Relationships between Polyphosphate Chemistry, Biochemistry and Apatite Biomineralization

Sidney J. Omelon and Marc D. Grynpas*

Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Avenue, Toronto, Canada

Received February 4, 2008

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1. Introduction

Biomineralization processes provide astonishingly complex and functional solid structures that are composed of materials with remarkable properties providing advantages to numerous

* To whom correspondence should be addressed. Mailing address: Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Room 840, Toronto, Ontario M5G 1X5, Canada. Tel: (416) 586-4800, ext. 4464. Fax: (416) 586-8844. E-mail: grynpas@lunenfeld.ca.

10.1021/cr0782527 CCC: $71.00
Published on Web 11/01/2008

Sidney Omelon received a B.Eng (chemical engineering) from McGill University (1994). Interspersing her graduate work with industrial work as a process engineer, she studied calcium sulfate precipitation (M.Eng., mining and metallurgical engineering, McGill University, 1998) then calcium polyphosphates (Ph.D., materials science and engineering and biomedical engineering, University of Toronto, 2006) under Marc Grynpas. She spent a year as a postdoctoral fellow at the Samuel Lunenfeld Research Institute working on bone mineralization under Marc Grynpas. Currently she is working with carbonate minerals for a start-up company in Northern California. Her research interests continue to be inspired by mineral precipitation and dissolution.

Marc Grynpas, Ph.D., is a professor in the Department of Laboratory Medicine and Pathobiology and a member of the Institute for Biomaterial and Biomedical Engineering at the University of Toronto. He is also a Senior Scientist at the Samuel Lunenfeld Research Institute of Mount Sinai Hospital and the Director of the Bone and Mineral Research Group at the University of Toronto. Dr. Grynpas graduated from the Free University of Brussels with an undergraduate degree in Physics. At the University of London, he completed his Ph.D. in Crystallography and Biophysics on the structure of bone. After a postdoctoral fellowship at Queen Mary College (University of London) on the relation between bone structure and bone mechanical properties, he joined the laboratory of Professor Melvin Glimcher at the Children's Hospital in Boston where he worked on the nature of bone mineral. The research in his laboratory is focused on the nature of bone mineral, animal models of osteoporosis and osteoarthritis, the effects of drugs and trace elements on bone quality, and the determinants of bone fragility and bone fatigue.

life forms.[1] Some invertebrates control the crystallization of calcium carbonate polymorphs (aragonite or calcite) within organic matrices, for example. Marine sponges and diatoms biomineralize intricate silicate structures by controlling the nucleation and condensation of silicate ions. The mineral in the vertebrate skeleton, apatite ($Ca_5(PO_4)_3(F,OH,Cl)$), is predominately composed of phosphate and calcium.[1] Biomineralized materials composed of carbonate, silicate, or phosphate provide structure, protection, and tools for predation. However, the vertebrate skeleton also satisfies a wider range of demands such as growth, self-repair, and sequestration of heavier elements, and is a source of calcium, phosphate, and pH control to assist in maintaining homeostasis. Until recently, the reason why apatite is the mineral component of the vertebrate skeleton was not understood.[2]

Biomineralization of apatite is not limited to vertebrates; it is also associated with marine, oral, and other bacteria and has been observed within mitochondria. Previously, the biomineralization of vertebrate apatite was associated with unstable, amorphous, calcium- and phosphate-containing "electron-dense granules". The role or the composition of these electron-dense granules was not understood; they were associated with apatite mineralization by their colocation with mineralizing skeletal tissues. Electron-dense granules[3] have been found in many organisms and are proposed to have a range of elemental compositions and roles, including calcium, phosphate, and carbonate storage and transport. Calcium- and phosphate-containing granules have been identified within calcium carbonate mineralizing orthopods; they were proposed to store and transport calcium between molting. Granules containing calcium and phosphate have been located on the inside and outside of mitochondria. It has been postulated that mitochondria serve a role in biomineralization, but that role has not been identified.

Researchers outside the field of biomineralization have determined that mitochondria can produce polyphosphate $(PO_3^-)_n$, as well as store calcium in a calcium−polyphosphate complex. In recent years, marine microorganisms were shown to produce marine apatite deposits through the formation and degradation of polyphosphates.[4−6] This association between polyphosphate biochemistry and apatite biomineralization is new. Recent identification of polyphosphates in mineralizing cartilage and bone suggests that the electron-dense granules previously detected in and related to apatite formation in the vertebrate skeleton may contain calcium and polyphosphate.

The study of polyphosphate chemistry remains on the fringe of chemistry and biochemistry.[7] Polyphosphates, also known as condensed phosphates, are polymers of phosphate connected through a phosphoanhydride bond. Polyphosphates serve many biological roles, including storage of phosphate, a source of energy in anoxic environments, and the sequestration of multivalent cations. Polyphosphates have only relatively recently been identified in a wide range of life forms, including bacteria, plants, animals, yeasts, and fungi.[8] The biochemical roles of polyphosphates continue to be discovered; the enzymes responsible for polyphosphate condensation and hydrolytic degradation also continue to be identified.[9] Three important attributes of polyphosphates that render it a useful resource for the biomineralization of apatite are its affinity for calcium, its ability to sequester a local high concentration of orthophosphates while maintaining a low orthophosphate activity, and its ability to be synthesized and degraded by enzymes.[9]

Inorganic or enzymatic hydrolytic degradation of calcium polyphosphate complexes can result in the precipitation or biomineralization of apatite. In an elegant reversal of this process, where apatite biominerals are reabsorbed, as occurs in the vertebrate skeleton, the phosphate from dissolved apatite can be enzymatically condensed into polyphosphates. The formation of polyphosphates, effectively reducing the free orthophosphate activity, stores orthophosphate ions in a local, high concentration and provides a counterion for calcium sequestration; polyphosphate chemistry provides a valuable tool for apatite biomineralization.

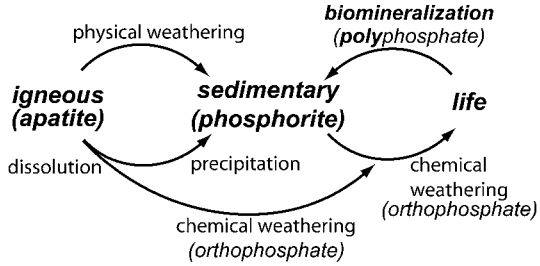

Figure 1. Simplified schematic of the phosphorus cycle from apatitic igneous rock to phosphorite sedimentary rock through chemical or physical weathering. Life forms accumulate soluble phosphorus species and can produce apatite through biomineralization.

The purpose of this thematic and qualitative review is to provide a brief introduction to apatite chemistry and survey relevant publications in geomicrobiology, geology, mineralogy, microbiology, pathology, and skeletal biology from as early as the 19th century as they relate to the mineralization and biomineralization of apatite. The links in the literature between electron-dense granules and biomineralization, as well as the relationship between polyphosphate chemistry and apatite formation are surveyed. Following a brief introduction to polyphosphate chemistry, the recent concept of vertebrate apatite biomineralization control though the enzymatic formation and destruction of polyphosphates will be reviewed and thoughts on future research directions presented.

2. Phosphate Chemistry

Decades ago, phosphorus chemistry was an active area of research. Between 1958 and 1983, the 11 volume series *Topics in Phosphorus Chemistry*[10] and two texts on general phosphorus chemistry[11,12] were published. Much of the sections on phosphate and polyphosphate chemistry reviewed here are sourced from works produced by the late E. J. Griffith, who wrote and edited many phosphate chemistry journal articles as well as books on phosphate chemistry.

2.1. Phosphates on Earth

Phosphorus is approximated to have the ninth largest total concentration in igneous rocks (rocks formed from cooled magma), excluding oxygen and nitrogen. Most naturally occurring phosphate on Earth is found in the phosphate mineral "apatite" ($Ca_5(PO_4)_3(F,OH,Cl)$)[13] Approximately half of the phosphate reserves in the lithosphere of the Earth are igneous rock (apatite), while the other half is a component of sedimentary rock.[14] Sedimentary rock, also known as phosphorite, can be formed by weathering of igneous rock into smaller particles.[15] It can also be composed of minerals precipitated from the dissolution products of igneous rock or minerals produced by biomineralization (Figure 1). The dissolution of phosphate rock can produce soluble orthophosphate (PO_4^{3-}) ions that can be incorporated into life forms.

On land, phosphorus is a limiting nutrient for biological productivity; this productivity is related to the dissolution of apatite. Apatite is a major source of phosphorus; it is an ore mined to produce fertilizers, detergents, and phosphoric acid.[13] Dissolution and transport of terrestrial apatite to the oceans is the major marine source of phosphate.[16]

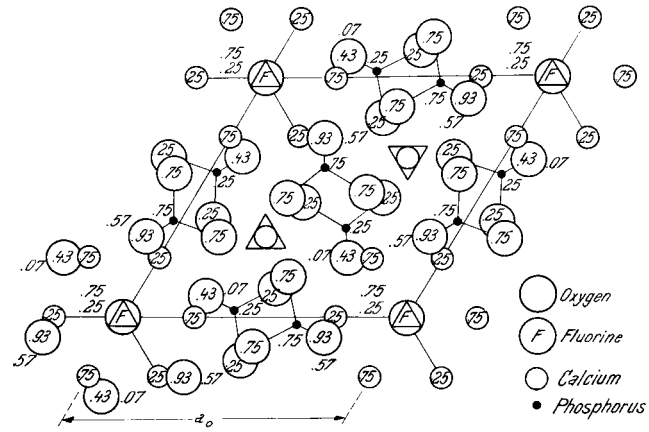

Figure 2. Projection of the apatite structure on (0001). Elevations are represented as fractions of *c*; *m* planes are located at 0.25 and 0.75 *c*, which represents 3-fold axes on which Ca atoms are located; the origin is the 6_3 axis (lower right). Reprinted from ref 23, Copyright 1973, with permission from Springer-Verlag.

2.2. The Importance of Phosphates to Living Systems

Phosphate is argued to be one of the essential elements for life on Earth.[16,17] Griffith claimed that it is not possible to know whether the properties of phosphates dictated the characteristics of life or life on Earth required some of the unique properties of phosphate.[18] Some of these important properties include a stable oxidation state (+5) and a buffering capacity and range near pH 7. Phosphorylation and dephosphorylation are important biochemical mechanisms. Enzymatic condensation of phosphates and hydrolytic degradation of condensed phosphates and phosphate esters occur in biochemical and inorganic processes.[11,19] Phosphate mineral solubility ranges from higher values for minerals such as brushite ($CaHPO_4 \cdot 2H_2O$) to very low values such as the apatite minerals found in bones and teeth.

3. Apatite

Different calcium phosphate minerals are stable at different pHs; however, apatite is the most common "natural" calcium phosphate mineral and is also the biomineral found in the vertebrate skeleton. Due to their differences, geological apatite[15,20] and biological apatite[21] will be introduced independently.

3.1. Geological Apatite

Apatite is named from the Greek απαταω (to deceive) because it was commonly confused with other minerals (reported by Gerhard in 1786, cited in ref 22). The state of the apatite literature in 1973 was noted to be voluminous and not always rigorous.[23] Interested readers are directed to a special issue of *Reviews in Mineralogy and Geochemistry*[13] and the text by McConnell.[23]

The basic structure of apatite was first determined by both Náray-Szabó[24] and Mehmel.[25] Apatite is often described by the formula $Ca_5(PO_4)_3(F,OH,Cl)$ because F (fluorapatite), OH (hydroxylapatite), and Cl (chlorapatite) are the most common subsitutions.[22] More generally, apatite is described as $A_5(TO_4)_3Z$ in which A = cations Ca, Sr, Pb, Ba, etc., T = anions of oxide groups P, As, or V, and Z = F, OH, or Cl.[26] The apatite unit cell is large; the fluorapatite ($Ca_{10}F_2(PO_4)_6$) unit cell is composed of 42 atoms (Figure 2).[27]

Table 1. Possible Substitutions in the Francolite Structure[a])

constituent ion	substituting ion[b]
Ca^{2+}	**Na^{+}**, K^{+}, Ag^{+} **Mg^{2+}**, **Sr^{2+}**, Ba^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+} Bi^{3+}, Sc^{3+}, Y^{3+}, REE^{3+} U^{4+}
PO_4^{3-}	**CO_3^{2-}**, **SO_4^{2-}**, CrO_4^{2-} **$CO_3 \cdot F^{3-}$**, $CO_3.OH^{3-}$, AsO_4^{3-}, VO_4^{3-} SiO_4^{4-}
F^{-}	OH^{-}, Cl^{-}, Br^{-} O^{2-}

[a] Adapted with permission from ref 29. Copyright 1994 Swiss Geological Society. [b] REE = rare-earth elements; compositionally significant substitutions are indicated in bold.

A characteristic of apatite mineral that is relevant to this review is the tolerance of the apatite structure to substitutions. In 1938, McConnell stated that "The structure of apatite seems to be remarkably stable, permitting a number of rather unusual types of substitution and involving a considerable number of ions".[27] Apatite is able to incorporate half of the elements in the periodic table within its crystal structure.[28] Table 1 presents a list of known substitutions for a common carbonated, fluoridated apatite (francolite).[29] Ease of atomic substitution for apatite leaves this mineral open to a wide array of compositions.

Substitutions influence many mineral properties, including solubility. For example, F^{-} substitutions will decrease apatite solubility, while CO_3^{2-} substitutions will increase its solubility.[30] The carbonate ion is another common substitution.[22] Synthetic carbonate apatites have also been prepared and characterized.[22] Carbonated apatite is not a very common naturally occurring mineral but is most similar to the mineral in bones and teeth.[31] Carbonated apatite containing less than 1 wt % fluoride has been named dahllite.[27] Francolite[32] is a another commonly occurring carbonated apatite mineral that contains a higher weight percent of fluoride.[33]

The list of review papers[22] and conference proceedings[22,29] on the subject of apatite mineral is far longer than is referenced in this review. Here, it is sufficient to introduce apatite as a commonly occurring mineral composed mostly of calcium and phosphate, with significant carbonate and fluoride content. Apatite minerals generally exhibit low solubility at neutral to high pH and hold a high potential for ionic substitutions. Apatite therefore has a wide range of compositions and properties. As noted by Gruner[33] "The apatite group seems to enjoy the questionable distinction of having produced fewer reliable analyses than almost any other group of minerals."

3.2. Biological Apatite

Apatite mineral is not only found within our physical landscape but also generated within or by the action of biological organisms through processes called biomineralization.[1] Lowenstam[34] first grouped "biological mineralization" into two processes. By one method, mineralization is orchestrated by the organism through an "organic matrix-mediated" process. The organism produces an organic structure within which ions are located. This is followed by the induction of crystal nucleation and growth. The other method is "characterized by bulk extracellular and/or intercellular mineral formation"; this biological process results in crystal products similar to those expected by crystallization from solution.[34,35] Biomineralized apatite has been associated with many life forms. The mechanisms for biological apatite production are varied and still under investigation. The references on these topics are much more extensive than presented here. This review will focus on microbiological and vertebrate apatite biomineralization.[36]

3.2.1. Microbial Apatite

Some smaller organisms, including a few invertebrates,[37,38] oral bacteria,[36,39] mitochondria,[40] *Escherichia coli*,[41] and other bacteria[42−45] form apatitic calcifications, which can form in both intra- and extracellular locations.[46] Marine bacteria, oral bacteria, and some other bacteria such as *E. coli* have been identified as organisms that are capable of metabolizing phosphorus and mineralizing apatite. Large submarine phosphorite deposits have recently been associated with bacterial activity.[4]

3.2.2. Vertebrate Apatite

Interest in vertebrate mineralization processes has resulted in many reviews and publications on the formation of apatite in bone,[26,47−53] cartilage,[54−58] enamel,[59−62] and dentin,[63,64] as well as pathological calcification.[65−67]

De Jong was the first to provide evidence of the similarity of the apatite structure to the mineral of bone,[68] suggesting that bone mineral could be carbonated apatite. Optical and X-ray analysis of bone and other mineralized tissues by Taylor and Sheard matched analyses of two apatites: fluorapatite and dahllite.[69] The opinion that bone mineral is a substituted carbonated apatite similar to dahllite is commonly held by mineralogists and scientists trained in the physical sciences.[51,70−74]

However, biochemists first approached the problem of characterizing the mineral in bone by its in vivo solubility. Neuman[75] stated that "the hydroxy apatite is the only solid phase of the $Ca-PO_4-H_2O$ system which is stable at neutral pH". The fact that hydroxyapatite is the most stable calcium phosphate phase at physiological pH[76] seems to have led to the idea that it is probably the mineral component in biological systems such as bone. This in turn has led to a large and current literature referring to the apatitic biomineral as hydroxyapatite.[77] Hydroxyapatite is commonly used as a model for biological mineralization and demineralization[78] and orthopedic biomaterials.[79]

The staunch mineralogist McConnell passionately decried this association of bone with hydroxyapatite,[71] emphasizing that "the substance of teeth and bones should properly be called a carbonate hydroxy-apatite or dahllite".[31] McConnell pulled no punches, writing, "The literature (Carlström lists 195 references) is burdened with meaningless data, faulty logic and *non sequiturs*. Many papers which have been written on the problem of carbonate apatites show no evidence that the authors were cognizant of previous investigations on closely related segments of the problem."[71] It was later shown that hydroxyl groups are not detectable in bone apatite crystals[80] but that has not necessarily corrected the common terminology for bone mineral. The uncertainty in both the initial phases[81,82] and the process of bone mineralization are still not clear and are under debate.

For the purposes of this review, it is important to be aware that biological carbonated apatite is typically composed of very small crystallites (on the order of tens of angstroms[83]), can be highly substituted,[23] and is poorly crystalline.[84] The high specific surface area and poor crystallinity of biological apatite promote rapid dissolution when required for biological

processes.[53] The genesis of biological apatite has been postulated to be a result of biochemical activity, such as the action of carbonic anhydrase[85,86] and alkaline phosphatase,[87] a phosphoesterase.[88]

3.2.3. Enyzmatic Formation of Apatite

Alkaline phosphatase has been implicated in the apatite mineralization of or by bacteria.[89,90] However, studies of calcifying bacteria such as *Ramlibacter tataouinesis* conclude that "The mechanism and, in particular, the source of phosphate leading to the biomineralization inside the cells are still unclear".[44] It has been postulated that microorganisms that store high intracellular concentrations of phosphates as polyphosphates[91] may consequently release phosphate as dissolved inorganic orthophosphate (PO_4^{3-}) after they die.[92] The release of orthophosphate from polyphosphates could be by spontaneous hydrolytic degradation or by enzymatic action of phosphatase enzymes such as alkaline phosphatase, 5′ nucleotidase, or cyclic phosphodiesterase.[89] This dissolved orthophosphate may reprecipitate as another phase, such as apatite, which is a generally insoluble form of phosphorus at neutral to alkaline conditions.[93] A relationship between the biochemistry of inorganic polyphosphates and the biomineralization of apatite may be related to an intermediate, concentrated, biological storage of calcium with phosphate, with structures identified in various fields by various names. In this review, these storage structures will be referred to as "electron-dense granules".

4. Electron-Dense Granules

Metal-containing granules have been reported in a wide range of organisms.[94] Reviewed recently within the context of kidney stones, Ryall commented that a wide range of organisms store calcium in "electron-dense granules".[3] Other names for these calcium-containing electron-dense granules include metal-containing granules, calcium granules, storage vesicles, calcium spherules, mineral concretions, mineral granules, spherocrystals, intracellular granules, calcium phosphate (CaP) granules, CaP spherites, and acidocalcisomes.[3] Four groups of granules (types A−D) have been identified; the list of proposed functions includes waste disposal, osmoregulation, excretion of excess ions, calcium/phosphate/carbonate storage and mobilization, skeletal function, and detoxification of poisonous heavy metals.[3] One of the four groups is described as typically spherical with a poorly crystalline or amorphous content composed of calcium, phosphate, magnesium, and some carbonate.[3]

Amorphous, electron-dense granules containing calcium and phosphate within arthropods that molt and bear a calcium carbonate skeleton was a puzzling discovery.[3] Noted to be "considerably more soluble, and hence, more rapidly mobilized, than crystalline mineral",[3] the calcium phosphate granules within an organ (hepatopancreas) of the arthopods were proposed to be a storage form of calcium and phosphate during the intermolt period. The role and speciation of phosphate in these calcium-mobilizing electron-dense granules is unclear in the literature.

4.1. Electron-Dense Granules, Mitochondria, Calcium Carbonate Biomineralization, and Polyphosphate?

In order to grow, the crustaceans with calcium carbonate skeletons, such as the terrestrial isopod *Porcellio scaber* (also commonly known as the potato bug) and the blue crab *Callinectes sapidus*, demineralize, molt, rebuild, and then remineralize their exoskeleton. Within the molting potato bug, Ziegler identified electron-dense granules with high calcium and phosphorus content and observed that "more than half of the Ca within the granules could be bound to phosphate and/or phosphorylated proteins."[95] The phosphorus concentration in the granules was much larger than the calcium carbonate containing sternal deposits.[96,97]

Similar to the potato bug, before the growing crab molts its skeleton, the skeletal mineral (calcium carbonate) is resorbed, and the calcium is retained within an organ known as the hepatopancreas. The "mineral" stored in the hepatopancreas is returned to the new exoskeleton. Becker et al. detected electron-dense calcium-, magnesium-, and phosphate-containing granules within the hepatopancreas and theorized that they were a mechanism for calcium mobilization in the crab.[98] Although the crab produces a calcium carbonate mineral skeleton, the carbonate content of the electron-dense granules was no more than 1% of the mineral weight. Interestingly, it was noted that considerable phosphorus, "undetectable as orthophosphate, was released in inorganic form by wet ashing (with sulfuric acid) and was thus present in covalent linkage, possibly to some organic moiety".[98] Although qualified as circumstantial, the role of mitochondria in the blue crab mineralization phenomenon was suggested, as was a possible relationship between the electron-dense granules and crab and vertebrate skeletal mineralization. The same group reported blue crab mitochondrial respiration and phosphorylation,[99] as well as mitochondrial accumulation of large amounts of calcium and phosphate.[100] In the yeast literature, it was shown that mitochondria produce polyphosphates to store phosphate;[101] it is possible that these previously observed electron-dense granules also contain polyphosphate. Given these observations of low orthophosphate content before hot acidic hydrolytic degradation, it is possible that this group observed calcium−polyphosphate granules as hot, acidic conditions catalyze the production of orthophosphates from polyphosphates.

4.2. Electron-Dense Granules, Mitochondria, Calcium Phosphate Biomineralization, and Polyphosphate?

Amorphous, electron-dense granules containing calcium and phosphate were discovered in the vertebrate skeleton.[102,103] Figure 3 shows calcium- and phosphate-containing electron-dense granules, termed mitochondrial granules, detected in bone.

Landis performed a careful electron microprobe analysis of different calcium phosphate minerals, calcium pyrophosphate, electron-dense granules, and mineral found in vertebrate bone (Figure 4).[103] The electron-dense granules found in mitochondria exhibited a Ca/P ratio close to that of calcium polyphosphate ($Ca(PO_3)_2$). It is possible that Landis also observed calcium- and polyphosphate-containing granules in bone prepared in anhydrous conditions.

This review will next provide a brief introduction to the chemistry and biochemistry of polyphosphates, with a focus on calcium polyphosphates. This will be followed by a

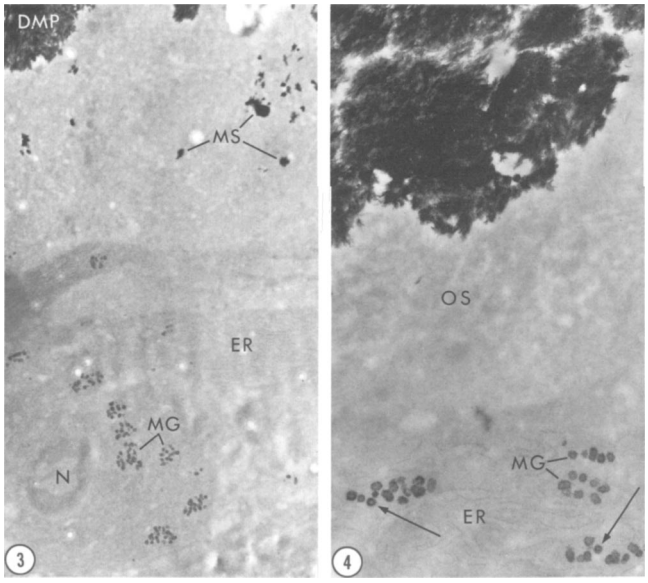

Figure 3. Unstained ultrastructure from a 6-day mouse rib, fixed in glutaraldehyde−paraformaldehyde at ×9500 magnification (left, "3"). Dense mitochondrial granules (MG) are observed in the absence of heavy metal counterstaining. Mineral clusters (MS) and dense masses of mineral phase (DMP) are also apparent. Nuclei (N) and endoplasmic reticulum (ER) are less distinct. At higher magnification (×21000, right, "4"), mitochondria remain relatively indistinct but can be defined by the presences of granule (MG) and surrounding endoplasmic reticulum (ER). Some granules appear with a less dense central region (arrows). Collagen in the osteoid (OS) is not distinguishable unstained. Reprinted from ref 102, Copyright 1977, with permission from Elsevier.

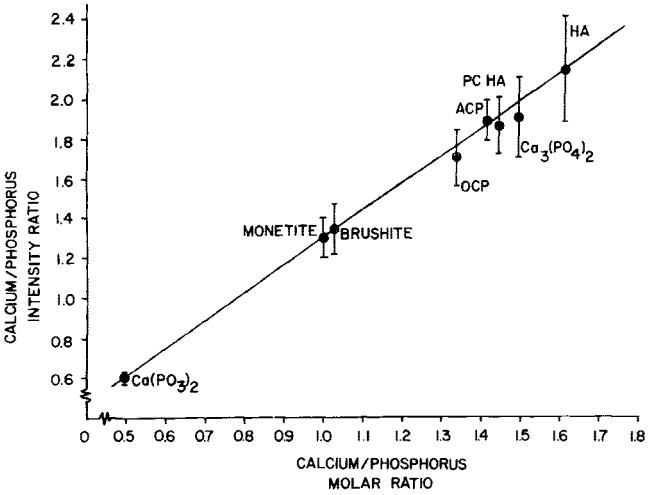

Figure 4. Ca/P intensity ratio as a function of Ca/P molar ratio for calcium phosphate solid phases prepared in vitro. Molar ratios corresponding to the intensity ratios for tibiae were determined by interpolation and found to be in the range of approximately 0.8−1.1 (mitochondrial granules), 1.2−1.3 (clusters of solid phase particles in the osteoid), and 1.4−1.5 (dense solid phase masses). Abbreviations: OCP, octacalcium phosphate; ACP, amorphous calcium phosphate; PC HA, poorly crystalline hydroxyapatite; HA, hydroxyapatite. Reprinted from ref 103, Copyright 1978, with permission from Elsevier.

review of the literature concerning biomineralization of apatite, electron-dense bodies, mitochondria, and polyphosphates.

5. Inorganic Polyphosphate Chemistry

The *Topics in Phosphorus Chemistry* series[104] and a book by Griffith[18] offer detailed reference for inorganic polyphosphate chemistry. The application of the chemistry of polyphosphates in the mineral processing industry was also reviewed.[105] This introduction to inorganic polyphosphate chemistry will be restricted to a description of the characteristics relevant to this review on apatite mineralization.

5.1. History

In 1827, Clark reported that when a phosphate of soda that was "dried at a red heat" was used in a double displacement reaction with nitrate of silver, a white solid product that was different than the previously reported yellow product (a phosphate of silver) was formed. He first named the pyrolized, condensed phosphates "pyrophosphates".[106] This term is currently used to describe the smallest polyphosphate: the product of the condensation of two orthophosphates ($P_2O_7^{4-}$).

5.2. Definition

Polyphosphates are derived from the parent orthophosphoric acid by the elimination of water between hydroxyl groups and the formation of a phosphoanhydride bond. Condensed phosphates may be described empirically as $H_{(n+2)}P_nO_{(3n+1)}$ or $n(H_3PO_4)\cdot(n-1)H_2O$, where n is the number of phosphorus atoms in the chain. Polyphosphates are also termed "condensed phosphates" and traditionally as "metaphosphates". The metaphosphate nomenclature is now reserved for referring to cyclic polyphosphate species, which will not be considered in this review. The linear polymer is usually the most common polyphosphate species in hydrated environments. Branched polyphosphates are stable in anhydrous conditions.

5.3. Formation

Polyphosphates can be formed by dehydration at high temperatures[18] or via condensation by select kinase enzymes.[9] The biochemistry of inorganic polyphosphates will be briefly reviewed in a succeeding section.

5.4. Hydrolytic Degradation (Hydrolysis)

Griffith made a special point of clarifying the terms "hydrolysis" and "hydrolytic degradation",[18] because they are both used in the literature to describe the process of breaking the P−O−P bond of polyphosphates. Hydrolysis is often used to describe the reaction of water with an organic ester. Griffith noted that because phosphates are excellent buffering agents, "they react with water as a reversal of neutralization resulting from the slight ionization of water"; he argued this is the usual meaning of hydrolysis.[18] In this work, hydrolytic degradation will be used specifically to describe P−O−P bond breakage by reaction with water. The literature includes mechanistic,[107] kinetic, and product definition studies[108−110] of polyphosphate hydrolytic degradation, which will not be reviewed here.

Griffith summarized "reliable generalizations" about polyphosphate hydrolytic degradation;[18] some of them are listed because they are relevant to this review. The P−O−P linkage that is characteristic of polyphosphates is subject to hydrolytic degradation. The rate of degradation in inorganic aqueous media depends on the polyphosphate species, pH, temperature,[11] counterion type,[111−116] and concentration. Hydrogen ions catalyze polyphosphate hydrolytic degradation.[117,118]

Most hydrolytic degradation obeys first-order kinetics with respect to the phosphate. There are at least two routes for hydrolytic degradation. Hydrolytic degradation of the end-group phosphate of polyphosphate produces orthophosphates. Polyphosphates composed of four or more phosphorus atoms per molecule may degrade by reversion to a ring metaphosphate, typically a trimetaphosphate; technically this is not hydrolytic degradation because water is not a reactant. Longer-chain molecules may undergo hydrolytic degradation at a middle P−O−P linkage, resulting in the production of two smaller polyphosphate chains. Although thermodynamically favored, the kinetics of polyphosphate hydrolytic degradation are slow at neutral pH and ambient temperatures.[104] This property suggests that the phosphoanhydride bond is an excellent candidate for enzymatic control.

5.5. Analytical Methods

A detailed review of polyphosphate extraction techniques and measurement assays may be found in the book *The Biochemistry of Inorganic Polyphosphates* by Kulaev, Vagabov, and Kulakovskaya[9] and *Methods for Investigation of Inorganic Polyphosphates and Polyphosphate-Metabolizing Enzymes* by Lorenz and Schröder.[119] Van Wazer surveyed the history of differential analysis of phosphate mixtures.[11]

The following methodologies are employed on free polyphosphate ions or polyphosphate ion fractions that can be extracted from or identified within a biological sample. Pure polyphosphate solids may also be analyzed in the solid state or after dissolution by these methods.

5.5.1. Chromatography

Early separation technology involved paper chromatography.[120,121] However, paper chromatography is a difficult technique and separates polyphosphate species below a linear chain length of 10 only. The two-dimensional paper chromatography technique can separate linear polyphosphates from cyclophosphates.[122] Ion exchange resins can separate polyphosphates of different chain lengths.[9] Liquid chromatographic systems also separate and detect cyclic and linear polyphosphates[114,123] with polyphosphate chains <10 phosphate units long.

5.5.2. Hydrolytic Degradation Assay

An assay of orthophosphate released after acid hydrolytic degradation (1 M HCl at 90 °C for 10 min)[9] can be used to determine the presence and concentration of polyphosphates. Orthophosphate concentration can be measured by colorimetry.[124]

5.5.3. Titration

Linear polyphosphate chains in solution have one titratable strong acid hydrogen associated with each phosphorus atom. There is one titratable weak acid hydrogen associated with each of the terminal (end-group) phosphorus atoms in a linear chain. Titration of any dissolved linear polyphosphates between the end point near pH 4.5 and 9 is a method of quantifying the number of orthophosphate end groups and middle groups.[111,125]

5.5.4. Colorimetry

Complexes of toluidine blue and polyphosphate complexes in solution can be used to estimate the concentration of polyphosphate species with chain lengths longer than 10 phosphate units in simple solutions.[119] Shorter polyphosphate chains give weak or no metochromatic reaction.[9] A Mn^{2+}-induced quenching of the calcium fluorescent indicator Fura-2 in solutions with lower polyphosphate concentrations can also measure polyphosphate concentration.[126]

5.5.5. Solubility Fractionation

Van Wazer[125] used the method of solubility fractionation to separate polyphosphate species of different sizes. Water-soluble organic phases such as acetone or salts such as sodium chloride can be used to precipitate the longer polyphosphate species in a fractional extraction process.[11]

5.5.6. Gel Electrophoresis

Gel electrophoresis separation of polyphosphate species is commonly used.[127−129] After separation, polyphosphate species (greater than ~5 phosphate units) can be identified with toluidine blue staining. In situ hydrolytic degradation followed by staining for phosphate can detect all linear polyphosphate species, cyclic trimetaphosphate, and the original phosphate content after PAGE separation.[130] Polyphosphate species containing ^{32}P can also be detected by autoradiography.[8]

5.5.7. ^{31}P NMR

Nuclear magnetic resonance of phosphorus was first used to detect end and middle chain phosphorus atoms as anions in solution and melted glass by Van Wazer.[131] It can be used in aqueous and solid states to provide a very precise analysis of "visible" polyphosphate speciation, because it resolves end-chain phosphorus, penultimate end-chain phosphorus, and middle chain phosphorus atoms. However, not all polyphosphate within a sample is "visible" by ^{31}P NMR, because NMR detection requires high concentration and may not detect polyphosphate species that are aggregated or in metal complexes.[8,9] The interested reader is directed to reviews of ^{31}P NMR polyphosphate spectroscopy.[9,132]

5.5.8. Infrared

Infrared methods for polyphosphate analysis include FTIR[133,134] and Raman spectroscopy. Raman spectroscopy has been used to measure polyphosphate chain hydrolytic degradation rates in vitro.[117]

5.5.9. X-ray Diffraction and X-ray Energy-Dispersive Analysis

X-ray diffraction by powder diffraction or through transmission electron diffraction may produce unique diffraction patterns that can be attributed to solid phases that exhibit a certain range of periodicity in their structure. McIntosh and Jablonski[135] presented X-ray diffraction data for many calcium phosphate phases. X-ray diffraction analysis of a solid material that has no long-range order will result in a diffuse signal, which is attributed to an "amorphous" material.

Transmission or scanning electron microscopy allows for the detection of electron-dense regions within unstained specimen; when coupled with X-ray energy-dispersive analysis, the detection of high concentrations of phosphorus and counterion cations is possible. This analytical strategy

is not a specific detector of polyphosphates, but it has been used[9] to study polyphosphate granules and the cations with which they are associated within various organisms.[136]

Methods requiring extensive sample preparation such as TEM should be anhydrous, because hydrous sample preparation may hydrolytically degrade polyphosphates. As polyphosphates form complexes with osmium,[137] lead,[138] and iron,[136] staining with these elements with minimal water contact may also indicate the presence of polyphosphates with TEM, although these stains are not necessarily unique for polyphosphates.

5.5.10. Cytochemical Methods

Staining of cells and tissues is one method of identifying polyphosphate anions within biological structures. Dyes used to identify polyphosphates include toluidine blue, neutral red, and methylene blue.[9] For toluidine blue, the concentration of polyanionic charges affects the size of the metochromatic shift.[139,140] The fluorescent reporter DAPI has also been used to detect polyphosphates within biological samples[9,141−143] with fluorescence microscopy. DAPI is a fluorescent reporter that is commonly used to detect DNA but may be used to unambiguously identify polyphosphates, because there is sufficient spectral separation between the DAPI−DNA complex emission peak (465 nm) and the DAPI−polyphosphate complex peak (520 nm).

5.6. Sensitivity to Sample Preparation

Early work on the detection of early apatite biomineralization events noted the sensitivity of biological sample preparation.[103,144,145] Some detection and sample preparation methods require the use of aqueous solutions. Exposure of polyphosphates to aqueous solutions can result in spontaneous polyphosphate hydrolytic degradation and the production of orthophosphates. In particular, the electron-dense granules were only detected in skeletal tissue specimens prepared under anhydrous conditions.[54,102,103,146] It is possible that the identification of apatite in some specimens may be an artifact due to inadvertent polyphosphate hydrolytic degradation. Ashing is another sample preparation technique used for bone sample preparation; the high-temperature processing may also affect amorphous polyphosphates, causing them to crystallize (section 8.1.2). Heat treatment in the presence of acid would also be expected to increase the polyphosphate hydrolytic degradation rate, as noted in section 4.1.

5.7. Solubility

Since polyphosphates are unstable with respect to hydrolytic degradation, a solubility product for polyphosphates is impossible to determine. It is not possible to characterize a polyphosphate-containing, solid−aqueous system at equilibrium because the polyphosphates will react with the water.[18] Rates of solution for solids such as calcium polyphosphate or calcium sodium polyphosphate have been measured, but these measurements are difficult to reproduce due to the number of factors that can affect the experiment. Griffith listed some of the variables that can change the outcome; they include temperature, particle size, enzymes,[147] amorphous content, dislocations in the crystal structure, and sample age.

5.8. Adsorption

There is a larger literature on the chemistry of pyrophosphates and their adsorption on apatite mineral; this review will focus solely on longer chain polyphosphates. Polyphosphates are known to adsorb to the minerals zincite[148] and titania.[149] Long-chain polyphosphates were also observed to adsorb to synthetic hydroxyapatite.[150]

5.9. Formation of Soluble Complexes with Metal Ions (Sequestration)

Both ortho- and polyphosphates form soluble complexes with metal ions. The orthophosphate complexes are very weak for the alkali and alkaline earth metals and only become important for transition metals such as iron. Polyphosphates form relatively strong complexes with alkali and alkaline earth metals as well as with transition metals.

Polyphosphates chelate metal cations,[136] forming complexes with metal ions such as Fe^{3+}, Fe^{2+}, UO_2^{2+}, and Ca^{2+}, the strength of which is related to the pH change of the polyphosphoric acid solution (Figure 5).

Sodium polyphosphate is used to inhibit scale (calcium sulfate, calcium carbonate, and magnesium carbonate formation).[151] The earliest developments in this technology were made by Hall in the 1930s.[152] The method of action is thought to be reduction of the calcium activity by the formation of a calcium−polyphosphate complex.

Uranium stabilization through polyphosphate injection is under investigation by the Pacific Northwest National Laboratory.[153] Vazquez et al. measured the interactions of uranium with polyphosphate and concluded that uranium−polyphosphate complexes are formed between pH 3 and 10. If the pH is less than 6, an insoluble uranium−polyphosphate complex forms. Above pH 6, a soluble uranium−polyphosphate complex predominated.[154] The sparingly soluble minerals apatite and autunite ($Ca[(UO_2)(PO_4)_2 \cdot 11H_2O)$[155] are proposed as minerals for uranium sequestration.[153] The in situ immobilization strategy involves the application of tripolyphoshate to contaminated groundwater; following hydrolytic degradation, precipitation of uranium phosphate solids,[156] autunite, and apatite results.[153] The hydrolytic degradation of polyphosphates in the presence of calcium has also been linked to the formation of apatite mineral in other fields of study.

6. Calcium, Polyphosphates, and Inorganic Apatite Mineralization

This section will provide a brief review of inorganic calcium polyphosphate solids, because their composition, approaching $[Ca(PO_3)_2]_n$ for long chain polyphosphates, is close to that of apatite ($Ca_{10}(OH)_2(PO_4)_6$) and they have been linked to apatite formation. More information on the chemistry of calcium polyphosphate solids are found in books by Griffith[18] and Ropp.[157]

6.1. Calcium Polyphosphate Transforms to Apatite in Aqueous Solutions

If precipitated from solution, the first calcium polyphosphate solid phase formed is an amorphous polyphosphate gel. Upon further drying, the gel solidifies as a glass with a high water content.[158] With further heating, water is lost, and depending on the heating regime, the calcium polyphos-

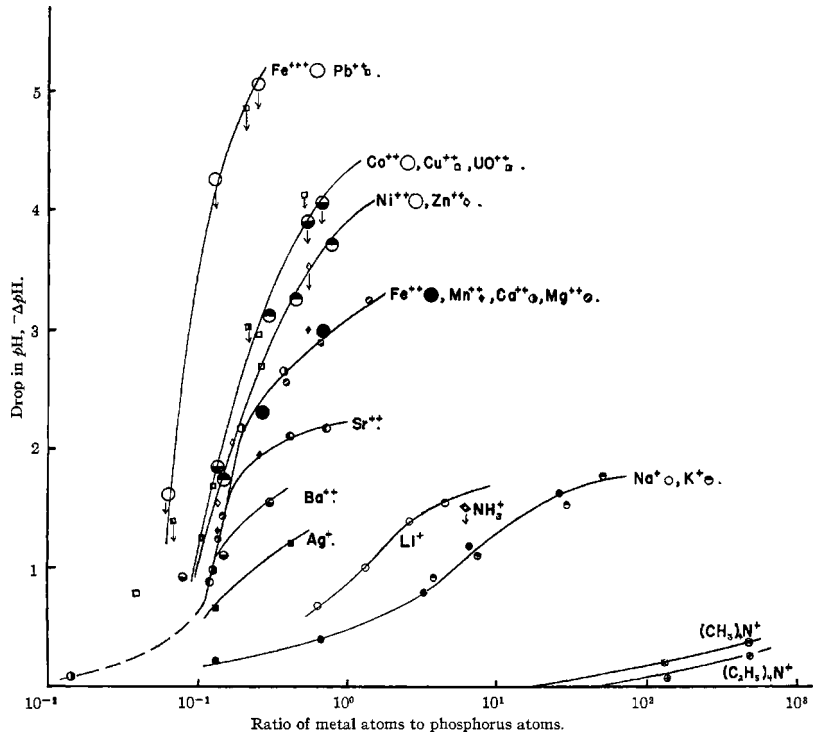

Figure 5. The effect of various salts on the pH of a polyphosphoric acid (from a sodium phosphate glass for which $n = 5.0$) with the weak acid half-neutralized and at a concentration of 15×10^{-4} atomic weights of phosphorus per liter. Reprinted with permission from ref 136. Copyright 1950 American Chemical Society.

phate may crystallize or may remain amorphous until it melts.[157,158] Polyphosphate glasses can also be fabricated directly from phosphate melts.[159,160] There are many crystal structures of calcium polyphosphate; McIntosh and Jablonski[135] listed 10 pyro- and polyphosphate phases. Seeded, controlled crystallization from the melt can result in the formation of crystalline calcium polyphosphate fibers.[161]

Calcium polyphosphate glass dissolved in basic pH solutions was observed to release hydrated calcium and polyphosphate ions into solution.[162] The total dissolved calcium and orthophosphate concentrations were reported to be over 3 orders of magnitude greater than those required to saturate the solution with respect to hydroxyapatite. The absence of hydroxyapatite was explained by the presence of phosphate in solution as polyphosphate.[162] The dissolved polyphosphate ions underwent hydrolytic degradation in solution, increasing the concentration of orthophosphate ions. Through dissolution and reprecipitation, the calcium and polyphosphate-containing glass eventually transformed into hydroxyapatite,[162] because it is the least soluble calcium orthophosphate phase in basic solutions.[76] Before the production of apatite, the total phosphate and total calcium values may have exceeded apatite solubility, but the actual orthophosphate and calcium activities may have predicted an apatite undersaturated system.

6.2. Polyphosphates Reduce Hydroxyapatite Saturation

The saturation of hydroxyapatite is defined by its solubility product and the activity of its component ions. For hydroxyapatite, the saturation is defined by the activities of calcium, orthophosphate, and hydroxyl groups and the solubility product at a given temperature,[30]

$$\sigma_{HAP} = \frac{((\alpha_{Ca^{2+}})^{10}(\alpha_{PO_4^{3-}})^{6}(\alpha_{OH^-})^{2} - K_{sp})}{K_{sp}} = S - 1 \quad (1)$$

Polyphosphate ion formation and hydrolytic degradation can affect the saturation of hydroxyapatite by affecting the activity of its component ions. Production or hydrolytic degradation of polyphosphates within a closed system changes the speciation and therefore the activity of orthophosphate. Polyphosphates chelate calcium ions and would consequently reduce the activity of calcium in solution. If the orthophosphate concentration is measured with an assay that hydrolyzes polyphosphates, then the measured orthophosphate concentration will be higher than the actual initial orthophosphate concentration due to the production of orthophosphates by polyphosphate hydrolytic degradation. Calcium ions that are strongly sequestered to polyphosphate ions in solution may not be detected by calcium assays that involve complexation with a detection agent such as *ortho*-cresolphthalein. The effect of polyphosphate on some measurement techniques for orthophosphate or calcium concentrations may skew calculations of the apatite saturation value. Polyphosphates may also affect the formation of apatite through the inhibition of crystal nucleation or growth.

6.3. Polyphosphates Inhibit in Vitro Apatite Precipitation

Francis[163] noted the reduced activity of polyphosphates to inhibit the crystal growth of apatite was caused by their "hydrolytic instability". An interesting result from his double displacement apatite crystallization experiments (mixing solutions of sodium phosphate and calcium chloride at constant pH (7.4 or 12) under nitrogen) was the formation of an amorphous solid if sodium tripolyphosphate was added

to the orthophosphate solution before addition of the calcium chloride, suggesting that polyphosphates also interfere with the nucleation of apatite from solution.

The ability of polyphosphate (and pyrophosphate, $P_2O_7^{4-}$) to inhibit in vitro formation of the mineral found in bone (apatite) was revealed by Fleisch and Neuman.[164] These two fathers of bisphosphonate chemistry understood that the serum within the vertebrate skeleton is supersaturated with respect to apatite and that the formation of bone mineral was a temporally and spatially controlled phenomenon. Armed with their in vitro results, they postulated that bone mineral formed only when two conditions were met: the presence of a suitable nucleation site (hypothesized to be "nucleating collagen") and the destruction of an inhibitor ("perhaps a polyphosphate"). Fleish later reported that alkaline phosphatase, an enzyme known to hydrolyze organic phosphates, inactivated an inhibitor of calcium phosphate nucleation in plasma ultrafiltrate. Although not identified in urine or plasma, Fleisch suggested the inhibitor was a polyphosphate or pyrophosphate in 1962.[165]

6.4. Polyphosphates Inhibit In Vivo Apatite Precipitation

Refocusing their efforts on an in vivo system, Fleisch's group later induced aortic calcification in a rat with excessive vitamin D and prevented the calcification with subcutaneous (under the skin) injection of pyrophosphate or long-chain polyphosphate.[166,167] When incubated with chick embryo femurs, the higher concentrations of polyphosphates and pyrophosphates tested (4 and 16 μg P/mL) inhibited bone mineralization, while the lowest concentration (1 μg P/mL) increased calcium phosphate deposition, suggesting a dose response.[168] They concluded that inhibition of mineralization was by blocking apatite crystal growth; however, the mechanism of increased apatite formation at lower polyphosphate and pyrophosphate concentrations was not known. Injected polyphosphates were also shown to inhibit skin calcification in the calciphylaxis model in rats.[169] Further work in the vitamin D-induced rat calcification model showed that although aortic calcifications were inhibited by polyphosphates and pyrophosphates, calcifications in the kidney were not inhibited.[170]

This lack of calcification inhibition observed within the kidney was attributed to a high concentration of alkaline phosphatase within the kidney, which is known to act as a pyrophosphatase and therefore is expected to hydrolyze the polyphosphate inhibitors. It was concluded that the calcium-chelation ability of polyphosphates was not responsible for the reduction in the induced aortic calcification model. It was proposed that the condensed phosphates might have a direct action on cells, inhibiting calcification. However, it was also concluded that the most probable explanation for the observed effect was the inhibition of calcium phosphate crystal growth, as observed in vitro. The stability of the polyphosphate molecule was noted to affect its ability to inhibit apatite mineralization.

The theory that the destruction of an apatite mineralization inhibitor was required for biomineralization to occur was moved forward in 2005, when it was shown that tissue nonspecific alkaline phosphatase (TNAP) cleaves pyrophosphate, an inhibitor of mineralization.[171] It was proposed that mineralization of bone and pathological mineralization were linked to the coexpression of collagen and TNAP.

The combined properties of hydrolytic instability, metal ion sequestration, susceptibility to hydrolytic degradation by enzymes, and the reluctance to form its own crystalline solid from solution could make polyphosphates an interesting precursor for biomineralized apatite. This field of polyphosphate biochemistry is not mainstream;[7] the next section presents a brief review of topics relevant to this review.

7. Polyphosphate Biochemistry

7.1. History

Inorganic polyphosphates were first found in living organisms more than 100 years ago.[172] Pioneers of the biochemistry of high molecular weight polyphosphates include Ebel,[173] Wiame,[174] Belozersky,[175] Drews,[176] Lohmann,[177] Kornberg,[178] Kulaev,[179] and Harold.[180] Besides being attributed to the origin of life[181,182] or not,[183] the wide functionality of inorganic polyphosphates in life forms ranging from prokaryotes to higher eukaryotes has been related to various stages of cell evolution.[184] The interested reader is also directed to extensive reviews on the subject.[185−193]

Inorganic polyphosphates receive a mere two paragraphs in *Lehninger Principles of Biochemistry*[194] (2000), where they are introduced as a linear polymers of orthophosphate residues linked through phosphoanhydride bonds that are potential phosphoryl group donors. Their biological role "remains uncertain". Kulaev et al. have outlined the current understanding of the biochemistry of polyphosphates;[9] the following sections summarize the biochemical roles of polyphosphate relevant to this review from this text.

7.2. Polyphosphate Kinases

The polyphosphate kinase family of enzymes can condense orthophosphates, forming polyphosphates. Polyphosphate kinase (polyphosphate:ADP phosphotransferase, EC 2.7.4.1) can transfer phosphate residues from ATP to polyphosphates, as discovered by Kornberg et al.[178,195,196] 3-Phospho-D-glyceroyl-phosphate:polyphosphate phosphotransferase (EC 2.7.4.17), also known as 1,3-diphosphoglycerate−polyphosphate phosphotransferase[179] catalyzes the transfer of an orthophosphate residue from 3-phospho-D-glyceroyl-1-phosphate to polyphosphate. Dolichyl-diphosphate:polyphosphotransferase (EC 2.7.4.20) found in the membrane of yeast cells[197,198] catalyzes the exchange of an orthophosphate residue from dolichyl diphosphate to polyphosphate.

7.3. Polyphosphatases

In the late 1950s, Karl-Kroupa measured the stability of polyphosphate ions in aqueous solutions containing various common living organisms, having noted that enzymatic hydrolytic degradation of polyphosphates can be extremely rapid.[147] Since then, many polyphosphatase enzymes have been identified that degrade polyphosphates by either transferring an orthophosphate residue to another molecule, cleaving a free orthophosphate residue from the end of the chain (exopolyphosphatase), or hydrolyzing the polyphosphate chain within the middle of the chain, producing two shorter polyphosphate chains (endopolyphosphatase).

Polyphosphate−gluclose phosphotransferase (EC 2.7.1.63) catalyzes the phosphorylation of glucose using ATP or polyphosphate as the orthophosphate donor. Exopolyphosphatase (polyphosphate phosphohydrolase, EC 3.6.1.11)

hydrolyzes an orthophosphate from the end of a polyphosphate ion. The first report of this enzyme was by Kitasato[199] and others through the 1940s to 1960s.[9] Adenosine-tetraphosphate phosphohydrolase (EC 3.6.1.14) is considered to be a property of exopolyphosphatase PPX1, hydrolyzing adenosine-5′-tetraphosphate and guanosine-5′-tetraphosphate.[200] Triphosphatase (also known as tripolyphosphatase, EC 3.6.1.25) can catalyze the hydrolytic degradation of a polyphosphate ion composed of three orthophosphate residues into one free orthophosphate residue and a pyrophosphate ($P_2O_7^{4-}$) ion.[201] Endopolyphosphatase (such as polyphosphate depolymerase, EC 3.6.1.10) cleaves polyphosphate chains at a P−O−P bond in the middle of the polyphosphate chain, producing two smaller (4−5 orthophosphate residues long) polyphosphate molecules. This reaction was first recorded by Malmgen[202] and Mattenheimer.[203] The ability of PolyP:AMP phosphotransferase to transfer an orthophosphate residue from a polyphosphate molecule to AMP, producing ADP and a shorter polyphosphate molecule, was first found by Dirheimer and Ebel.[204]

There are enzymes that are designed to build and selectively hydrolytically degrade polyphosphate molecules, producing different reaction products, including an orthophosphate residue. The enzymatic flexibility of processing polyphosphate ions leads to a number of proposed biological roles for polyphosphate ions.

7.4. Proposed Biological Roles of Polyphosphates

The proposed biological roles of polyphosphates are the subject of many papers and review articles. The thorough text by Kulaev[9] includes the following proposed biological functions of polyphosphate ions, most of which have been identified in microorganisms.

7.4.1. Phosphate Storage

Polyphosphates were identified as a constituent of microbial volutin granules by the 1950s.[205] Volutin granules, also known as metachromatic granules, are observed by light microscopy in many microorganisms when stained with toluidine blue. The production of polyphosphates as an orthophosphate store that can be drawn upon intermittently between periods of orthophosphate starvation and excess orthophosphate availability is a strategy that has been proposed since the 1960s.[180,193,206−208] Harold[180] suggested that polymers of phosphate were very efficient species for the storage of orthophosphate because high concentrations of polyphosphate have little effect on osmotic pressure. Such stores of polyphosphate were identified within the vacuoles of *Saccharomyces cerevisiae* in the late 1970s.[209] Phosphate is viewed as a limiting nutrient in the marine environment.[210−213] The collection and storage of orthophosphate as polyphosphate may also be a strategy for limnetic and marine organisms, because polyphosphate has been proposed to be part of the biogeochemical cycling of phosphorus in marine ecosystems[214] and has been detected in the uppermost sediment layer of limnetic and marine sediments.[5,132]

Alkaline phosphatase, an enzyme specific for the monophosphate ester bond, is found in the water column and is a major enzyme class of marine bacteria. Plants and soil microbes also release phosphatase to produce bioavailable phosphate.[16] Phosphatase activity was hypothesized to be related to phosphorus deficiency within bacteria; its activity has been proven to be inversely related to phosphate concentration.[214] Microbial communities studied in the Canadian arctic also contained polyphosphate granules[215] that were suggested to be stores of phosphorus, an essential nutrient for microbial growth.[216]

Bacteria that accumulate large stores of phosphate under oxic conditions are under active investigation as biological agents to remove phosphorus from wastewaters (see Seviour[217] for a review of this field). One of these phosphate-accumulating bacteria, *Acinetobacter johnsonii* 210A, was observed to uptake and then reduce the orthophosphate levels to micromolar concentrations by generating polyphosphate.[218,219] Mitochondria have detectable polyphosphate stores[101,220] that may act as orthophosphate reservoirs to meet the orthophosphate demand.

Energy-dispersive X-ray analysis has been used to identify polyphosphates within biological "electron-dense bodies".[180,205] More recently, identification of polyphosphates within polyphosphate-accumulating bacteria has also been accomplished *in situ* by dual staining with rRNA-targeted oligonucleotide probes and DAPI.[221] Energy-dispersive X-ray analysis has also been used to identify polyphosphate granules in polyphosphate-accumulating *Acinetobacter* strain 210A and show that the cations present in the granules can be Ca^{2+}, Mg^{2+}, or both, depending on the intracellular Mg/Ca ratio.[222] The colocation of cations such as Ca^{2+} and Na^{+} within polyphosphate granules has been identified with both energy-dispersive X-ray analysis and staining with orthophosphate in polyphosphate-accumulating microbial populations used for phosphorus removal in wastewater.[223]

7.4.2. Chelation of Divalent Cations

Although any cation is a candidate for balancing the anionic charges of polyphosphates, sequestration of divalent ions such as Ca^{2+}, Mg^{2+}, and other metal ions, including complexes such as the uranyl ion $(UO_2)^{2+}$ with polyphosphates has received particular research attention.

Some microorganisms display passive tolerance to heavy metals; it was suggested that this tolerance may arise from intracellular sequestration with polyphosphate.[209,224] Polyphosphate-assisted tolerance to lead,[225] aluminum,[226] cadmium,[227−229] copper,[230] manganese,[231] barium,[232] calcium,[233] and uranium[234,235] have been reported. Although intracellular sequestration of these metals within polyphosphate-containing granules would reduce intracellular free metal concentration, it has also been proposed that metal phosphate complexes are produced by the hydrolytic degradation of polyphosphates and transported out of the cell by the inorganic phosphate transport system,[236] as evidenced with uranium.[237]

Storage of high concentrations of orthophosphate as polyphosphate reduces the concentration of free orthophosphate in solution; in a system that includes calcium ions, which are sequestered by polyphosphates, the saturation level of apatite mineral is reduced. This allows for the containment of large total concentrations of calcium and phosphate at neutral pH without the precipitation of apatite. Specific Ca^{2+} and polyphosphate-containing storage organelles named acidocalcisomes[189] have been identified in the cells of protozoa[238] and algae,[239] as well as within platelets,[240,241] which contain high concentrations of calcium and phosphate but do not form apatite mineral.

Siderus et al. studied calcium uptake and release by electron-dense, calcium-containing polyphosphate granules

in an alga (*Chlamydomonas eugametos*) identified with both DAPI and X-ray microelement analysis. They concluded that "polyphosphate stores represent a source of calcium that can be mobilized and not just a calcium trap".[242] They noted that Ca^{2+} release from the polyphosphate granules was coupled to an influx of K^+. They suggested that polyphosphate bodies may preferentially accumulate some cations but could also accumulate others. Polyphosphate extraction from *Propionibacterium shermanii*[243] required two different extraction processes to isolate all of the polyphosphate. Clark et al. concluded that one fraction was soluble and the other fraction was tightly complexed within granules, suggesting that polyphosphates were not limited to existing within storage granules and may have other biological roles.

7.4.3. Energy Source

The phosphoanhydride bonds of polyphosphate are related to the phosphoanhydride bonds of ATP, possessing an intermediate free energy on the scale of phosphorylated compounds.[9] Polyphosphates can act as both a donor and an acceptor of orthophosphate; Belozersky was the first to suggest that primitive organisms could employ polyphosphates as an evolutionary precursor of ATP (cited within ref 9). Polyphosphate molecules have since been shown to be involved in energy production via different pathways.

Phosphate-accumulating bacteria used in wastewater treatment are capable of accumulating "an excessive amount of phosphate as polyphosphate",[218] also known as "luxury uptake". These bacteria consume polyphosphate as an energy source in anaerobic conditions, releasing orthophosphate as a byproduct of anoxic energy production (reviewed by Seviour[217]). *Acinotobacter johnsonii* produces a neutral $MeHPO_4$ complex as a biproduct of energy production. This complex can be excreted with H^+, generating a proton motive force.[219] Phosphate transport in prokaryotes, including proposed roles of polyphosphates, have been reviewed by van Veen.[244] The metabolism of polyphosphates by these polyphosphate-accumulating bacteria in activated sludge was theorized to have ecological significance in the phosphorus dynamics of freshwater ecosystems as well.[245]

Benthic microorganisms have also been suggested to metabolize phosphate in alternating redox environments that exist close to the water/sediment interface. In oxic conditions, phosphate is accumulated as polyphosphate. When exposed to an anoxic environment, polyphosphates undergo hydrolytic degradation as an "auxiliary metabolism", releasing orthophosphate.[4,246] The precipitation of calcium phosphate minerals by microorganisms was previously suggested to be related to their ability to change the chemical composition of the pore water within sediments (reviewed by Krajewski et al.[247]). Recently, a direct link was made between the biological hydrolytic degradation of polyphosphates and phosphate release by the sulfur microorganisms *Thiomargarita namibiensis* and the production of large stores of apatite (phosphorite) mineral within marine sediments,[4] suggesting that this large-scale apatite precipitation was the result of biomineralization processes.

8. Calcium, Electron-Dense Granules, Mitochondria, Polyphosphates, and Apatite Biomineralization

This last section reviews some of the literature on the biomineralization of apatite, the links between calcium- and phosphate-containing electron-dense granules and apatite biomineralization, mitochondria, and biomineralization, and the more recent link between polyphosphates and apatite biomineralization.

8.1. Biomineralization of Apatite

Referring to the terminology of Lowenstam, biological apatite formed by a living organism could be biologically induced, whereby bulk extracellular or intercellular minerals precipitate, or biologically controlled. Mineral formation could be also be orchestrated by an "organic matrix-mediated" process.[34] There is evidence that bulk extracellular apatite biomineralization occurs due to an increasing concentration of orthophosphate generated by biologically controlled polyphosphate hydrolytic degradation. Examples of extracellular and intracellular and possibly biologically controlled processes of biological apatite formation that involve polyphosphates will be reviewed, including the evolution of a theory proposed almost four decades ago of apatite production within the vertebrate skeleton.

8.1.1. Bacterial Induction of Apatite Mineralization

Large phosphorite (apatite) deposits in the sea have been attributed to the hydrolytic degradation of polyphosphate (originially identified as electron-dense bodies and by staining with toluidine blue) by bacteria as an energy source during alternating anoxic−oxic conditions.[4] By use of soft X-ray fluorescence spectromicrosopy, 1-μm-sized, concentrated polyphosphate bodies, as well as apatite mineral, were confirmed major phosphate phases within a sample of bacteria and surface marine sediment from another site located close to anoxic and oxic basins,[248] although no conclusions were drawn between the presence of the polyphosphate bodies and the apatite mineral in the study. However, the combination of high byproduct phosphate concentrations within the pores of the sediment with the high calcium concentration and slightly alkaline pH of seawater would suggest the possibility of localized apatite precipitation.

Polyphosphate stores within dead or lysed microorganisms may also be a source of phosphate for apatite formation. Konhauser[215] postulated that the decomposition of polyphosphate-containing bacteria by heterotrophs may result in the assimilation of part of the phosphate by other microorganisms, while another part may be released as dissolved orthophosphate. Polyphosphate hydrolytic degradation and apatite formation may take place spontaneously, because they are thermodynamically favored in aqueous, neutral to basic pH environments within a dead organism. Apatite crystals were observed subsequent to cell death within *E. coli*;[41] *E. coli* were later discovered to produce polyphosphates to support resistance and survival during its stationary phase.[249]

In the reverse process, geomicrobiologists have postulated that bacterial acidification and therefore dissolution of environmental apatite mineral[216] may serve as a source of orthophosphate, which can be scavenged and stored within the microorganisms as polyphosphate.[215]

8.1.2. Bacterial Control of Apatite Mineralization?

In 1967, Ennever and Creamer wrote an editorial focusing on bacteria that were associated with the mineralization of apatite.[250] Calcifying bacteria of interest to the editors included those that generate dental calculi. In particular, they summarized three studies of *Bacteronema matruchotii* that identified intracellular calcium hydroxyapatite. Because the mineralization is internal to the living organism and occurs within a regular time period, it was assumed that this apatite mineralization is biologically controlled. Granules of polyphosphate were later identified within *B. matruchotii*; the ability of polyphosphates to inhibit apatite mineralization was discussed by the authors in the editorial. The editorial concluded with an expression of interest in using the bacterial formation of apatite as a potential tool for understanding vertebrate bone mineralization.

Takazoe et al.[251] revisited their earlier detection of polyphosphate granules within *B. matruchotii* and studied its calcification within unfixed, frozen specimens using electron microscopy and elemental analysis. They concluded that there were two areas of mineral deposition: calcium-, phosphorus-, and magnesium-containing metachromatic granules and the cell membrane. Because no other mineral phases were detected, they postulated that amorphous calcium phosphate transformed directly to apatite. This work refuted an earlier proposition[252] that *B. matruchotii* mineralization involved an orderly sequence of mineral phases, terminating with apatite formation. As noted by Takazoe et al., the samples that lead to the orderly sequence theory were analyzed after high-temperature ashing (heating in a furnace), which may have altered the solid state of the specimen.

The refuted work concluded that an "early mineral phase" (EMP) precedes apatite formation within *B. matruchotii.* This material was identified by X-ray diffraction after ashing, although it appeared amorphous by in situ electron microscopy. The published EMP X-ray diffraction data was compared with published X-ray diffraction patterns of different calcium phosphate phases,[135] as well as those in the International Centre for Diffraction Data (ICDD) powder diffraction file by the authors of this review. It is possible that the EMP is a calcium polyphosphate phase (γ-calcium metaphosphate, 09-0345 $Ca_2P_2O_7$ or 33-0297 $Ca_2P_2O_7$) that could have crystallized from an amorphous state with the additional heat provided by the ashing process. Although the biological process that controlled the staged, apatite mineralization was not identified, it was later proposed to be related to the presence of unidentified proteins.[253]

The presence of amorphous, electron-opaque material and apatite was also observed in the eukaryote *Candida albicans*.[254] Although the composition of the electron-opaque material was not identified, polyphosphate was later identified in *C. albicans* and proposed to act as a magnesium store.[255]

8.2. Mitochondria, Calcium, Polyphosphates, and Apatite

Lowenstam wrote that the "most commonly identified sites (of intracellular mineral deposits) are the mitochondria, the Golgi complex, or vesicles, which are intimately associated with the latter."[34] This section will summarize some of the mitochondrial literature related to calcium, polyphosphate, or calcification.

Mitochondria were proposed to act as "buffer" structures for cellular Ca^{2+}, accumulating or releasing it when required. Maximal uptake of Ca^{2+} by rat diaphragm mitochondria was understood to require the presence of Mg^{2+} and orthophosphate in the media;[256] however, no analysis was made for polyphosphates in this study. Electron-dense granules in mitochondria were determined to contain calcium and phosphate at concentrations exceeding the solubility product of calcium phosphate salts at neutral pH.[257] However, these dense, calcium- and phosphate-containing granules were surprisingly noncrystalline.[258]

Polyphosphates were identified as an alternate "but very active" pathway for labile phosphate metabolism in rat liver mitochondria in the 1960s.[259] Polyphosphates were more recently identified within the mitochondria of the yeast *Saccharomyces cerevisiae*.[101,220] Two exopolyphosphatases have been indentified within the mitochondria of the yeast *Saccharomyces cerevisiae*.[260] It is possible that mitochondria control both the production and the hydrolytic degradation of polyphosphates.

Recently proposed physiological roles of polyphosphate in mammalian mitochondria include playing a part in energy metabolism and ion transport, as well as a possible effect on mitochondrial Ca^{2+}-buffering capacity.[261] Greenawalt noted that the stores of electron-dense, calcium- and phosphate-containing granules in rat liver mitochondria discharged their contents when exposed to 2,4-dinitrophenol, suggesting that interfering with oxidative phosphorylation was somehow related to the ability for mitochondria to maintain intramitochondrial calcium phosphate reserves.[258] The authors wondered whether the dense granules observed in the rat liver mitochondria were equivalent to the electron-opaque mitochondrial granules seen in "mitochondrial profiles in thin sections of osteoclasts in healing fractures of bone, as described by Gonzales and Karnovsky[262]".

Mitochondria are known to accumulate mineral if they are incubated in Ca^{2+}-containing solutions, as well as in vivo in a range of physiological and pathological conditions (references in Bonucci[40]). Bonucci used electron microscopy to study calcifying mitochondria in damaged rat liver, heart and muscle because calcium is contained in "electron-dense aggregates".[40] Two types of electron-dense bodies were identified in the mitochondria: crystals similar to that of bones and teeth (most common in the muscular and myocardial cells) and amorphous "granular aggregates" (most common in hepatic cells) both of which were removed after formic acid treatment. The granular aggregates exhibited the same ultrastructure as inorganic inclusions found within in vitro calcium phosphate-loaded mitochondria and were occasionally seen in the same mitochondrion or cells as crystalline aggregates. Bonucci mused on the role of the granular materials, referring to previous work suggesting that inorganic granules found in normal and challenged mitochondria were a colloidal, subcrystalline precursor of calcium-deficient hydroxyapatite[263] and wondering whether it might be a similar precursor for the calcification of bone. He found it difficult to explain why such a local supersaturation of calcium and phosphate does not invariably induce the formation of crystal structures. It was noted that intramitochondrial inorganic granules stained with toluidine blue[264] were found in both normal and challenged mitochondria. Although polyphosphates were not mentioned in this work, the reported characteristics of the granules suggest that polyphosphates are components of the amorphous, electron-dense, calcium- and phosphate-containing granules that are unstable when exposed to acid.

In 1969, electron microscopic analysis of the formation of apatite mineral within mitochondria led to the postulation that mitochondria might be involved in biological mineralization.[265,266] Shapiro and Greenspan[266] postulated that mineralization is under strict cellular control and that the mitochondria concentrate calcium and phosphate and then these ions are transported to the extracellular matrix where mineralization occurs. A report of mitochondrial electron-dense granules detected in the calcifying cartilage of rats and mice was published that same year.[267]

Although not generally accepted, Halstead built the argument that marine organisms may have, in seasonal abundance of phosphate, reduced their intracellular calcium concentrations by extracellular deposition of apatite. In conditions of reduced phosphate availability, extracellular apatite could serve as a phosphate store. It was theorized that the first mineralized tissue in the first marine vertebrates may have been an apatite-containing calcified cartilage, which eventually evolved into an endoskeleton that was gradually replaced by bone. Once on land, vertebrates bore an apatitic skeleton that served as a useful calcium store.

8.3. Cartilage Calcification and Polyphosphates

The vertebrate skeleton is composed largely of mineralized bone tissue. Bone is formed by one of two processes: intramembranous ossification (skull and other flat bones) or endochondral ossification (long and weight-bearing bones). In endochondral ossification, a cartilage model of the bone is made then it is replaced by bone. Before being replaced, the cartilage calcifies. In this section, a brief review of cartilage calcification in the growth plate will be followed by the presentation of some of the literature on detection and analysis of electron-dense granules in calcifying cartilage. Previous work that detected the transient staining of an unidentified compound in specific regions of the growth plate of polyphosphates will be followed by recent work identifying polyphosphates in these regions.

8.3.1. Calcification in the Growth Plate

One of the processes by which bones grow in size is called endochondral ossification. This process occurs in the expanding growth plate, which is located at the ends of growing long bones. The growth plate lies below the soft cartilage that caps the ends of the growing bones. The growth plate includes the zone of calcifying cartilage located below the cartilage, and ends where mineralized bone begins. Four zones are identified in the growth plate: from the end of the cartilage (resting zone), progressing to the proliferative zone (soft cartilge), through the hypertrophic zone, where calcification begins to occur, and finally ending at the resorption zone, where growing bone replaces the calcified cartilage. Long bone growth along the long axis of the bone is accomplished through the progressive production of new cartilage, calcification of older cartilage, and replacement of the calcified cartilage with bone.[268] The active growth plate is an excellent area to study the calcification of cartilage, because it is a progressive process with many time points caught within one longitudinal section cut from the growth plate.

8.3.2. Detection and Analysis of Electron-Dense Granules

Electron microscopic analysis of the rat and mouse growth plate by Martin et al. showed a gradient of mitochondrial electron-dense granules along the different cartilage zones.[267] Chondrocytes in the proliferative zone had few mitochondrial granules; the number and size increased toward the zone of calcification. In this zone, there was a decrease in the number and size of mitochondrial granules. Granules were located over the endoplasmic reticulum and most of the mitochondria, which were associated with the endoplasmic reticulum in the zone of provisional calcification. Granules that stained with osmic acid but not calcium were less electron-dense than those stained with both osmic acid and calcium, suggesting that these granules may retain the ability to take up calcium or may stabilize the granules. These amorphous granules were suggested to be candidates for the nucleating sites of mineralization, because the mitochondria appear to discharge their granules into the calcifying matrix.[267]

Outside of the growth plate, Kim noted that the osmiophilic spherules observed in calcifying cartilage were similar to electron-dense, osmiophilic granules that stained with toluidine blue and were colocated with apatite calcification in human heart valves.[269] More electron-dense bodies were observed with age in these otherwise normal cadaver valves. This suggests that cells located in or in proximity to heart valve tissue may be producing biomineralized apatite.

Electron-dense "spherules" ranging in size from 200−800 Å were also identified within mitochondria of the chondrocytes (cartilage cells) in the proliferative and hypertrophic zones of fresh, stained tibial epiphyseal plates (Figure 6) and occipital bones of mice and rats as well as the extracellular regions of the proliferative and hypertrophic zone.[54] The granules were amorphous and contained Ca and P (Figure 7).

A quantitative analysis of the Ca/P molar ratio of the observed amorphous granules and known calcium- and phosphate-containing solids proved that granules found in the proliferating and hypertrophic cartilage cells exhibited Ca/P molar ratios ranging from 0.80 ± 0.05 to 1.07 ± 0.24. These values lie between those of calcium polyphosphate (0.5) and brushite ($CaHPO_4$, 1.62).[54] The Ca/P ratio of the electron-dense solids increased with proximity to the calcified cartilage (up to 1.51 ± 0.09) toward the value for hydroxyapatite (1.62). These ratios suggest that polyphosphates are produced in the noncalcified cartilage and are eventually hydrolytically degraded into orthophosphate, which precipitates as apatite in the calcium-rich calcifying cartilage matrix.

8.3.3. Role of Phosphatases

It was proposed that the rate-limiting step for cartilage calcification was the transport of P_i from the cartilage cells to the calcium-rich calcifying cartilage matrix.[270] In 1923, Robison[88] suggested that a phosphatase enzyme located within calcifying cartilage yielded free phosphate ions, implying that phosphatase is "omnipresent and essential in calcifying areas".[75] Alkaline phosphatase is expressed by hypertrophic chondrocytes[271] and is presumed to be a factor in skeletal mineralization,[272] possibly by controlling the concentration of orthophosphate available for apatite formation.[273]

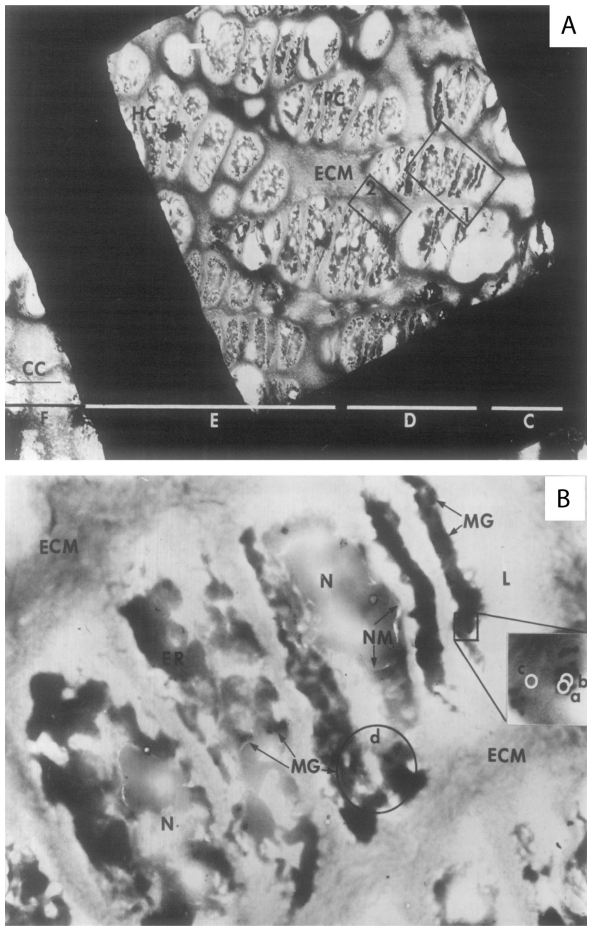

Figure 6. Ultrastructure, electron diffraction, and X-ray microanalysis of unstained sections of tibial epiphyseal growth plate cartilage from normal 4-week-old rats, prepared by dry ultramicrotomy at −80 °C. Unembedded sections were mounted on carbon-reinforced, Parlodion-coated copper grids. (A) Survey of a portion of a thin cryosection of the growth plate (×800 magnification). The full section actually extends over many other adjacent grid squares so that in some cases much of the epiphyseal plate may be examined on a single grid. The particular region shown includes proliferating chondrocytes (PC) organized in columns of cells and larger hypertrophic chondrocytes (HC) near the calcifying cartilage (CC). Divisions (C−F) of the cartilage corresponding to the zones defined in panel A are marked for reference purposes. Boxes (1, 2) denote areas illustrated elsewhere at higher magnifications. ECM denotes extracellular matrix. C = middle proliferative zone; D = lower proliferative zone; E = upper hypertrophic zone. (B) Enlargement of region 1 (D in panel A), the lower proliferative cartilage zone (×5200 magnification). Unfixed, unstained, proliferating chondrocytes may be identified by their size, shape, electron density, and columnar arrangement. Lacunae (L) are relatively electron transparent. Perilacunar and extracellular matrices (ECM) are variable in electron density and fibrillar in appearance. Nuclei (N) in some cells may be distinguished by a uniform electron density within the nuclear membrane (NM) which appears in negative contrast. Endoplasmic reticulum (ER) can be identified by the regular pattern of alternate density contributed by composed membranes and cisternae. Numbers of mitochondrial granules (MG), ranging between 500 and 1000 Å in diameter, are conspicuously dense. Circled regions were examined by X-ray microanalysis (a−c, inset, ×24 000 magnification) and selected area diffraction (d). Reprinted from ref 54, Copyright 1982, with permission from Elsevier.

8.3.4. Detection of Polyphosphates

Hirschman published a transient toluidine blue staining of fresh rat epiphyseal cartilage in the matrices of the resting and hypertrophic zones, as well as the proliferating and hypertrophic chondrocytes in the 1960s.[274,275] The toluidine blue staining was only observed with fresh specimens; its transient nature was unexpected and unexplained. Metachromatic shifts of toluidine blue have been reported for polyphosphate ions of different chain lengths and concentrations.[139,140] The observed toluidine blue fading stain may have been caused by hydrolytic degradation of polyphosphates when the tissue sections were exposed to water.

Fresh sections of the growth plate of rat long bones also stained with neutral red identified osmiphilic granules and vacuoles; the size and number of these stained bodies increased the closer the cells were to the zone of calcification.[276] More granules were also reported within the mitrochondria that were closer to the zone of hypertrophy. While unknown at the time, neutral red also stains for polyphosphates.[9] It was hypothesized that the cells close to the calcifying cartilage could be discharging some "physiological function, influencing or related to the deposition of calcium salts in the cartilage matrix?" Recent analysis of murine growth plates with the fluorescent reporter DAPI (4′,6-diamidino-2-phenylindole) detected polyphosphates in the same locations as those reported by Hirschman.[150]

Application of DAPI to dry-cut sections of EDTA-decalcified three-month-old murine (mouse) vertebrae showed the formation of DAPI−polyphosphate complexes within the growth plate. Polyphosphates were detected within the hypertrophic zone by displaying the characteristic DAPI−polyphosphate fluorescence at 520 nm, which is convoluted with the emission spectrum for DAPI−DNA (Figure 8). Application of alkaline phosphatase to the growth plate section showed a decrease in the DAPI−polyphosphate emission component of the emission profile, further proving the presence of polyphosphates.[150]

Both theories that the rate-limiting step of cartilage calcification is phosphate transport to the sites of calcification[270] and that mitochondria play a role in biomineralization[266] are supported by the controlled mitochondrial production, transport, and hydrolytic degradation of polyphosphates by alkaline phosphatase within the growth plate.

8.4. Bone (De)mineralization and Polyphosphates

Lowenstam noted that minerals formed in a particular site may be either (i) retained in place, (ii) transferred intact to other sites, (iii) excreted, (iv) dissolved and replaced continuously, periodically, or only occasionally, or (v) continuously reconstituted. The mineral in bone is subject to continual active and passive dissolution and replacement; Lowenstam outlined that these remodeled minerals serve "in part, as a reservoir for cations or anions to be used for a variety of functions".[1] Vertebrate bone is an actively remodeled tissue; the calcium, phosphate, and carbonate ions all have a variety of functions for the organism. This section will give a brief background to bone, present the detection of electron-dense granules in resorbing and mineralizing bone, and finally, present the recent detection of polyphosphates in resorbing bone.

8.4.1. Bone Mineralization

The vertebrate skeleton must satisfy a wide range of demands, including growth, protection, structural support, a source of calcium and carbonate to maintain homeostasis, a

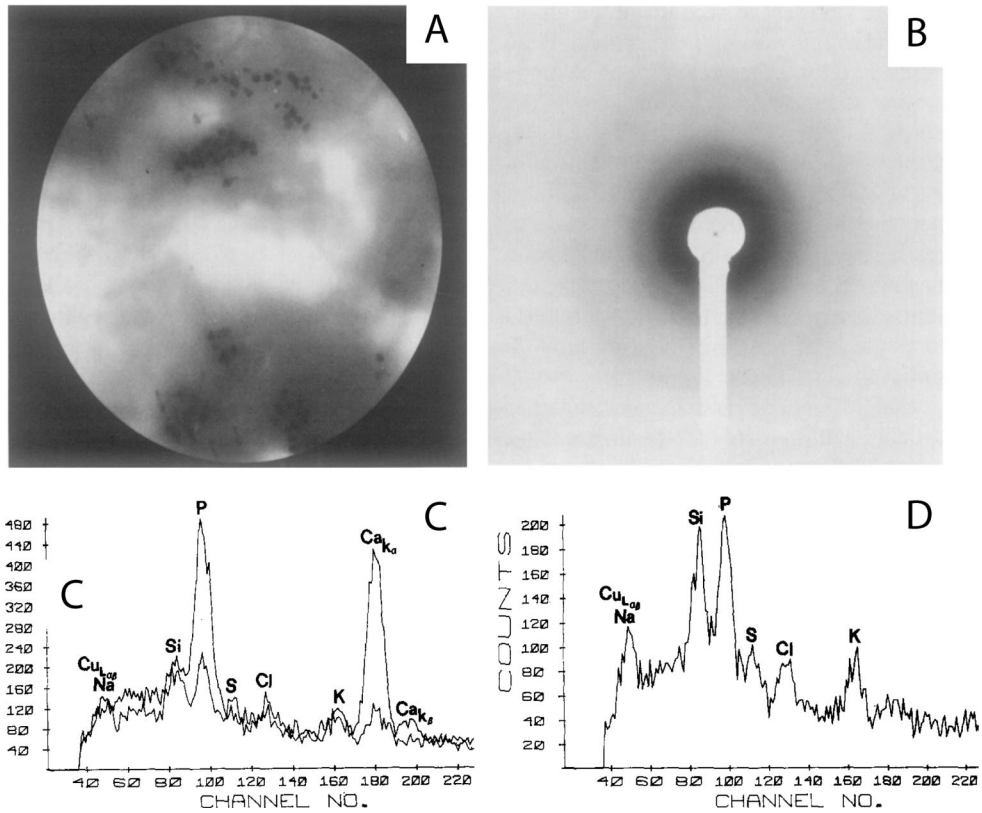

Figure 7. (A, B) Electron diffraction (100 kV) of mitochondrial granules from Figure 6B. The pattern from panel A (×15 000 magnification) has no distinct lines (B). (C) Superimposed X-ray spectra generated from a single unfixed, unstained mitochondrial granule (center of circle a, Figure 6B) and from the adjacent mitochondrial matrix (center of circle b). Significantly greater peaks of phosphorus and calcium are generated from the granule. (D) X-ray spectrum from cellular cytoplasm (c, Figure 6B, inset) near mitochondria. Reprinted from ref 54, Copyright 1982, with permission from Elsevier.

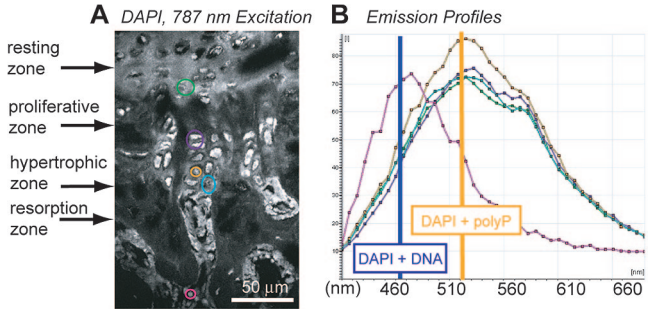

Figure 8. DAPI-identified polyphosphate (polyP) in vertebral growth plate sections (EDTA-decalcified, 3 month-old murine vertebral body) cut under anhydrous conditions: fluorescence emission (400−700 nm) (A) imaged upon multiphoton excitation with emission wavelength scan analysis and (B) by color-defined ROI. Red ROI in subchondral bone represents emission wavelength profile corresponding to that of DAPI bound to DNA, not polyphosphate. Other ROIs are emission wavelength profiles with a maximum at ∼520 nm, corresponding to that of DAPI bound to polyphosphate. Reproduced from ref 150.

sink for heavy metal ions, and the ability to continually repair damage caused by locomotion or trauma. These demands require that vertebrates continually resorb (remove) and remodel (rebuild) their skeleton throughout their life.

Bone is a composite of collagen, noncollagenous proteins, and poorly crystalline, highly substituted apatite crystals.[1] Remodeling is accomplished by the basic multicellular unit; it is composed of two coupled cell types: the bone resorbing osteoclast and the bone building osteoblast.[277] In the accepted model of bone resorption, the osteoclast forms a sealed "resorption zone" upon the surface of bone. Acidification of this resorption zone dissolves the apatite mineral, and subsequent release of collagenase proteins by the osteoclast digests the exposed collagen matrix. Osteoblasts follow the excavating osteoclasts, laying down a new collagen matrix (osteoid) that is later mineralized. There is an unexplained delay of 15−20 days in the mineralization of osteoid in humans, which is called the mineralization lag time.[278]

8.4.2. Detection and Analysis of Electron-Dense Granules

Bone formation is a sequential process; a clear understanding of its early mineralization stages has been elusive.[81,82] Previous electron microscopic examination of nonaqueous-processed, unstained, growing bone has revealed electron dense "mineral granules" within the mineralizing osteoid matrix, the mitochondria of osteoblasts, osteocytes (cells embedded in bone),[279] osteoclasts,[102,262] and the mitochondria of osteoclasts.[262] Gonzales et al. noted that the "crystals within osteoclasts are thought to be bone salts on the basis of morphology and not on the basis of electron diffraction".[262] Similar granules containing calcium and phosphate observed in osteoid examined by electron microscopy after anhydrous sample preparation were suggested to be stabilized by a crystallization inhibitor.[103] This interpretation has developed into some of the current theories of bone mineralization: that amorphous calcium phosphate is the first solid phase found in newly formed bone and that within matrix vesicles, amorphous calcium phosphate is transformed into apatite crystals.

Landis et al. measured the sizes of mitochondrial electron dense granules in anhydrously prepared, unstained bone, which ranged from 400−1000 Å in diameter. At higher

magnification, the granules revealed clusters of smaller particles approximately 50−100 Å in diameter that surrounded a less electron-dense core. The diameters of granular clusters in mineralizing osteoid ranged over a few thousand angstroms; these clusters were of intermediate electron density between unmineralized collagen and hydroxyapatite[102] (Figure 3).

Inorganic granules found in mineralizing bone were isolated by Aaron et al.[146] The authors pursued the characterization of the unstable components of the early stages of calcification, which have been variously described as "globules, vesicles, buds, nodules, clusters of crystallites, calcified spherules, and spherical aggregates of crystals" (from ref 145). Through a variety of techniques, they extracted ~1 μm sized spheres containing calcium and phosphate. They colocated acid phosphatase stained areas (associated with bone resorption) with the presence of granules. The isolated calcified microspheres were approximately 1 μm in diameter, close to the 1000 Å size of the electron-dense granules detected in the anhydrously prepared bone tissue by Landis et al.[103] Aaron reported mineral packets found within "filaments" of approximately 5 nm in width. The size of these packets correlates well with the 50−100 Å discrete, electron-dense elements within the granules observed by Landis et al.[103]

Electron-dense "spherules" were also identified within occipital (rear and rear-bottom skull) bones of mice and rats. These intracellular metachromatic spherules were shown to be labile and easily dissolved during tissue processing for embedding.[280] Changes in the chemistry of these granules located in cartilage and bone samples was induced by sample preparation;[54] therefore these granules were best observed in frozen, fresh, or anhydrously prepared samples. These unstable granules were associated with calcification by their localization. Their role in mineralization was not understood but was postulated to involve an organic component of the extracellular matrix.[54]

Using electron microprobe analysis, Landis et al. also measured the Ca/P molar ratio in electron-dense granules located within mitochondria (0.8−1.1), osteoid (1.2−1.3), and mineralized bone (1.4−1.5). They noted that the lower Ca/P molar ratio of the electron dense granules was similar to that measured for monetite ($CaHPO_4$) (molar Ca/P = 1.0) and calcium pyrophosphate ($Ca(PO_3)_2$) (molar Ca/P = 0.5) (Figure 6) but did not suggest that these electron-dense, calcium- and phosphate-containing granules might be composed of calcium polyphosphate.[103]

8.4.3. Role of Phosphatases

Aaron et al. commented that rudimentary biochemistry "in the form of extracellular acid phosphatase activity and possibly also carbonic anhydrase (unpublished results) seems to be associated with the calcified microspheres".[146] This postulation that carbonic anyhydrase is associated with an apatite precursor echoes an earlier apatite biomineralization hypothesis involving a role for carbonic anhydrase presented by the mineralogist McConnell in 1961.[281] Alkaline phosphatase, which is expressed by hypertrophic chondrocytes[271] and osteoblast cells,[282] is presumed to be involved in skeletal mineralization,[272] although its substrate(s) is not well-defined. Alkaline phosphatase has been linked to the destruction of pyrophosphate, a mineralization inhibitor.[171] In 1961, Fleisch and Neuman proposed that the role of phosphatase in bone was the destruction of a mineralization inhibitor, "perhaps a polyphosphate".[164]

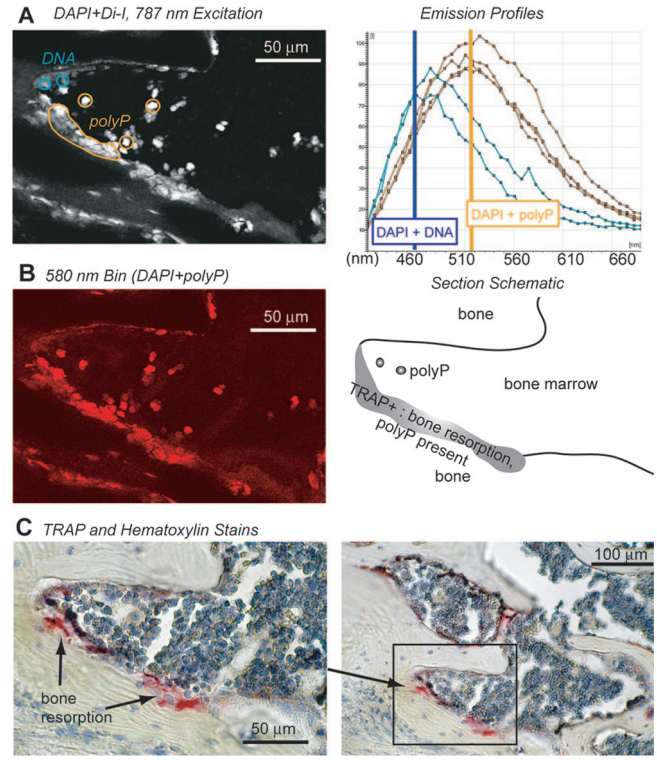

Figure 9. Detection of polyP in a bone resorption site of an EDTA-decalcified, three-month-old murine vertebra. (A, left) Confocal fluorescence image (400−700 nm) from a 5−10 μm bone section stained with DAPI and exposed to multiphoton excitation (787 nm). (A, right) Spectral scans of imaged region (A, left) were acquired in 20 nm bins. Emission intensity was plotted for each of the indicated ROI. Blue ROI = DAPI−DNA emission. Yellow ROI = DAPI−polyphosphate (polyP) emission. (B, left) The 580 nm bin emission for the same image captured in panel A spatially resolves DAPI−polyP distribution. (B, right) Schematic identifies relevant fluorescent regions (A, left) within the resorption zone. (C) The same bone section was subsequently stained for TRAP and counterstained with hematoxylin (an aqueous process, thought to accelerate hydrolytic degradation of polyP) to confirm the presence of osteoclasts (red staining) at the resorption site (left and right images correspond to high and low magnification, respectively). Reproduced from ref 150.

8.4.4. Detection of Polyphosphates

Polyphosphates were previously detected in osteoblasts (bone-forming cells).[283] Polyphosphates were also recently identified within a resorption pit of bone.[150] To detect polyphosphates within a section of bone tissue, dry-cut, un-dewaxed, cold EDTA-demineralizing processing methods were preferred because hot or acidic conditions are known to accelerate the kinetics of polyphosphate hydrolytic degradation.[110] Sections of three-month-old murine vertebrate bone were labeled with DAPI. DAPI−polyphosphate complexes were identified within granules located in regions of bone resorption by their respective emission fluorescence wavelength (520 nm)[141,143] (Figure 9A). DAPI also binds to DNA; however, the emission wavelength for the DAPI−DNA complex is shifted toward 460 nm. Figure 9B shows the 580 nm emission bin for the image captured in panel A. This highlights the regions containing DAPI−polyphosphate. The imaged regions were confirmed to be bone resorption zones by subsequently staining the same section for tartrate resistant acid phosphatase (TRAP, red), a marker

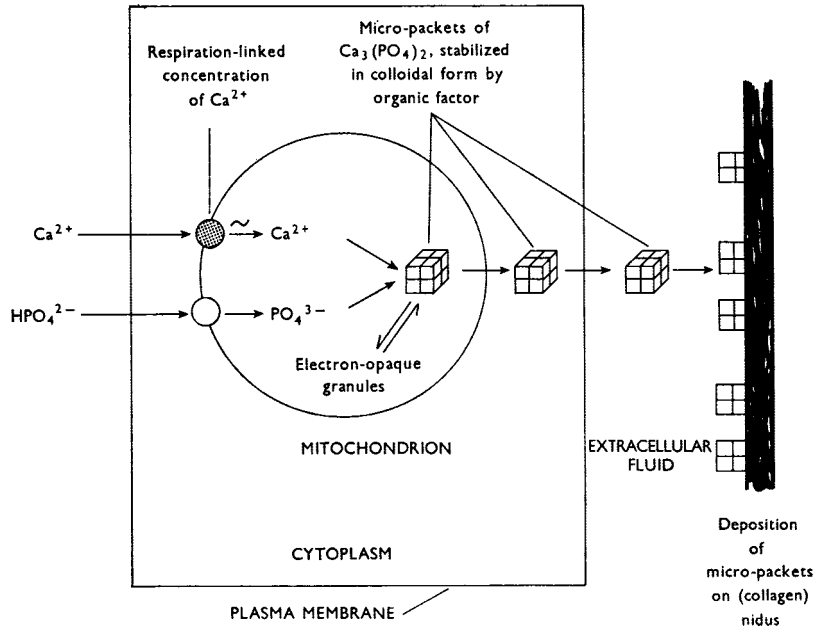

Figure 10. Working hypothesis for the role of mitochondria in biological calcification. Reprinted from ref 286, Copyright 1970, with permission from The Biochemical Society and Portland Press.

for osteoclasts[143] and counterstaining with hematoxylin (Figure 9C), indicating that polyphosphates are colocated with resorbing bone.

The cells that resorb vertebrate bone (osteoclasts) are rich in mitochondria.[262,284,285] A mitochondrial strategy of storing high concentrations of orthophosphate by condensing orthophosphate ions into polyphosphates may be utilized by the osteoclasts in order to scavenge and store the free orthophosphate released during bone mineral resorption in the acidic resorption zone in which apatite mineral is not stable. Calcium polyphosphate granules produced by the mitochondria within the bone-resorbing osteoclast could serve as a labile, noncrystalline (and therefore bioavailable), high concentration store of calcium and phosphate. These granules would then be available for transport to sites requiring the component ions of apatite mineralization, such as new bone.

The detection of electron-dense granules within anhydrously prepared, new, mineralizing bone[102,103] suggests that these granules may also contain calcium and polyphosphate. The expression of alkaline phosphates by osteoblasts and the correlation of alkaline phosphatase activity with bone mineralization[272] indicate that further research may identify polyphosphate metabolism in bone mineralization.

Another hypothesis for bone mineralization was proposed by the biochemist Lehninger. In 1970, Lehninger penned a "Jubilee Lecture" that built the case for mitochondrial control of biomineralization.[286] He postulated that the electron-dense, calcium- and phosphate-containing, amorphous granules observed in mitochondria were amorphous tricalcium phosphate, stabilized as micropackets by biological factor(s). These amorphous packets are transported to mineralization sites and eventually undergo nonreversible hydrolytic degradation, resulting in apatitic bone mineral (Figure 10).

In conclusion, we postulate that these micropackets contain calcium and polyphosphate that undergoes hydrolytic degradation, increasing the local calcium and orthophosphate concentrations and precipitating apatite, probably under the control of tissue nonspecific alkaline phosphatase. Our current understanding of the biochemistry of polyphosphates supports Lehninger's theory that "mitochondria may also function as packaging plants, in which are manufactured the bricks and mortar of hard tissue; moreover, mitochondria may also harbor the secret as to why we do not all turn into stone."[286]

9. Summary and Thoughts on Possible Future Research Directions

9.1. Summary

The objective of this review is to show that biomineralization of apatite appears to have a close relationship to the metabolism of phosphate via bioavailable polyphosphates, the production of phosphate-containing, electron-dense granules, and the action of mitochondria. We do not claim to cover all of the theories of apatite mineralization but have tried to collect historical work in apatite biomineralization research and show how the observations in the literature may be tied together by polyphosphate chemistry and biochemistry. Enzymatic polymerization of phosphate enables an organism to store high concentrations of phosphate and calcium without risking an undesired precipitation of apatite. Enzymatic hydrolytic degradation allows for the controlled increase of orthophosphate by the destruction of polyphosphate.

Polyphosphates also serve as storage sites for cations, such as calcium, and as alternate energy sources for some organisms who are exposed to anoxic environments. The sequestration power of the calcium−polyphosphate complex offers an excellent chemical method for biological control and transport of free calcium as well as phosphate concentrations within amorphous electron-dense bodies.

We conclude this review by presenting the theory that the hydrolytic degradation of polyphosphates, either by spontaneous hydrolytic degradation or by controlled enzymatic hydrolytic degradation with enzymes such as alkaline phosphatase, links the formation of apatite mineral to both induced and controlled biomineralization processes. A phosphatic mineral within the vertebrate skeleton offers more flexibility for metabolic processing because it can be dissolved by biologically controlled acidification and its component ions

can be stored as bioavailable calcium polyphosphate complexes. This bioavailable store of the building blocks of a metabolically active apatite skeleton offers more biological processing flexibility than a skeleton composed of carbonate minerals, which do not polymerize, or a silicate skeleton, which polymerizes but is not hydrolyzed by any known biological action.

9.2. Thoughts for Possible Future Directions

Enzymatic production and destruction of polyphosphate ions provides another explanation for the vertebrate mineralization process that has been debated in the literature over the last four decades. It has been difficult to detect and quantify polyphosphates in mineralized tissue due to the instability of the polyphosphate molecule in aqueous systems; anhydrous techniques are required to prevent polyphosphate hydrolytic degradation. Refinement and increased use of anhydrous bone tissue preparation techniques may lead to a further understanding of the metabolism of polyphosphates within bone tissue. This understanding may offer new angles of research for clinically relevant bone diseases such as osteoporosis. Other inappropriate apatitic precipitations such as cardiovascular calcifications[269] and kidney stones[3] may be further understood and alleviated if a role of calcium polyphosphates in these calcifications could be determined.

Another example of a pathological calcification is the formation of minerals within the joint capsule. The concentration of calcium-containing crystals within the synovial fluid has been noted to increase with the severity of osteoarthritis.[287] Osteoarthritis is a degenerative disease of the joint. The analytical techniques used to better analyze the "hydroxyapatite" and calcium pyrophosphate dihydrate crystals found in joint synovial fluid was recently reviewed.[288] The paper suggests that organelles that contain electron-dense granules may be responsible for the undesirable mineral formation. Further investigation may identify calcium and polyphosphate within these precursor granules that may form the minerals attributed to osteoarthritis.

The production of polyphosphates during bone resorption might serve as a source of polyphosphates for other cells. The microvilli observed on the distal side of the osteoclast cell body[262] suggest that calcium- and polyphosphate-containing granules could be exported by the osteoclasts into the marrow space for use by other cells. One possible example is the recent identification of polyphosphates in platelets[240] and their role in fibrin clot structure.[241]

Polyphosphates were recently found to have an effect on breast cancer cell survival.[289] Calcium- and phosphate-containing electron-dense granules and hydroxyapatite were detected in human breast carcinomas.[290] Calcium phosphate microcalcifications have been associated with mammary malignant lesions;[291] solids with P/Ca ratios ranging from 0.5−1.5 were measured in "type 2" (calcium phosphate mineral) microcalcifications.[292] A P/Ca ratio of 1.5 is close to a Ca/P ratio of 0.7, suggestive of calcium polyphosphate. Perhaps an investigation may show a polyphosphate metabolism related to calcifying malignant lesions.

A drug group used to treat osteoporosis is based on the chemistry of bisphosphonates. The clinical effect of bisphosphonate is an increase in bone mineral density. Bisphosphonates are compounds based on a P−C−P moiety that is similar to the phosphoanydride bond but is not hydrolyzed by enzymes.[293] The effect of bisphosphonates on polyphosphate metabolism and its consequent effect on apatite biomineralization is currently unknown. Bisphosphonates have been used to reduce skeletal morbidity in women with advanced breast cancer and bone metastases. There is evidence that bisphosphonates induce death in a human breast cancer cell line[294] and inhibit the development and progression of bone cancer in a mouse model of breast cancer.[295] With a known history of affecting apatite biomineralization, perhaps bisphosphonates may be discovered to play a role in affecting polyphosphate metabolism.

10. Acknowledgments

Dr. Zachary Henneman is thanked for initial contributions to the manuscript. ChrystiaWynnyckyj, Peter Alexander Robinson, Jennifer Tinning, and Jon Campbell are thanked for contributing their editorial expertise. Dr. Christopher Omelon is acknowledged for manuscript assistance. The reviewers are thanked for their constructive feedback. The Natural Sciences and Engineering Research Council of Canada is acknowledged for funding this research and S.O.

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CR0782527