Introduction

Milestones in the study of biominerals

A summary of the discontinuous and somewhat erratic path of research on their formation and properties

The microscopic study of living organisms did not develop until the first half of the nineteenth century, in spite of earlier observation of "cells" by Robert Hooke (1665). It was only in 1831 that Robert Brown described a new structure in plant cells: the "nucleus." In transferring microscopic observations of Mathias Schleiden on plant tissues (1838) to his own studies on animal tissues, Theodor Schwann (1839) made a major contribution by proposing the theory of universal cellular organization in living organisms. In this same year the Microscopical Society of London was founded, and among the founding members was James Scott Bowerbank.

In his paper, "On the structure of the shells of molluscan and conchyferous animals" (1844), Bowerbank showed that far from being simple aggregates of unorganized mineral particles, these shells are formed of sharply distinct units, each possessing a definite geometry whose regular arrangement demonstrates that their order is completely controlled by the producing organism. The rapid diffusion of this new type of analysis is attested to by the publication the following year of Carpenter's paper, "On the microscopic structure of shells" (1845, with a second part in 1847).

These two studies represent much more than a simple change in the scale of observation. For two or three decades the microscope had already been used to observe fossil morphology, but now, by focusing on the organization of the mineral constituents themselves, beneath the surface of the sample, study began of the functions of the cells producing structures capable of being fossilized (e.g., the mantle of molluscs, the basal epithelium of coral polyps, etc.).

The extracellular formation of crystalline structures

In confirming the high level of control exerted by living organisms on their mineralized hard parts, microscopic observation indicates equally that these structures form exceptions to the theory that regards cells as basic components of all parts of metazoans. This is particularly obvious for the category of macroscopic calcareous structures produced in various invertebrate phyla, such as shells of molluscs, carapaces of arthropods, or scleractinian corallites. It is an impressive paradox that Bowerbank, as a founding member of the Microscopical Society of London, published the first such studies, and these resulted in a conclusion that was in complete opposition to the fundamental working concept of his colleagues; namely, his conclusion that mineralized structures of living animals are not themselves cellular.

This surprising conclusion was progressively extended to other groups of organisms, but was not always readily accepted. Thus, when the fibrous organization of the coral skeleton (Scleractinia) was discovered by Pratz in 1882, a lively controversy developed as to whether the cells of the polyp (in its basal ectoderm, in contact with the skeleton) were themselves calcified (the position of von Heider, 1886), or if indeed they only directed external calcification (the position of von Koch, 1886). It is interesting that it was the best-informed investigator of coral structures at that time who opposed the second hypothesis most vehemently. In 1896, M. Ogilvie published her seminal analysis of the scleractinian corals, showing the possibility of systematic treatment based on precise observations of the spatial arrangements of the calcareous crystallites that form the coral skeleton. At the same time, however, she accepted the erroneous cellular hypothesis of von Heider (and one could find adherents until much more recently). It seemed impossible to her that these fiber orientations, so precise that one derived from them basic criteria for taxonomic identification, could be developed by different mechanisms from those ensuring the precise coordination of cellular tissues during the ontogenetic development of any organism. This is the key point in understanding these features. Shells and other calcified structures must be interpreted as resulting from secretion processes, and crystallization occurs external to the secreting cells.

As to the terminology used, although its use is quite widespread, the expression "mineralized tissue" should *not* be applied to calcified biogenic structures, since these structures themselves are not formed of cells. This can be argued concerning bones, as the secreting cells are included within the mineralized material (this is why fossil nucleic acids can be found in fossil bones), but the mineralized material itself is actually extracellular. More readily visible in mollusc shells, for instance, the cellular layers producing mineralized structures move freely over the mineral surface (for example, the oyster's mantle or gastropods that retract within their shells).

The heavily calcified tests of the Foraminifera may appear to be obvious exceptions to this rule, as here the calcareous structures are located within the cell membrane. However, these calcified structures are carefully isolated by continuous organic envelopes from the cell compartment where metabolic reactions occur. Perhaps more significant is the case of the unicellular algae of the prymnesiophyte Coccolithophoridae, in which the individual coccoliths are formed within vesicles issuing from the Golgi apparatus. Little is known about the molecular mechanism providing for the transit of Ca-carbonate though cell cytoplasm to the vesicles, but during the entire crystallization process organic membranes prevent contact between crystallized Ca-carbonate and living cytoplasm.

The corallinacean algae (Rhodophycaea) also appear to be exceptions to this carefully maintained exclusion between intracellular physiological processes and biocalcification. Here the cellular frame itself is incrusted by carbonate, leading to the formation of a wall completely separating adjacent cells. This process leads to the death of these cell layers at a short distance below the surface of the organism.

In addition to descriptions of calcified materials, the great importance of another mineral component was established during these early nineteenth-century decades: biomineralization based on silica. In the instructions formulated by Alexander von Humboldt

for exploration of the Antarctic seas (for an expedition led by J. C. Ross in 1839), the methodical sampling of Si content of marine waters was recommended. Just previously, in 1838, C. G. Ehrenberg had shown the geological importance of siliceous unicellular algae by his microscopic observation of sands known as the "Kieselgur." J. D. Hooker participated in the Ross expedition, and his "Flora Antarctica" (1847) constitutes the first analysis of the production of biogenic silica in the ocean.

Thanks to microscopy, siliceous biomineralization, principally associated with sponges, radiolaria, and diatoms, rapidly was recognized as second in importance quantitatively, only outranked by carbonates. Although it occurs both in animals (sponges and radiolaria) and plants (diatoms), the essential character common to all siliceous biomineralization is that the silica is always present in an amorphous state, just the opposite of the two combinations of calcium (carbonate and phosphate) that are always produced in a crystalline form. The term "amorphous" applies to the molecular level of organization of the mineral material, in that the silicon–oxygen tetrahedra are associated through the intermediary of water and organic molecules to form a specific mineral named *opal*. It does not indicate that the siliceous structures are not controlled at the level of their overall morphology. Just the opposite, the extreme precision with which Radiolaria, Diatomacea, and Porifera build their mineralized structures, all with specific morphologies, has been known for a long time.

There exists a strict correlation between the nature of the material utilized and overall crystallographic characteristics of the structures produced, a situation all the more striking in that it totally ignores their taxonomic position, even at the highest level (above the phylum level). On a practical level, the result of this constant contrast between crystalline Ca-carbonate and amorphous silica biomineralization is that only the former can be the subject of detailed microstructural analysis.

The third major group of common mineralized biomaterials, far behind Ca-carbonate and silica from a quantitative standpoint, are those composed of calcium phosphate. These occupy a somewhat ambiguous position between calcareous and siliceous mineralization. Due to their resulting from extracellular crystallization in the greatest part of vertebrate bone, they are crystalline and thus parallel invertebrate carbonates somewhat. There are important differences between groups, however, and the most obvious is the dimensional scale of crystallized units. Calcareous systems can produce units with a monocrystalline aspect capable of reaching great size, commonly several hundreds of micrometers. In mineralized phosphates, crystals are always much reduced in size, so that characterization of their morphology is difficult even at present. Therefore, it is not surprising that early observations of microstructure were made on carbonate biominerals.

This situation does not rely on particular taxa. Phosphates are almost exclusively the skeletal material of the Vertebrata, but they also are present in some invertebrates. In the latter, crystalline units never attain dimensions comparable to those in carbonates. Conversely, some vertebrates also produce extremely interesting calcareous structures, e.g., otoliths and eggs. In these cases, their structures present crystallization aspects (size of microstructural units and layered growth mode) that are comparable to those characteristic of invertebrates.

Factors retarding the study of biominerals

The study of the mineralized structures formed by organisms began at practically the same time (the decades around 1840), and in the same circles as the study of purely organic tissues and cells. This simultaneity of the time of origin is noteworthy because it underlines the striking difference between the more rapid rate of progress in understanding cellular structures, as compared to that of the mineralized components of organisms. The origin of this is readily apparent. The techniques of microtomy, complemented by numerous and varied staining techniques for the characterization of tissues and cell organelles, rapidly made possible the analysis of histological and cytological phases, but have proved to be much less applicable to mineralized structures.

Preparation of rock thin sections and polarization prisms, both due to the work of William Nicol (1815 and 1828, respectively), were available already at the time of Bowerbank, and during the following century, only the increasing quality of optical microscopes provided marked improvement of the study of mineralized hard parts of organisms. In terms of methodology, prior to the 1950s no significant innovation was introduced to change the concepts of biogenetic mineralization of structures. Even X-ray diffraction (W. L. and W. H. Bragg 1912) did not have the same fundamental importance for biominerals that it did in the development of general mineralogy, because the true originality of the biominerals is not mineralogical diversity.

Optical studies carried out during the nineteenth century resulted in some high quality syntheses during the first part of the twentieth century, by Schmidt (1924), Bøggild (1930), and Taylor *et al*. (1969–1973). These firmly established that living organisms exert such precise control on the organization of their skeletal structures that families, genera, and sometimes species can be identified by reconstructing their three-dimensional arrangements of calcareous skeletal units. Bøggild, for example, succeeded in distinguishing genera of the Mollusca by their characteristic shell microstructures. However, from the standpoint of methodology, compared to Bowerbank's pioneering observations, no significant innovations were yet introduced to generate changes in existing concepts regarding the formation and, specifically for Ca-carbonate, the mode of growth of the crystal-like mineral units forming the mineralized structures of most invertebrates.

The geochemical era

It is fascinating to realize that, in this stagnant situation, the first event that contributed to a major renewal in research on biominerals resulted from the development of a specialty that until then was unknown in biology: thermodynamics, and more particularly, the thermodynamics of isotopic fractionation. In 1947, H. C. Urey, the chemist already famous for his discovery of deuterium (1934 Nobel Prize), produced a mathematical expression for the difference that would be established during chemical reactions between proportions of isotopes of a chemical element *before* it entered into the reaction, and the proportions of isotopes of this same element *after* the reaction, i.e., within new molecules resulting from the reaction. Urey showed that *this change of proportion varies as a function of the temperature* at which the reaction producing the compound has occurred, and suggested that,

reciprocally, the measure of isotopic ratios in a given material could provide an indication of the temperature at which it was formed. Urey stated: "I suddenly found myself with a geological thermometer in my hands." In 1950, the technology of mass spectrometry permitted the measurement of a difference of 0.2 parts per thousand, which is the variation of the ratio between stable isotopes of oxygen per degree centigrade during precipitation of Ca-carbonate. By carrying out a series of in vitro Ca-carbonate precipitations, McCrea (1950) experimentally checked Urey's calculations and, in 1951, the result of the first practical application of the isotope-based paleotemperature reconstruction was published (Urey *et al*. 1951).

In the history of paleontological research, there are no other examples of innovation having resulted in such marked reverberations, as did this presentation in 1951 of the reconstruction of the life of a Jurassic belemnite coming from the Isle of Skye (Scotland). Belemnites are representatives of an extinct group of the Cephalopoda (Mollusca). Their most common structure is a massive conical rostrum made of radially diverging carbonate units in which concentric growth layering is easily visible. By carrying out regularly spaced sampling of carbonate material along an identifiable ray in the section, they could thus measure the ratios of $^{12}C/^{13}C$ and $^{16}O/^{18}O$ isotopes recorded in the successive layers produced by the belemnite during its lifetime. In a few lines, the announcement of a truly astonishing precision was enunciated thus: "This Jurassic belemnite has recorded three summers and four winters . . . It lived during its youth in warmer water than in its adulthood, and died in the spring at an age of about four years. The mean seasonal variation of the water was approximately 6 °C, with a mean temperature of 17.6 °C." Never before had any fossil been made the object of such detailed analysis. This opened the age that has been called that of "isotopic paleontology" (Wefer and Berger 1991).

The question of a "vital effect" – establishing an overriding taxonomic influence in the mineral realm

Chemical paleontology may be a more appropriate name for studies that began in the middle of the twentieth century. With more accurate mass spectrometers providing better measurement of isotopic fractionation, chemical analysis made additional striking progress with the establishment of atomic absorption spectroscopy (Walsh 1955). As a result, it became possible to make multiple and important series of measurements of concentrations of minor chemical elements associated with biogenic minerals, such as strontium found in aragonitic material and magnesium in a calcitic one. These notable phenomena now became the focus of interest for numerous investigators, as experiments of coprecipitation showed that concentrations of minor elements incorporated in carbonates vary as a function of temperature. Thus, sampling this variation permits using concentrations of magnesium, strontium, and numerous other elements as sources of information on environmental parameters. Methods of paleoenvironmental reconstruction now appeared that were much more rigorous than traditional and older qualitative ecological observations.

Starting in the 1950s, Chave, Lowenstam, and numerous others undertook the exploration of this phenomenon in comparing results of experimental chemical precipitation with measurements on biominerals of organisms originating in natural environments. During

the following decades, the growth in sensitivity of analytical techniques has responded very well to the needs of researchers seeking to accumulate data on the composition of biogenic mineralized materials. At the same time, the results obtained also bring with them a conclusion that contrasts markedly to views that had initially been proposed. Through innumerable measurements carried out on the chemical and isotopic properties of biominerals, a biological influence has been progressively established with a strength and precision comparable to those that had been shown by their optical characterization during the initial phase of biomineralization studies.

The conclusion now imposed by modern data is that the chemical compositions and the isotopic fractionations observed in biominerals cannot be explained by straightforward precipitation phenomena. Chemical partitioning and isotopic fractionation that occur during formation of biominerals are undoubtedly sensitive to temperature and other environmental parameters, but they have been shown to be primarily dependent on taxonomy.

This conclusion regarding chemical composition and isotopic fractionation in biominerals not being explicable by ordinary precipitation phenomena was foreseen from the beginning. It reflects the foresightedness of the initial authors of isotopic methods to have formulated explicitly the existence of a "vital effect" (Urey *et al*. 1951, p. 402). It is perhaps also of interest to note that, prior to becoming oriented towards physical chemistry, H. C. Urey had acquired a zoology degree at the University of Montana in 1917. Possibly his initial education determined his particular sensitivity towards the properties of living systems, a quality lacking in some of those who later applied chemical/isotopic methods to biological minerals. Forty years after Urey's remark, Morse and Mackenzie indicated that the progress of knowledge concerning the mechanisms for precipitation of carbonates had not led to development of a satisfying overall theory (1990, p. 602). It is of note that this conclusion was formulated with regard to sedimentary carbonates, regarded as reflecting purely physical and chemical mechanisms, as it is far more obvious concerning their biogenic forms.

In practice, this property has led to the necessity for "calibration" of each species utilized, and for doing this for each environmental parameter analyzed in ancient biomineralization. Inversely to the rational and rigorous views that motivated technical applications during the decades of the 1950s to 1970s, a purely empirical practice has developed to deal with this. Simply stated, the methods of formation of biominerals do not correspond to conditions observed in laboratory precipitation experiments. It is clear that the progress of instrumentation, which seemed to be the only limitation during initial phases of research on the chemical composition of biominerals, can now only be properly exploited when accompanied by better understanding of the fundamental biological processes that control the recording of environmental conditions in biominerals.

The development of biochemistry in biomineral studies: a bottom-up approach leading to an hypothesis of biochemically driven crystallization

The period during which the first applications were established for measurement of isotopic ratios and trace elements in biogenic minerals is also the time when research began that would progressively establish the *biochemical diversity as well as the taxonomic specificity*

of the organic materials that are always associated with mineral structures produced by living organisms.

The presence of organic compounds in biogenic mineralized materials was recognized shortly after the observations of Bowerbank. The word "conchyolin," coined as early as 1855 by Frémy, has long been used as a general designation for these compounds as a whole. However, their concentrations are in general low enough that for a long time the analysis of these organic components was hardly possible. From a practical viewpoint, it was only a century later that electrophoresis and chromatography became the main sources of biochemical data, enabling consistent analyses to be made of these organic compounds, and accurate biochemical comparisons carried out on them.

At the same time, the number and diversity of known biominerals has increased rapidly. When Lowenstam began his methodical investigation of biomineral diversity (1963), the number of mineral combinations known in living organisms was roughly ten. In 1989, Lowenstam and Weiner recorded more than 60 biologically produced minerals, of which approximately half are combinations based on calcium, and a quarter are phosphates. Of these, 24 combinations are produced by procaryotes, 11 by plants, 10 by fungi, and an additional 10 more by the Protista and 37 by other animals. It is impressive that all these minerals appear as "ordinary" compounds in the sense that no unique or peculiar mineral combinations seem to have been developed by living organisms. This leads to a paradoxical conclusion, which is that, *properly speaking, there are no biominerals* per se.

Progress in our knowledge of the organic compounds associated with these various mineral features has resulted in a shift of research interest away from the minerals themselves to the properties of their associated biochemical compounds. During decades, investigators have focused on the analysis of skeletal "matrices." Progressively, these organic compounds have been shown as diverse and specific as the shapes and compositions of the mineralized structures from which they were isolated.

This led to the suggestion made by Wada (1961), and a little later by Watabe (1965), that deposition of mineral material could only be carried out *because* of substrates formed by preexisting organic compounds. This hypothesis has been invariably confirmed by analytical advances that have been progressively extended to cover a whole array of biogenic mineralized structures, and reinforce the conclusion that biological control is always exercised by the previous emplacement of an ensemble of organic compounds.

This organic phase shows singular variety in its relative proportions of glucidic and proteic compounds, and this is clearly dependent on the taxonomic position of the producing organisms, even though environmental conditions also influence its general composition. To model the organic architectures that can furnish sites capable of attracting mineral ions is presently a fundamental approach to biocrystallization at the molecular level. Even though numerous points remain unexplained that pertain to the mechanisms by which these organic materials are able to control the growth of biogenic mineral materials, there is no longer any doubt that they do constitute an essential agent of the phenomenon called biomineralization.

It is also important to note that studies developed at the molecular scale have not yet explained the process of crystallization as it results in forming species-specific morphologies

of biocrystal units. The recent development of new methods of observation and analysis, providing access to submicrometer-scale imaging, suggests that, in addition to the chemical bottom-up approach, high-resolution studies of skeletal structures can contribute to bridging the gap between traditional optical microscopy and interpretations based on the molecular approach.

Collecting information on mineral and organic components at micrometer to nanometer scales by use of the "new microscopes"

The first of these new instruments relies on production of "new light," i.e., the electromagnetic spectra that are produced when rapidly moving particles (accelerated close to the speed of light) are obliged to change their direction of propagation. This is obtained in synchrotrons, which are actually magnet-driven polygonal circuits for particles, each angle being the source of a wide electromagnetic spectrum with highly specific properties. Associated with high-resolution monochromators, selected X-ray wavelengths allow characterization not only of chemical elements but, additionally, of the chemical state of given elements. For instance, distinction can be made between the sulfur in proteins (sulfated amino acids) and the sulfur in polysaccharides, both distinct from the sulfate form in gypsum. Synchrotron mapping has established a correlation between mineral growth zonation (made visible by etching and SEM observation) and biochemical zonation. It is through this type of experiment that a mechanism has been found to produce stratified growth simultaneously involving organic and mineral phases. Taken together, this insures organic control of developing microstructural units.

Conformity between the mineral and organic phases within a given growth layer implies that the relationship between the two components occurs at a submicrometer level. The instruments grouped under the designation of "atomic force microscopes" or "near-field microscopes" now acquire images of spatial arrangements of the two components within micrometer-thick growth layers. Actually, this is presently a family of instruments that are still undergoing important development and diversification, yet they provide a method and perspective that suggests that we can look forward to acquiring data at extremely fine scales (nanometer to molecular). Different modes of observation (tapping or contact, for example) and different signals that can be acquired simultaneously provide descriptions of microstructural characteristics and distributions of associated organic phases on a submicrometer scale. The result has profoundly changed our reconstruction of biomineral structures, with important consequences to our understanding of the biomineralization process itself.

Looking for a working model to explain the variously scaled properties of calcareous biocrystals

The decade of the 1950s epitomized the disparity of scale in research dealing with calcareous biominerals. At that time, they were described as ordered crystals as seen in polarizing microscopes complemented by X-ray diffraction, both techniques emphasizing their crystalline patterns. But, simultaneously, the beginnings of studies carried out at the isotopic level

revealed species-specific fractionation that suggested enigmatic growth mechanisms. Only Urey as a Nobel Prize chemist was able to hypothesize such an obscure mechanism as that referred to as the "vital effect," that is possibly involved in what was (and sometimes still is) represented as simple crystallization due to precipitation from a saturated solution. More than a half-century later, our data has been improved greatly, but in spite of the considerable influx of this new information, diversity remains the dominant impression. A consistent integrated scheme cannot yet be established, owing to the diverse interpretations concerning the respective proportions of crystallographic processes and biochemically driven mechanisms in the formation of these materials.The scale gap of the 1950s between optical morphology and isotopic and molecular levels is still yet to be totally removed. Therefore we here investigate the major types of calcareous structures in order to examine what their level of commonality may be at which similarities occur between these very different structures. Through a top-down approach and specific preparative processes, this investigation results in a different view of growth mode for most of these structures, directly linked to the cyclical secretional functioning of mineralizing organs, allowing neither free space nor time available for uncontrolled crystallization. Truly, the micrometer-thick growth layer appears to be the common characteristic of biogenic calcareous structures, whatever their morphology. As an example of multiple consequences, shaping of skeletal units by the controlled development of growing single crystals, long admitted as a key process in biocrystal formation, no longer seems an operative concept.

This growth layer is also the domain where the interplay between organic and mineral materials actually occurs. Because it is formed in direct contact to the mineralizing organ, the micrometer-thick growth layer is also a most essential location, where molecules resulting from intracellular biochemical processes are transferred to the cell exterior and become operative. Atomic force microscopy associated with transmission electron microscopy (imaging and diffraction) provides us with initial information about the sequential development of the biocrystallization process at the elementary growth level. In-depth understanding of the difference between those crystalline structures generated by living organisms, and crystalline materials that result from chemical precipitation relies on a reliable reconstruction of this stepping mode of crystallization.

From an environmental viewpoint, analyzing biochemical consequences of gene regulation that make the biomineralization process sensitive to environmental conditions will thus establish a more precise relationship between oceanic waters and various taxonomy-linked calcified structures. Understanding the relationships between environments and biomineralization offers paleontologists a new basis for re-examination of the overall evolution of fossil faunas over millions of years. The reactions of different phyla to long-term (and large-scale) environmental variation may well have influenced their capabilities during skeletogenesis, with additional consequences in the character and intensity of diagenetic processes. Not only is it the interpretation of individual fossil samples that can benefit from fine scale study of their calcareous microstructures. By applying the methods used to characterize biocrystals of related extant species, understanding of faunal development throughout the fossiliferous portion of the geological column can be improved.

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The concept of *microstructural sequence* exemplified by mollusc shells and coral skeletons

Similarity of growth mode and skeletogenesis at the micrometer scale

At the time that Heinz Lowenstam began an investigation bearing on the different minerals that living organisms can produce, only a dozen or so biogenic minerals were known. Thirty years later, their number surpasses 60. Well before the appearance of the synthesis uniting the essential data that had been established during the 1980s (Lowenstam and Weiner 1989), Lowenstam had proposed (1981) a fundamental distinction concerning the mode of formation of biogenic minerals, and more particularly the precision of the controls on their deposition exerted by the producing organism. Lowenstam proposed then to distinguish the "matrix-mediated minerals," characterized by formation very precisely controlled by the action of an organic component specifically produced, and those that, although equally produced by a living organism, are developed in a more autonomous way; they are only "biologically induced." In these last, mineral elements can be developed according to methods and arrangements quite close to those that can be observed in purely chemical precipitated materials.

Among the calcified structures belonging to this category were placed the calcareous skeletons produced by corals. This opposition between molluscan shells, examples of "matrix-mediated minerals," and coral skeletal carbonate has remained generally accepted and very recently has still been formulated in reference journals (Veis 2005). This is however, truly surprising. The precision of the biological control on the skeleton by the coral polyps had been well established at the end of the nineteenth century by M. Ogilvie (1895, 1896), who carried out a pioneering study of the specific arrangements of the fibrous aragonite units there. But it is true that the difference between these and mollusc shells, the almost exclusive focus of research on methods of biomineralization has long remained poorly established. One should note in addition that this concept of being "biologically induced" in the formation of the coral skeleton is perfectly convenient for geochemists utilizing these materials as archives of ancient environments, because they can thus base their interpretations on a chemical theory lacking any biological influence, in spite of contradictory evidence long since published (i.e., Weber and Woodhead 1972).

During recent years, a convergent set of observations and analyses led to re-examination of this view. An additional objective of this chapter is to explain the concept of the microstructural sequence and its relationships with the mode of growth of calcified structures, beginning with two groups whose anatomical and physiological organizations are