B. Moore), Springer-Verlag, New York, pp. 351-395 (1984).
- [113] S. Mann, *Biomineralization: Principles and Concepts in Bioinorganic Materials*, Oxford University Press, Oxford (2001).
- [114] S. V. Dorozhkin, *Acta Biomaterialia* 6, 4457 (2010).
- [115] L. Bertinetti, C. Drouet, C. Combes, C. Rey, A. Tampieri, S. Coluccia, and G. Martra, *Langmuir* 25, 5647 (2009).
- [116] K. A. Gross and C. C. Berndt, in *Phosphates -- Geochemical, Geobiological and Material Importance*, 48 (Eds.: M. J. Kohn, J. Rakovan, and J. M. Hughes), Mineralogical Society of America, Chantilly, Virginia, pp. 631-672 (2002).
- [117] Y. Hong, H. Fan, B. Li, B. Guo, M. Liu, and X. Zhang, *Materials Science and Engineering R* 70, 225 (2010).
- [118] M. Lombardi, P. Palmero, K. Haberko, W. Pyda, and L. Montanaro, *Journal of the European Ceramic Society* 31, 2513 (2011).
- [119] R. M. Urban, *Clinical Orthopaedics and Related Research* 459, 110 (2007).
- [120] L. Podaropoulos, A. A. Veis, S. Papadimitriou, and C. Alexandridis, *The Journal of Oral Implantology* 35, 28 (2009).
- [121] R. Crespi, P. Capparé, and E. Gherlone, *Journal of Periodontology* 80, 210 (2009).
- [122] H.-Y. Song, A. H. M. E. Raman, and B.-T. Lee, *Journal of Materials Science: Materials in Medicine* 20, 935 (2009).