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General Palaeontology (Palaeobiochemistry) The renewal of ideas about biomineralisations

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Abstract

Biomineralogy is an ancient discipline, based initially on techniques that were those of classical crystallography and histology, in light or in electron microscopy. Recently, the research landscape has been modified by new methodologies coming from molecular genetics and also from inorganic chemistry, driven by hopes to create a genuine ‘chimie douce’, close to physiological conditions, in which biomineralisations would provide useful models. Historically, belief preceded basic understanding. Today, mineralised tissues appear to be highly structured composites at nanoscales and their morphogenesis might be a model system for genetics. **To cite this article:** *Y. Bouligand, C.R. Palevol 3 (2004).*

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Résumé

Le renouvellement des idées à propos des biominéralisations. La biominéralogie est une discipline ancienne, basée initialement sur des techniques qui furent celles de la cristallographie et de l’histologie classiques, en microscopie photonique ou électronique. Récemment, le paysage de la recherche s’est modifié en raison de nouvelles méthodologies venues de la génétique moléculaire et aussi de la chimie minérale, avec l’espoir de créer une authentique « chimie douce », proche des conditions physiologiques : les biominéralisations constitueraient d’utiles modèles de ce point de vue. Historiquement, de vieilles croyances ont précédé les premières connaissances. Aujourd’hui, les tissus minéralisés sont perçus en tant que composites hautement structurés, dont la morphogenèse pourrait être un système modèle pour la génétique. **Pour citer cet article :** *Y. Bouligand, C.R. Palevol 3 (2004).*

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1. A primordial interface: minerals and life

Most advances in biomineralogy have come from the emergence of new chemical and physical techni-

ques. However, the main concepts were based on geometric considerations, as in crystallography, but differently, since biological shapes are curved in general, whereas straight lines and planes dominate in crystals. This aspect was developed in several chapters of a famous book written by D’Arcy Thompson [54]. In recent years, the context was different for two reasons.

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Molecular biology and genetics have proven to be of considerable interest in the study of biominerals, and this trend is illustrated in several works presented in this issue. Today also, biomineralisations represent an attractive topic for specialists of inorganic chemistry, because life is able to produce various chemicals in large quantities, under physiological conditions, whereas the same materials are manufactured throughout the world, using procedures that often remain deleterious for people and environment.

These differences between chemistry in factories and chemistry in biological systems were considered very early, but the dream is now the possibility of a genuine ‘chimie douce’ as suggested by Livage [32], different from the usual chemistry and even from biotechnologies that are not more innocent. This new chemistry would involve soft conditions, replacing furnaces and reactors in factories by tiny melting pots resembling enzymatic sites and other systems at work in cell machineries, but the main point would be to build these nanoreactors with the very atoms of inorganic chemistry, so that confusions with true biological processes could be avoided, a way to circumvent some difficulties encountered in recent biotechnologies. The mineral world and the living world are compatible, since the latter arose from the former, and still coexist, so that the study of mineral metabolism and of skeleton formation could provide ideas for experiments in pure inorganic chemistry.

Organizing this meeting on biomineralisation was a splendid initiative of two professors at the ‘Collège de France’, from the very different disciplines of inorganic chemistry and palaeontology. Their long-term views were not limited to these nanofactories for the future, since the topic covers large domains in biological sciences and life history at the surface of the Earth. The fossil record is based mainly on mineralised tissues. Recognizable remains of cells and molecules of biological interest were found in various rocks, including the most ancient ones. Purely inorganic nanofactories were possibly at work for long periods before the very beginning of biosphere and biomineralisations probably began with life itself, but were certainly preceded by innumerable mineral-organic associations.

2. Ancient paradoxes and beliefs

Simple but difficult questions have appeared regularly about the mineralised parts of living beings but,

even today, in many cases, answers are not on the horizon (see Marin in this issue). Some words suffice to explain this situation, say ‘the paradox of the sculptor and the mollusc’ for instance. To create a statue, an artist uses hard materials such as marble or wood, and hard tools to carve them, whilst soft living bodies, as molluscs, do not need such instruments and nevertheless produce solid shells comparable to sculptures, with precise geometries and decorations; there are also soft matters such as clay plus water, shaped by fingers, but hardening requires high temperatures in an oven, whereas the mollusc ‘works’ at the water temperature, in normal environmental conditions.

So, we return to Livage’s hope of a ‘chimie douce’ with morphogenetic capabilities, but the route will be long, as suggested by the state of the art related in some optimistic articles, which however cannot hide the distances separating the genuine shells or other elaborated biological materials from the products of some beautiful experiments [38,33].

Many good ideas were debated by people for centuries, but did not survive simply, because various wrong conceptions could provide easy answers to innumerable questions, and spread among people and even scholars, by a kind of natural selection. Examples of such convenient beliefs were reported in a book by Metzger [34], dealing with the intellectual context at the birth of crystallography [35]. For instance, in the 17th century and later, water was often told to turn into stone, as ‘proven’ by petrifying sources and stalactites or stalagmites in caves, so that ice was a solid state of water among others, as some rocks that also return to water, but less easily than ice... Stones were said to be alive and even to reproduce, despite evidence of the contrary, whilst it was admitted that certain rocks were products of life, which remains true in many cases, but this depends on the rock itself and on the precise meaning of the term product. Note that the interpretation of ancient texts is not easy and, for instance, the word ‘water’ often meant a solution or even any other liquid. The expression ‘mother water’ of a crystal is still in use, and applies to aqueous supersaturated solutions, which give rise to crystal germs and their growth. Today, this term is often replaced by that of ‘mother liquor’.

Ancient observations and ideas on biominerals were presented in general in books and articles about pearls [12,51]. Pliny the Elder wrote that pearls ‘formed around drops of rain entering the oyster’, what seems

to be pure poetry, but Pliny possibly believed also that water could turn into stone, so that the end of the story could be correct... In China, in the 13th century, pearls were really produced by inserting leaden images of the Buddha between the inner surface of the shell and the mantle of a mussel *Cristaria plicata*, and then waiting for some years. In the 18th century, Carl von Linné himself proposed a process to prepare artificial pearls. They were also generated by simple intrusion of sand grains or parasites, or by displaced ova. Remarkable works on pearls came from Japan.

3. Minerals are present in most groups of living beings

The first studies on biominerals were conducted by zoologists who described skeletons, either *external* like carapaces, shells, and concretions formed by invertebrates and protozoa, or *internal* like bones in vertebrates and spicules in the form of long needles or that of fenestrated stones in many invertebrates. The vertebrate teeth are first internal and grow outside to be functional. The involved minerals are poorly soluble salts as calcium phosphates, carbonates, and sulphates, or oxides as silica and magnetite.

Most animals were considered as machines producing sculptured stones, with an excellent efficiency, whereas plants were at the origin of woods and related fibrous materials. However, corals represented a major exception, since they were first classified in the plant kingdom by Tournefort, for instance, as recalled in [35]. They were later recognized as animals, but in a group named *Phytozoa*. Indeed, their polyps are beautiful as flowers and the term Anthozoa is still in use, but polyps are not flowers. Corals form a calcified skeleton, at the origin of considerable calcareous rocks in reefs. The first naturalists were not completely wrong about the plant nature of corals, because they contain innumerable xanthellae, which are unicellular algae, with a mass of organic matter superior to that of the animal tissues in which they are enclosed. Through their chloroplasts and photosynthesis, xanthellae afford nutrients used by the coral and conversely these symbionts are protected against predators. Xanthellae also contribute to the calcium metabolism and mineralisation (see Allemand, in this issue).

Some seaweed as *Lithothamniae*, a family of red algae, also produce hard calcareous concretions, the

maerl for instance, the incrusting plates of *Lithophyllum*, and the pretty corallines, in ponds between rocks at the sea-shore, but these very special seaweeds are undoubtedly plants. Terrestrial plants contain minerals in their tissues, for instance silica in many species, at low percentages, but concentrated here and there.

Amorphous silica forms hard deposits in spicules of most sponges and also in the skeleton of unicellular organisms as diatoms and radiolaria, often at the origin of rocks as diatomites and radiolarites (see [53] and Coradin in this issue). Some sponges form calcium carbonate spicules and there are considerable sediments due to protozoa, for instance the coccoliths, which form the chalk [60].

Diverse bacteria also produce inorganic precipitates and their nature depends on their anabolism. Stromatolites, for instance, have been present on diverse seashores since the earliest times of life, and superpose calcareous layers, produced by intercalated cyanobacteria, first confused with blue–green algae. Some bacteria produce a hard calcite patina at the surface of calcareous stones, the biocalcin, which is a natural protection against erosion [31,45]. Many oral bacteria form the dental plaque and concretions like the tartar. Other bacterial species are the main actors in the cycle of diverse salts and oxides in nature, in particular the remarkable magnetobacterias. Mineral deposits are often important at the periphery of bacteria and in their cytoplasm. High concentrations of calcium are found also in mitochondria and in the endoplasmic reticulum of eukaryotes.

4. Early recognized properties of mineralised tissues

At the Renaissance and later, important collections were created by zoologists, botanists and also in hospitals. Conservation methods in appropriate liquids were improved to prepare long-term samples in tight flasks. Shells and skeletons were washed and ordered in boxes or in specialized pieces of furniture. Comparative observations and dissections led to reasoned descriptions, drawings, and paintings. Among ideas from these past times, several still prevail.

4.1. Thick mineralised materials are produced by thin living sheets

Metres of calcareous deposits in the bulk of coral reefs are formed by a thin external layer of living

matter (some millimetres thick), associating polyps and tissues in between. We know today that ions in the sea, Ca^{2+} and HCO_3^- , enter the complex metabolism of this thin layer, and finally cross it to assemble into a calcareous mineral (Allemand in this issue). At a smaller scale, the periost also is a thin living layer producing compact bone.

4.2. Accretive growth and intussusceptive growth

Measurements of lengths, weights, and growth rates began mainly in the 18th century and also physiological investigations. Two distinct types of growth were considered by Buffon and contemporaries: *accretion* was observed in crystals and shells, and consisted in peripheral additions of new materials, with visible growth lines in many cases. A second type, called *intussusception*, corresponded to an intercalary growth, generally studied in soft tissues of plants and animals. In the 18th century, Hales delicately inscribed a series of equidistant marks on young plant shoots and, after twenty-four hours, he ‘found a gradual scale of unequal extensions, those parts extending most which were tenderest’. This citation is reproduced from D’Arcy Thompson [54] and offers a clear example of intussusception. Hales also implanted silver nails at definite distances in long bones of young animals, some ones in the median and narrow diaphysis, which is more compact, and the other ones in the epiphyses, at the wider extremities. Some years later, distances were conserved in the diaphysis, but had increased between nails lying in diaphysis and those in epiphyses, showing that bone development was complex, associating both types of growth.

4.3. Organic matrices

In the 19th century, considerable advances on biominerals came from collaborations with chemists, affording their new chemicals, often well purified, and their analytical methods. The organic matrix of bone, the ‘osseine’, was discovered by a simple demineralisation in solutions of hydrochloric acid, and this led also to recognize the mineral itself, a hydrated calcium phosphate. Shells, carapaces, spicules, and most mineralised tissues, pathological or not, presented an organic matrix.

5. Mineralised tissues observed at microscales

The technical advances in microscopy and microtomy in the 19th century led to the discovery of most cell types and their assembly in diverse tissues. An extraordinary set of techniques was invented to see the organization of living matter through series of thin sections, where the order was remarkably preserved. This was the origin of two associated disciplines, named histology and cytology, since they considered tissues and cells. Their methods were adapted to mineralised organs by devising procedures of demineralisation, which did not alter strongly the matrix and the cells themselves, as can do hydrochloric acid solutions, replaced by trichloroacetic acid for instance. Calcium chelators and acid salts were used later, as diluted ammonium citrate, preserving some enzymes and most proteins. The polarizing microscope was essential in the study of minerals. X-ray radiographs and diffraction appeared during the following century.

Some stains were recognized to bind to precise components, and specific chemical reactions were observed at the level of cellular details. These histochemistry and cytochemistry have now their immunological and molecular prolongations in light or in electron microscopy. All these techniques are long and difficult, but often remain the sole way to define biomaterials, their nature, structure, shape, localization in cells, tissues and organisms, and to ask clear questions, a prerequisite to any further studies in X-ray and electron diffraction, or to apply microanalytical methods.

The concept of epithelium was established in the 19th century, from microscopical studies, and corresponds to single or multilayered sheets of densely packed cells. These epithelia delimit compartments, with distinct physiological properties, and are able of considerable works, for instance building carapaces in crustaceans and shells in molluscs. The carapace of crabs, or ‘cuticle’, can be one or two millimetres thick and is secreted by a single layered epidermis, which can be forty times thinner [57]. The mollusc shells also present a variable thickness, often several millimetres, and are produced by a single-layered epidermis or ‘mantle’. These animals secrete these remarkable shields by capturing the appropriate materials and processing them through their metabolism.

It appeared that matrices and mineral concretions were absent from cytoplasm in epithelial cells at the

origin of shells in molluscs or cuticles in crustaceans, so that it was concluded that cell membranes separating these cells from their external productions represented an essential part of the machinery elaborating the matrices. In bones, the collagen molecules assemble into fibrils at the close periphery of cells, the osteoblasts, and mineralisation occurs later, at a distance from membranes. The matrix assembly always precedes mineralisation. The synthesized matrix proteins are first packaged and exported in the reticulum and in Golgi vesicles, separated by a membrane from hyaloplasm, and they assemble into the matrix outside of the cell. This was made clear also from studies on mineralised skeletons of Protozoa, coccoliths for instance by Pienaar [39,40] and Young et al. [60], or on other unicellular organisms with silica tests [53] and on spicules in *Pluteus* larvae of sea urchins [1].

6. Biominerals are highly structured composites

The demineralisation methods even by hydrochloric solutions do not alter the bone shape but, after some days and washings, the bone becomes flexible and is easily cut by a razor blade, so that histological sections can be prepared. The mineral salts were first considered to correspond to hydrated $\text{Ca}_3(\text{PO}_4)_2$, with a small amount of carbonates, but more precisely it is an hydroxyapatite (see Rey in this issue). This demineralised bone is organic, with some mineral traces. This organic matter was called ossein, its main component being collagen, a fibrous protein also present in connective tissues. Heating of ossein or connective tissues in water leads to the production of a well-known glue, which is gelatine, and corresponds to denatured collagen. Organic matrices are present in most mineralised tissues and intracellular stones or spicules, but the mass ratio is highly variable between mineral, organic matter, and water. This ratio is not easily estimated, because a part of the organic matter is not the matrix, but comes from diverse associated cells and tissues, as nerves, vessels, etc.

Important matrices were found in vertebrate bones, in mineralised carapaces of crustaceans and in shells of molluscs, but are often less developed in spicules. Fibrous proteins and polysaccharides form these matrices, mainly proteins with collagen in bones and mainly chitin, a polysaccharide, in crustacean carapa-

ces. In both cases, the fibrils form a highly structured matrix, with a plywood-like arrangement, the fibril direction differing in successive layers, either by an angle close to 90° , in compact bone, or by a series of small angles leading to a continuously twisted packing, found in cholesteric liquid crystals, but stabilized as in crab carapaces, for instance, by the assembly of chitin chains into fibrils (see [5–9] and Giraud-Guille in this issue). Many fibrous matrices in biominerals present geometrical characteristics close to those found in liquid crystals formed by long polymers. This is the case of apodemes in crustaceans, these inner extensions of carapace for the attachment of certain muscles; they are made of aligned fibrils of chitin and proteins, with symmetries of nematic liquid crystals. Matrices in sponge spicules show symmetries analogous to those found in smectic liquid crystals (Garrone, in [53]).

Composites are materials made of distinct parts, differing by their physical and chemical properties, these parts being distributed according to a definite geometry. This term was introduced by Maxwell in very general terms. There is the example of materials associating transparent media of different refractive indices, for instance, a stack of layers of refractive index n_1 intercalated alternatively between layers of index n_2 , or also a set of parallel cylinders of index n_1 , separated by a medium of index n_2 . Such optical systems exist in muscles and in some mineralised tissues; in that case, both components are birefringent [23].

Skeletal tissues are ‘fibre-reinforced composites’, a kind of system where ‘the whole is more than the sum of its parts’, a conception often discussed by Plato himself. Such composites are realized in bones, carapaces, and shells. Separately, the mineral or the fibrils do not show ‘useful’ mechanical properties, the mineral being made of brittle crystals and the polymers being supple, but the composite structure resists strong constraints, since crystal fractures stop where they meet fibrils, and these latter do not bend, because the inter-distances are fixed in the mineral.

7. Structures and textures in biominerals

Goniometry applied to crystals began on the 18th century, polarizing microscopy on the 19th century and X-ray diffraction on the 20th century, so that there are three phases in the history of crystallography and also

in the exploration of biominerals. Chapter 9 in D'Arcy Thompson's book is a reasoned presentation of most concretions and spicules in unicellular organisms and invertebrates [54]. A book was published by Schmidt in 1924 about polarizing microscopy applied to minerals in the animal kingdom [49]. The inorganic deposits in tissues are either amorphous or crystallized. An amorphous state is confirmed by the absence of sharp spots in X-ray or electron diffraction patterns, and also the absence of transmitted light between crossed polars. When crystallized, the unit cells and symmetries, mainly recognized by X-ray diffraction, might differ for the same component. Calcium carbonate, for instance, gives either the rhombohedral calcite, or the less stable orthorhombic aragonite and hexagonal vaterite. The chemical and crystalline polymorphisms of biominerals and their distribution through the living world gave rise to innumerable articles. Main facts were known from works due to Biedermann, Bütschli, Kelly, Lacroix, Meigen, Rose and Schmidt, and all these references were recalled and discussed by Prenant [43], who also studied the determinism of the various crystalline forms of carbonates and demonstrated the roles of pH, $[Ca^{2+}]$, $[Mg^{2+}]$, P , the partial pressure in CO_2 and C , the total concentration of inorganic carbon in the system [43]. His results were presented in a tentative phase diagram and graphs of the main mineral transformations.

The crystal lattice of a mineral is never perfect and there are defects. Textures correspond to the arrangement of domains of uniform crystalline orientation, often separated by walls, which are narrow zones associating numerous defects. Definite textures were observed in biominerals. They were studied in artificial concretions and in spicules by Prenant [41–44], or in crustacean cuticle by Dudich [18] and Drach [17]. The presence of organic molecules or that of a matrix might be essential in the determinism of complex geometries in textures. Giard [25] had observed the aragonite spicules of *Didemnidae* (Tunicates) and found their shapes similar to those of calcium carbonate concretions obtained in vitro, without cells, by Harting in 1840 and 1872. Drops of $CaCl_2$ and CO_3Na_2 solutions were simply added to a colloidal gel [29,30]. Prenant multiplied such experiments in gelosis, to illustrate these similarities between in vitro and in vivo concretions [41,42]. They were confirmed later by Monniot [36].

Helical defects were observed in the nacre of molluscs [52,58], in brachiopod shells [59] and in hy-

droxyapatite crystals of tooth enamel [11,50]. These screw dislocations are involved in crystal growth and are therefore a factor of biomineralisation but, in the teeth, they represent an easy access for bacteria involved in caries [2–4]. Defects are present in the carapace matrix, as we have shown [6,8], and in calcite crystals of crabs ([27] and Giraud-Guille in this issue). The two types of defects and the twist are supposed to facilitate the calcite growth.

8. Mineralisation programs and factors

Mineralisation follows a program of precise events in skeletal structures. The construction of a coccolith for instance begins by the assembly of a thin fibrous matrix in the form of an elliptical disc [39,40], and then, according to a strict chronology, calcite pieces are built with sharply defined shapes [60].

Similarly, during their moult, crabs first absorb seawater, so that the body swells, unfolding the new skin, and they can leave their ancient carapace [17]. Mineralisation begins in the outer part of this new cuticle, which is alkaline, as indicated by a pH indicator, the Nile blue, in *Carcinus maenas* for instance [15]. This region also is rich in alkaline phosphatase [56], an enzyme removing phosphate groups, which attach to calcite and are poisons of calcite growth [51]. Calcite forms horizontal discs often centred on bristle ducts across the cuticle, and their radial growth leads to a mosaic, just below the outer epicuticle. This mosaic is thickened by further precipitations and crystallizations (some micrometres), which concentrate later along vertical walls forming a honeycomb pattern, a trace of the arrangement of epidermal cells [57]. These interprismatic walls contain carbonic anhydrase, which facilitates the precipitation of carbonates and accelerate the calcite growth, as shown by Travis [56] and Giraud-Guille (see this issue). The inner part of prisms remains poorly mineralised, but is filled by glycoproteins inserting between matrix fibrils. During these first steps of mineralisation, new matrix layers are secreted below those deposited before the moult, and will be mineralised later. We stop here this brief description of the first part of a complex program, certainly written in the genes, somewhere...

Experiments by Digby [14–16] indicated that this program was easily perturbed. The NaCl concentration is higher in the crab body than in seawater, due to an

active uptake of NaCl at the gill level, so that ions are expected to diffuse out across the carapace. Since Na⁺ diffuses more rapidly than Cl⁻, this was a simple explanation of the observed alkalinity in the outer part of the cuticle. To test this idea, recently moulted crabs were placed in seawater added with more NaCl, to obtain a reversed salinity gradient across the cuticle, and then mineralisation began along the inner part of the cuticle, far from the sites of phosphatases and carbonic anhydrase. This did not contradict the involvement of phosphatases and carbonic anhydrase, but shows that, in normal conditions, different factors cooperate. In these normal conditions, a potential difference was observed between both sides of the cuticle, but was only the half of the Nernst potential predicted from the salinity difference between seawater and the crab inside. This difference was attributed to an electron flow through the matrix, supposed to be a semiconductor, due to quinone tanned proteins, mainly in the epicuticle and its close neighbouring [14]. Indeed, a cuticle piece placed in a solution containing metallic ions and submitted to appropriate electric conditions can be copper or silver-plated in its strongly tanned regions, which also are the most salient and form the first nucleation sites of calcite discs, in normal conditions.

9. Environment, physiology and mineralisation

This electrochemical theory of biomineralisation due to Digby requires some enzymes, the phenol-oxidases involved in protein tanning, which add their effects to those of alkaline phosphatases and carbonic anhydrases to build the calcitic texture. Digby extended his interpretation to mineralisation of mollusc shells [14] and to other systems. He showed that mineralisations are highly sensitive to external factors. As indicated above, salinity variations might change the positions of the first calcified sites in the matrix. More generally, biomineralisations are modified by many external factors. For instance, a pH of 6.5 in seawater or rearing in lime free seawater suffices to prevent spicule mineralisation, but not the matrix differentiation in sea-urchin larvae. The same effect was observed in Foraminifera, which are Protozoa differentiating a calcified shell in normal conditions – see references in [43] and [54] (pp. 648–649).

Spicule demineralisation was obtained in living Alcyonaria by adding 0.7 g l⁻¹ of calcium glycerol-

phosphate to seawater, at pH 7.6 and at 17 °C, in well-aerated conditions [55]. Glycerol was released at the colony surface and calcareous granulations appeared in both epidermis and endodermis. These *in vivo* spicule demineralisations could be due to a local decrease of pH in mesoglea, since phosphatases separate glycerol from phosphates and these latter transform into phosphoric acid, whereas the calcium is precipitated into carbonates in other tissues with xanthellae. This early work proposed new methods that remain very interesting today.

Environment conditions often modify shell growth rates, either at the matrix level, or in the mineral progression, or both. Growth lines are visible in mollusc shells and in some crustacean mineralisations (see Marin and Luquet in this issue). In certain cases, they form age rings, as in otoliths (see Herbolme and Payan in this issue). Their existence is often related to periodic variations of environment, observed daily or yearly or something else. In vertebrates also, aquatic or not, bone mineralisation depends on nutrition and environment. Vitamin D and A are essential to a normal mineralisation of bones, with small doses of ultraviolet light to ‘activate’ sterols. This is a great chapter in mineral physiology with numerous metabolic steps, from absorption [19,20] to matrix mineralisation, and also resorptions due to pregnancy or lactation, with the lactose involved in the skeleton thickening [22] and its protecting role demonstrated by Fournier in 1954 [21], all that under endocrine control. Bone resorptions also occur in birds, when eggs are produced in number with their mineralised shells (see Nys in this issue). These problems about mineral metabolism could provide ideas to conceive control mechanisms applied to an inorganic ‘chimie douce’, but much ‘simpler’ questions will be asked before. How can organisms be able to capture inorganic ions, so highly diluted in their direct environment? These ions are internalised through channels or pumps, and calcium concentrates in compartments as mitochondria, endoplasmic reticulum and matrix, rather than in the hyaloplasm. All that is coordinated, for part by the thin cytoplasmic projections in bone or cuticle, so that mineral deposition occurs at chosen sites in the matrix, but how to investigate this control exerted by cells and membranes, and how can it be programmed for all these mineral and matrix re-handlings?

10. Nucleation and growth of biominerals

Biominerals are supposed to be deposited directly in a solid state, amorphous or crystalline. Nucleation depends on the existence of interfaces provided mainly by matrices, with diverse structures and defects from place to place, and also epitaxial possibilities [24].

About growth, one has two extreme situations and intermediate ones. Let us first consider the case of well-developed matrices, presenting a uniform distribution, at least locally. Nucleation begins here and there, at points that can correspond to singular structures of the matrix, and then growth is often radial. The main example considered above and studied by Prenant [41] is that of aragonitic spicules that form without cell intervention in the tunica of Didemnidae. Another example is the radial growth of calcite in many crustacean carapaces, often starting from canals associated to bristles or secretory cells but, instead of being spherulitic, the textures are cylindrical, with *c* axes of calcite radiating within the cuticle plane, and generally not parallel to fibril direction in this plane. The growth front is not in direct contact with the epidermis, since matrix formation precedes its mineralisation by a long time, from days to months, depending on body size. In crustacean cuticles also, we showed that the mineralisation does not change the distances separating the matrix fibrils, so that the mineral occupies the preformed spaces created during matrix differentiation a long time before [7]. It appears also that growth rates can depend on the angle between fibrils and the progressing mineral interface.

The situation is radically different in sea urchins, with a skeleton made of large calcite monocrystals (or close to be), and a reduced matrix [48]. These monocrystals do not have the usual shapes of crystals limited by planar faces, but those of sculptured plates or spines, and they show complex perforations, visible at microscales [37]. Numerous cylindrical holes are present in the crystal and curve to join here and there, so that the crystalline matter itself is divided into cylinders, which curve and join similarly. Two compartments interpenetrate, one made of monocrystalline calcite, and the other one made of cytoplasm, with a single cell membrane to separate them. These monocrystalline plates, spines, or spicules are produced within cells, in large and multiconnected vesicles, or between cells, but also in close contact with cell membranes

forming such complex surfaces. These topological arrangements of bilayers are known in bicontinuous phases of certain liquid crystals. Similar systems of membranes were also observed at very different scales, when chloroplasts transform into prolamellar bodies, in etiolated plants. The geometry of such etioplasts [28] and that of perforations in sea-urchin spines [37] are similar, but the scales strongly differ.

Mineral growth in sea-urchin plates is accretive for these calcite monocrystals, as demonstrated by Märkel [34], and this leads to suppose a rather complex behaviour of cytoplasm and of its limiting membrane, at the periphery of the crystal, to create new spaces available for crystal growth. This involves a complex program of membrane retractions and recombinations to build these highly perforated shapes, whereas the cytoplasm is maintained in perforations of the previously deposited crystal.

Now between these extreme cases, there are intermediate ones. In nacreous layers of molluscs, there are five to seven monocrystalline domains in each nacre plate, and the matrix seems almost absent from them, whereas each plate is completely surrounded by a dense matrix [48].

In bones, mineralisation begins within collagen fibrils, at a distance from osteoblasts, a situation similar to that of crustacean cuticles, but radial growth is absent and there are signs of epitaxy, the first apatite deposit nucleating within the gap zones of collagen fibrils. Later, the free spaces separating fibrils are filled by the mineral, apparently without changing the pre-existent inter-distances between fibrils, as in crustacean cuticle.

The matrix is not much developed in teeth, in the enamel mainly, but also in the dentin, or in the osseous rostrum of certain whales (see Goldberg and Zylberg in this issue). These tissues with large crystals and reduced matrix are very hard, but fragile. Usual pathologies as caries develop in enamel and dentin and can be stopped at best, but do not repair themselves, in contrast with other tissues, where wound healing is a natural process, and even in bones, fracture reparations are not excluded.

11. Liquid crystals and biominerals

We stressed some distinctive characters between non-biological minerals and skeletal structures (in the

first §). Minerals are often limited by planes and straight lines, whereas curved surfaces and lines are found in skeletal shapes. Similar differences separate true crystals from liquid crystals, which also show regularly curved lines and surfaces. Accretive and intussusceptive growth also separates true crystals from liquid crystals, since diffusion is a corollary of fluidity in liquid crystals. It was recalled also (in § 6) that fibrous matrices of mineralised organs presented the symmetries and other geometrical characteristics of liquid crystals, without being liquid, but their assembly was certainly related to that of liquid crystals. This particular state of matter seems today very essential in the study of biomineralisations.

11.1. Membranes are liquid crystalline

Important mineral deposits are produced by thin layers, and mainly by cell membranes themselves (as indicated in § 4 and 5). It is worth remembering that membranes are fluid and anisotropic, due to the parallel orientation of their components within this bilayer, phospholipids, and proteins mainly. This corresponds to the definition of liquid crystals, which are anisotropic liquids and most biominerals are precipitated in the close vicinity of membranes, in Golgi vesicles or at the periphery of cells or along their narrow projections within carapaces or bones. The liquid crystallinity of biological membranes confers remarkable morphogenetic capacities, from nanoscales to microscales and much larger ones when cells are associated in tissues. One knows also that long-range order arises spontaneously in liquid crystals.

11.2. Matrices originate from liquid crystalline sols or gels

Membranes are involved in the production of most macromolecules of the extracellular matrix. For instance, chitin synthetases are present in membranes that produce chitin in extracellular matrices, in mycelia and in arthropod carapaces, for example. Collagen also is produced as other proteins in the endoplasmic reticulum, at the contact point between a ribosome and the reticulum membrane. Glycosylation and triple-helix formation occur later in the Golgi and secretion follows. After cleavage of terminal peptides, collagen molecules assemble into fibrils and form highly orde-

red matrices, which are stabilized analogues of liquid crystals (as recalled in § 7). A brief passage through a liquid-crystalline phase is not excluded in the first steps of secretion, but the assembling matrix is rapidly gelled by the differentiation of fibrils [9]). Cholesteric phases of sonicated collagen were regularly obtained *in vitro* by Giraud-Guille [26,27] and this self-assembly was observed in cell-free conditions. On the contrary, *in situ*, the cells are expected to influence the orientations of the assembled fibrils, because membranes are associated to a subjacent cytoskeleton, actin filaments mainly, and can exert forces on a self-assembling matrix, slightly gelled, via certain molecules as integrins and other ones (rather numerous). Note also that membranes themselves can be more or less stabilized here and there, so that mechanical actions of cells on self-assembling matrices are highly plausible.

11.3. A fluid toolbox

Our idea is that liquid crystals could be an essential intermediate state of matter, well devised to accommodate things between the liquid state present in hyaloplasm, say the cytoplasm lying between organelles, and the stabilized state of matrices and even the solid state of minerals in the extrahyaloplasmic compartments, as Golgi vesicles for instance, or in the extracellular spaces. Liquid crystals could be the key of our paradox of the sculptor and the mollusc. The hard tools or the high temperatures used by the sculptor could be replaced by a very special machinery made of a liquid crystal at physiological temperatures, with inserted macromolecules considered as tools in their toolbox. The tools themselves are diverse macromolecules (or macromolecular systems) as receptors, ionic channels pumps, attached ribosomes, or enzymes involved in the production of matrix polymers for instance, or those essential to mineralisation. Most tools are designed to remain attached to their fluid toolbox, since they present amphiphilic properties and are not lost in general, because the liquid crystal is well separated from its aqueous environment, as can be membranes in cells and tissues. Note that certain enzymes are known to be attached to membranes, but are found also in the hyaloplasm (cytosol or cytogel), or in the extracellular matrix, and this can be the case of carbonic anhydrases or alkaline phosphatases, as indicated in § 8. This indicates that some tools, these enzymes, could be

released from the fluid toolbox at definite positions in the matrix, possibly with the help of cytoplasmic projections in pore canals, as in the carapace of crabs.

11.4. Some steps towards a 'chimie douce', the new challenge

Materials are said to be hybrid when they associate inorganic and organic parts, which strongly interpenetrate at nanoscales, both parts being linked by diverse bond types, covalent, ionic or van der Waals [46,47]. Recent advances in their synthesis came from the sol-gel process, leading to materials closely related to certain biominerals. For instance, metal-organic alkoxides were hydrolysed in the presence of organic molecules that are able to produce liquid-crystalline phases with water. The main example was that of $\text{Si}(\text{O}-\text{C}_2\text{H}_5)_4$ or TEOS, or tetraethoxysilane, which was added to a mixture of a cationic surfactant, $\text{CH}_3(\text{CH}_2)_{15}\text{N}^+(\text{CH}_3)_3$, (or CTACl, or cetyltrimethylammonium chloride) with water and HCl. For certain precise proportions of these components, the hydrolysis leads to the formation of amorphous silica deposited between the hexagonally packed cylindrical micelles of CTACl, which serve as template. The silica forms a honeycomb pattern within the liquid crystalline structure, and this was the prototype of a new type of materials called mesopores. A large variety was created, using aluminophosphates or other minerals in presence of templating organic components [33,38] (see also Coradin in this issue). This is an important advance towards systems resembling skeletal structures, but I observed that important differences remain, for instance, the fact that micelles are replaced in skeletal structures by cytoplasmic channels limited by membranes, which show similar shapes to those of cylindrical micelles, but are much larger [10].

Another domain of research is essential, that of inorganic liquid crystals discovered by Zocher in 1925 (see [61]) and revisited by Davidson et al. [13]. The hope now would be to create mineral amphiphilic molecules showing a polymorphism in structures and textures, comparable to that of organic liquid crystals.

12. Biomineralisations as model systems for molecular genetics

Techniques in molecular genetics were essential in morphogenetic studies and research on *Drosophila*

was extensive in this domain, leading to the discovery of many genes involved in body segmentation for instance. The morphology is highly complex, but precise, with some recognizable bristles in the adult, which can be given a name, as are the teeth in mammals or fingers, for instance. The distribution of bristles at the surface of *Drosophila* integument form remarkable patterns, with few individual variations at the same instar, and this suggests an elaborate subjacent geometry. Similar morphologies exist in crustaceans and most about growth and form in insects and in crustaceans is likely to have common origins at the genetic level. Minerals also form defined patterns in crustacean carapaces, less elaborated, but the involved enzymes are known, their positions also in the carapace architecture, as the main steps of the process, with several characteristics of the matrix that we recall:

- (1) defined fibril diameters and inter-distances in the organic matrix;
- (2) presence of phenoloxidasases in the epicuticle and in the twisted layers, just below the epicuticle;
- (3) very strong twist just below the epicuticle, creating screw dislocations;
- (4) presence of alkaline phosphatases just below the epicuticle;
- (5) presence of carbonic anhydrase along interprismatic walls.

These five characters are expressed according to a program defined in the matrix space and in the time of the inter-moult cycle. Points 1 to 5 do not exactly correspond to a chronology. Character 1 is expressed all along the secretion of the fibrous matrix and its expression is modulated according to the level in the cuticle. Character 2 is expressed during secretion of the epicuticle and begins a short time before 1, but stops much before the end of 1. Characters 3 and 4 are expressed at the beginning of the expression of character 1 and character 5 comes after 2, 3 and 4, but stops much before 1. The precise knowledge of the involved molecules and of these steps could provide the best access to a correct interpretation of genetic results.

Mollusc shells also present a subjacent geometry, with a system of coordinates made of intersecting growth lines and conical logarithmic spirals, both visible on the outer periostracum, with its characteristic plywood-like structure. These sharply defined architectures are continued below within the prismatic layer and the nacre, described in clear crystallographic

terms. The progressive assembly of these mineralised composites is known for its main aspects and some of the involved proteins and genes are studied today. Similar spatio-temporal programs of mineralisation are expected in most skeletal tissues, as illustrated in this issue by a promising series of results.

There are clear reasons that make biomineralisations very attractive today to geneticists and this can be understood with an example. Great therapeutic perspectives appeared twenty years ago with the progress of molecular techniques. Results accumulate now with large collections of genes, proteins, transcription factors, and some indisputable successes, but not so numerous, and one generally waits for applications. The origin of these difficulties is rather simple: pathologies often lead to slowly destructive effects that remain diffuse, not clearly visible and latent, so that there are neither sharply defined geometries, nor programs. To really grasp the force of these new methods in genetics, we need to apply them to model systems, as morphogenesis, but possibly less complex than the splendid patterns observed in adults of *Drosophila*. Biomineralisations could be the right model systems.

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