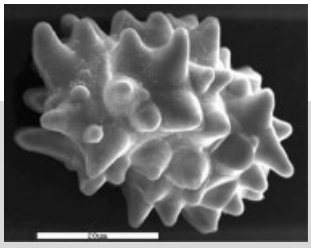
Taking Advantage of Disorder: Amorphous Calcium Carbonate and Its Roles in Biomineralization**

By *Lia Addadi*,* *Sefi Raz*,* and *Steve Weiner**



Amorphous calcium carbonate (ACC) in its pure form is highly unstable, yet some organisms produce stable ACC, and cases are known in which ACC functions as a transient precursor of more stable crystalline aragonite or calcite. Studies of biogenic ACC show that there are significant structural differences, including the observation that the stable forms are hydrated whereas the transient forms are not. The many different ways in which ACC can be formed in vitro shed light on the possible mechanisms involved in stabilization, destabilization, and transformation of ACC into crystalline forms of calcium carbonate. We show here that ACC is a fascinating form of calcium carbonate that may well be of much interest to materials science and biomineralization.

1. Introduction

Many mineralized tissues fulfill structural functions. The vast majority of these, such as bones, teeth, and shells, utilize crystalline minerals to stiffen and strengthen the tissue. The crystals often have unique shapes and are arranged in ordered arrays. Crystalline biogenic minerals are also used for other functions, including gravity sensing and navigation in the earth's magnetic field,[1,2] where organisms exploit the hardness and density of the crystalline mineral phases to their advantage. Organisms that produce mineralized skeletons in particular, may suffer from the anisotropic mechanical properties of the crystals, as these make them more vulnerable in certain directions. Crystalline minerals have a strong tendency to adopt specific shapes that reflect the molecular interactions and the symmetry within their structure. Thus molding these crystals into shapes more beneficial to their biological functions is not trivial. An alternative to using crystalline materials would be to use an amorphous mineral, as it is isotropic and can thus sustain mechanical challenges from all directions, and may be shaped more easily by the space in which it forms. Amorphous minerals, however, are less stable and hence more soluble than crystalline ones.

The amorphous mineral silica (opal) is widely used by many plants and animals for structural purposes.[3] Interestingly, most of the silica skeletons are formed by small single-celled organisms (mainly diatoms and radiolarians), or are present as small bodies distributed within larger multicellular tissues (e.g., sponge spicules and plant phytoliths). Thus, being relatively small, they are much stronger,[4] despite their glass-like properties. There are examples of organisms, particularly among the animals, that form relatively large structural tissues reinforced with amorphous minerals. Such minerals include silica,[5] amorphous calcium carbonate (ACC),[6] and amorphous calcium phosphate bearing tissues.[7] See Simkiss[8] for a review of biogenic amorphous minerals and Weiner et al.[9] for a review of ACC.

Table 1 shows the known distribution of ACC formed biologically. In only one major taxon, the crustaceans (crabs, lobsters etc.), is amorphous calcium carbonate widely used for structural purposes, namely to stiffen the exoskeletal cuticle.[6,10] In this case, it is conceivable that the main reason is not its isotropic mechanical properties, but rather its high solubility, as the mineral needs to be dissolved periodically during the molting phase.[11] We could thus conclude from the limited known distribution of ACC and other amorphous minerals (besides silica) among different taxonomic groups that although interesting in themselves, they do not fulfill important basic functions in biomineralization.

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Here, we will examine the possibility that this conclusion is incorrect, at least with regard to ACC. ACC may well be more widely distributed, but because it dissolves easily and is difficult to detect when associated with a crystalline form of calcium carbonate, its presence may well have been overlooked in many mineralized tissues. Furthermore, there is the possibility that ACC has an important basic function in many calcium carbonate formation processes as a transient precursor phase of calcite or aragonite. Although this review focuses on ACC, the same possibility may exist for other biologically produced minerals. This includes amorphous calcium phosphate, which may function as a precursor phase for crystalline carbonated apatite.

2. Biologically Produced Amorphous Calcium Carbonate (ACC)

ACC is one of six known forms of calcium carbonate, excluding high-temperature and -pressure forms. The other five are crystalline, and some contain water molecules as part of their lattice structure.[12] Calcite and aragonite are by far the most widely produced forms of calcium carbonate in biology.[1] Vaterite and monohydrocalcite, although less stable, are formed by a limited number of organisms. Calcium carbonate hexahydrate, also known by its geological name ikaite, along with the other two high-temperature forms, is not as yet known to be formed biologically. ACC is the only form of cal-

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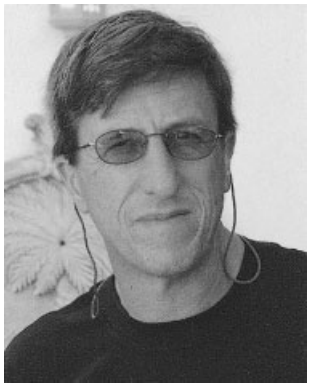

Prof. Steve Weiner studied chemistry and geology at the University of Cape Town and the Hebrew University of Jerusalem, and then obtained a Ph. D. at Caltech in the field of biomineralization. In 1977 he joined the Weizmann Institute of Science where he continued research on biomineralization. In the early 1980s the collaboration with Lia Addadi began and in the late 1980s he began an additional research project in the field of archaeological science.

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Table 1. The known distribution of biogenic ACC.

Kingdom/ Phylum	Form of deposit and references	Presumed function	Mg/Mg+Ca [a] [atom-%]	P/Mg+Ca [a] [atom-%]	All forms of $CaCO_3$ deposited by these organisms [b]
Plantae	Cystoliths in leaves [21,71,72]	?	0–26		Calcite, aragonite, vaterite, ACC
Arthropoda/ Crustacea	Cuticle [10,21,73,74]	Stiffens exocuticle	0–20	5-49	**ACC**, Calcite
	Gastroliths [74]	Temporary storage			**ACC**
	Storage structure [68]	Temporary storage	–	6–14	**ACC**
Porifera	Spicule core [13,18]	?	6–17		**Calcite**, ACC
Ascidiacea	Spicule (body) [19,75]	Stiffens internal tissues and tunic	0–16	45–55	Calcite, aragonite, vaterite, ACC, monohydrocalcite
Echinodermata	Larval spicule [14]	Precursor phase	5		**Calcite**, ACC
Cnidaria/ Gorgonacea	Spicules [76]	Mechanical support	18	12–13	**Calcite**, aragonite, ACC
Mollusca/ Bivalvia	Granules [77]	Temporary storage	?		**ACC**, vaterite
Bivalvia	Larval shell [15]	Precursor phase	?		Aragonite, ACC
Gastropoda (Nudibranchia)	Spicules [78]	Stiffens tissue?			ACC
Platyhelminthes	Corpuscles [79]	?			ACC

[a] The atomic percentages of the magnesium and phosphorous were normalized according to the total cations in the sample. This normalization was mainly performed due to lack of information on light elements such as carbon in various analytical techniques (e.g., energy-dispersive X-ray spectroscopy). Data from the literature was therefore treated in a similar manner even when additional information was available to allow comparison between the different organisms. [b] The most commonly formed calcium carbonate deposits are shown in bold.

cium carbonate that is isotropic in polarized light and does not diffract X-rays; hence it is described as being amorphous. We will, however, review data showing that this is a misleading term, and that this phase does indeed have short-range order that surprisingly varies between organisms and is, hence, presumably under genetic control.

Biogenic ACC was first identified at the beginning of the twentieth century when investigators noted that certain calcium carbonate deposits were isotropic when observed between crossed nichols using polarized light.[13] It was later shown that this phase does not diffract X-rays.[6] Despite more than a hundred years of investigations, the distribution of ACC in biology is still thought to be rather limited, except in one taxon, the *Crustacea*, where it is widely distributed (Table 1). Figure 1 shows scanning electron microscopy (SEM) images of some examples of stable biogenic ACC. Table 1 also lists the proposed functions of ACC, which are mainly as temporary storage deposits in various vesicles and other tissues (presumably because it is so soluble), and less obvious, as a stiffener for certain exoskeletons. In 1997, Beniash et al.[14] discovered that ACC functions as a transient precursor phase prior to the formation of the calcitic spicules of mature sea urchin larvae. More recently Weiss et al.[15] showed a similar function for ACC in the larvae of molluscan bivalves, except in this case it transforms into aragonite. Note that these are by no means the first-known examples of biogenic minerals being formed via transient precursors. Lowenstam and Weiner[1] list eight examples, including the formation of ferrihydrite as a precursor phase for magnetite,[16] and amorphous calcium phosphate as a precursor phase for carbonated apatite.[17]

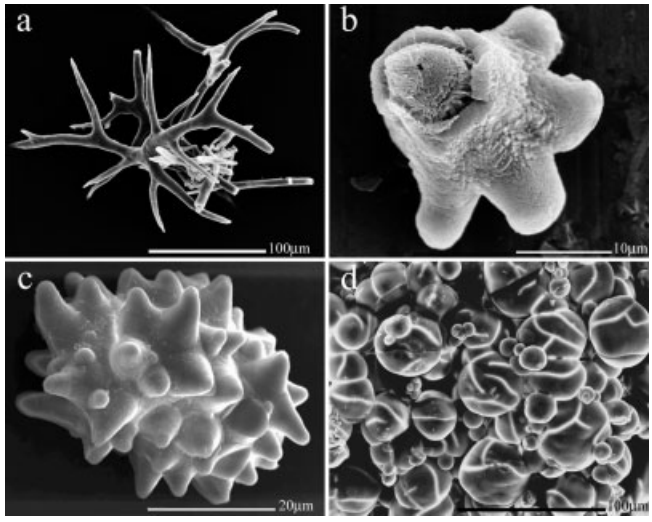

Fig. 1. SEM images of various skeletal parts composed of stable ACC: a) Body spicules from *Pyura pachydermatina*. b) Cross-section of a broken tunic spicule from *Pyura pachydermatina*. c) Cystolith from the leaves of *Ficus microcarpa*. d) Granule from storage structure of *Orchestia cavimana*. *Pyura pachydermatina* is a marine ascidian (Chordata). The body spicules are located in the branchial sac, while the tunic spicules are located in the thick layer (tunic), which envelops the body [66]. The body spicules, also called "antler spicules" because of their characteristic shape, are composed entirely of stable ACC. The tunic ("dogbone") spicules are composed of an external calcitic layer, separated by a membrane from an internal ACC core [19]. Cystoliths are irregular-shaped objects, a few tens of micrometers in length, which are found in the epidermis of leaves from plants of various families [69]. They are composed entirely of ACC, which is stable in vivo but crystallizes if extracted from the leaf in a humid environment. The terrestrial crustacean *Orchestia cavimana* temporarily stores calcium in its midgut during the moult stage. The calcium originates mainly from its old cuticle [67]. The calcium is stored in concretions composed entirely of stable ACC. The higher solubility of ACC makes it more easily available for mineralization of the new cuticle [68]. Note that Aizenberg et al. [20] published a similar photograph to that in (b).

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Below, we discuss the compositional and structural properties of biogenic ACC, and then assess the results of in-vitro studies that provide insights into how ACC forms and is stabilized. We will also review in some detail the two cases in which it functions as a precursor phase, and discuss what we think may be far-reaching consequences of these observations in terms of a more general strategy for calcium carbonate biomineralization processes.

2.1. Stable Biogenic ACC

A major distinction that can be made between different forms of biologically produced ACC is whether or not they are stable or transform with time into a crystalline form. The ACC deposits that function as mechanical stiffeners are stable, and even those that function as temporary storage sites, are stable until they dissolve. Two examples are known in which spicules comprise stable ACC side by side with a crystalline calcium carbonate form[18–20] (e.g., Fig. 1b). The two phases however are not mixed, but are located in distinct regions within the spicule, delimited by a membrane.

We have also noted that most biogenic ACC deposits that are stable in vivo, are also stable when extracted from the organism. This however is not always the case. For example the cystoliths from the leaves of *Ficus* sp. (Fig. 1c) rapidly convert to calcite once extracted in an aqueous environment.[21] It is thus of much interest to understand structurally what differentiates the various stable forms from each other, and from the transient precursor forms.

Limited information is available on the properties of most biogenic ACC phases, besides the fact that they are isotropic between crossed nichols. Chemical analyses of their mineral compositions reveal that most biogenic ACC deposits analyzed contain considerable quantities of Mg and/or phosphorous (Table 1). This may well imply that one or both of these components are involved in the formation and/or stabilization of this phase. In the cases that have been investigated spectroscopically in depth (see figure legends), ACC is always the major phase. It is not clear in which form the phosphorous is present. There is no spectroscopic evidence that it is in the form of a solid solution of amorphous calcium phosphate in ACC, namely as a homogeneous dispersion of phosphate ions substituting for carbonate ions in the bulk phase. Some of it may be in the form of a separate phase of amorphous calcium phosphate (see Fig. 2, legend).

Four forms of stable biogenic ACC have been studied using thermogravimetric and differential thermal analyses (TGA and DTA, respectively). All four stable forms have more or less the same stoichiometry, namely one mole of water per mole of calcium carbonate.[21]

The infrared spectra of all the stable ACC phases that we have studied are basically the same (Fig. 2). A most interesting feature of these spectra is that there are two main carbonate absorptions around 1450 cm^{-1}, as compared to a single

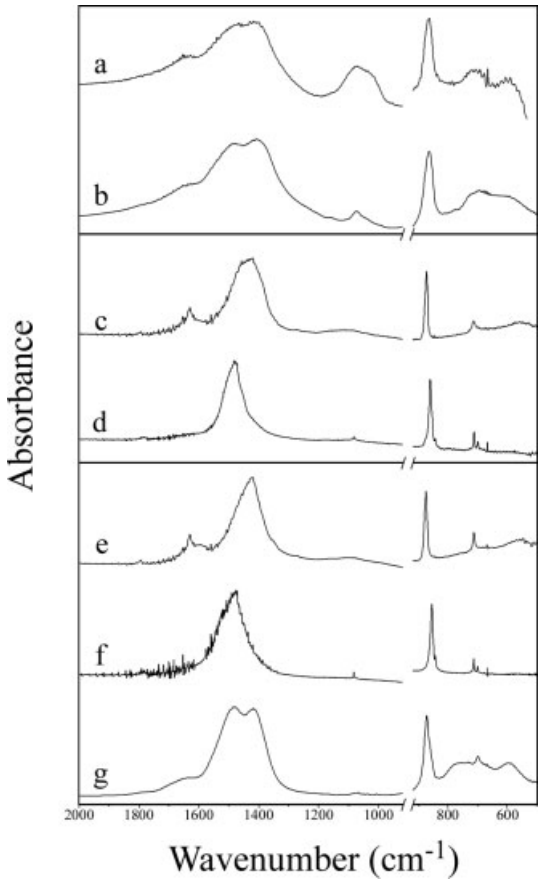

Fig. 2. Infrared spectra of: stable biogenic ACC from: a) *Pyura pachydermatina* body spicules; b) cystoliths extracted from *Ficus microcarpa.* Transient biogenic ACC from; c) spicules of *Strongylocentrotus purpuratus* extracted after 48 h in culture; d) six days old larval shell of *Mercenaria mercenaria*; Synthetic crystalline $CaCO_3$; e) calcite; f) aragonite; g) monohydrocalcite. In the region of 920–500 cm^{-1}, the intensities were normalized to the height of the ν_2 peak (866 cm^{-1} in ACC, 875 cm^{-1} in calcite and monohydrocalcite, 856 cm^{-1} in aragonite). The infrared spectra of the stable amorphous calcium carbonates are characterized by a split peak of the asymmetric stretch of the carbonate ion at 1420 and 1474 cm^{-1} (ν_3). This is indicative of a lack of symmetry in the environment of the carbonate ions. The lack of symmetry is also expressed by the presence of a broad peak at 1080 cm^{-1}, attributed to symmetric stretch (ν_1) in non-centrosymmetric structures [69]. The carbonate out-of-plane bending absorption at 866 cm^{-1} (ν_2) is broadened, compared to the crystalline forms. The in-plane bending at 713 cm^{-1} (ν_4) is so extensively broadened that it no longer appears as a discrete peak. The stable ACC spectra from different sources (e.g., (a,b)) are essentially identical in the region of the carbonate peaks. They differ mainly in the additional phosphate peak (1070 cm^{-1}) in samples containing phosphorous (e.g., *Pyura pachydermatina,* (a)). These peaks may indicate the presence of a relatively small amount of a calcium phosphate phase. The ACC portions of the spectra are most similar to monohydrocalcite, which has the same stoichiometry. In the latter structure the carbonate ions are also positioned at low symmetry sites (Fig. 5c). In the spectra of skeletal parts composed of transient ACC (c,d), all the peaks are sharper than the corresponding peaks in stable ACC (a,b), implying that the environment of the carbonate ions is more ordered. In sea urchin larval spicules, composed of transient ACC and calcite, the ratio between the maximum intensity of the peaks at 875 and 713 cm^{-1} increases proportionally to the percentage of ACC in the sample (compare (c) with (e)) [14]. In contrast, in larval mollusk shells, composed of transient ACC and aragonite, the ratio between the maximum intensity of the peaks at 856 and 713 cm^{-1} is substantially lower than in non-biogenic aragonite [15]. The values of these ratios depend on the relative broadening of the specific vibrations, and are clearly related to the amount of order around the carbonate. The extent to which each vibrational mode is affected is probably structure dependent as the environments of the carbonate in calcite and aragonite are different.

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absorption for calcite and aragonite. Monohydrocalcite and vaterite also have split peaks. The splitting is thus related to the asymmetry around the carbonates. The fact that this infrared absorption is split in ACC indicates that despite the "amorphous" term, these ACC stable phases do have some short-range atomic order. Stable ACC also has an absorption peak at 866 cm^{-1}, which is broader than the equivalent peak of the crystalline polymorphs (Fig. 2a). Stable ACC furthermore has a very broad and weak absorption at around 710 cm^{-1}, in contrast to calcite and aragonite that both have sharp peaks at 713 cm^{-1}. Thus the absence of a peak at 713 cm^{-1} indicates that well ordered calcite and/or aragonite are not present, but only ACC. This latter possibility can be discerned by measuring the ratio of the 875/713 peaks in the case of calcite[14] or 856/713 in the case of aragonite[15] (Fig. 2). It is interesting to note that the trends observed in these ratios for increasing proportions of calcite and aragonite relative to ACC, are opposite. We do not understand the reason for this. For more details, see the legend of Figure 2.

The Raman spectra of stable biogenic ACC phases are also similar to each other (Fig. 3). They essentially have only one major rather broad peak at around 1085 cm^{-1}, and usually a broad featureless hump around a 150–300 cm^{-1}. This is in contrast to the crystalline forms of calcium carbonate (biogenic or abiogenic) that all have a series of relatively sharp peaks. For more details, see the legend of Figure 3.

Perhaps the most powerful tool for probing structural variations of materials that do not diffract X-rays is extended X-ray absorption fine structure (EXAFS) analysis, as it provides information on the local short-range order around a selected atom.[22] This information relates to the number of atoms in each coordination sphere around a specific atom (calcium in our case), their symmetry and the distances from the calcium. The first EXAFS study of ACC was performed by Taylor et al.[23] on the ACC from cystoliths produced in the leaves of a *Ficus* tree. They studied the calcium edge and showed that there is indeed short-range order around the calcium ions both in terms of the number of coordinating atoms in the first and second shells, and their average distances from the calcium ions. Several additional studies reveal a fascinating picture. Comparative EXAFS studies of the first calcium coordination shells in the ACC from the body spicules of an ascidian (Fig. 1a), the cystoliths of another species of *Ficus* (Fig. 1c), and the cuticle of a lobster[21,24] show that they are all different. The differences are mainly, but not only in the number of calcium coordinating atoms present in the first, second, and third shells (Table 2, Fig. 4). The EXAFS spectral analysis involves comparing the measured data to known structures of related compounds. In this case all three stable ACC phases analyzed were most similar to monohydrocalcite, which also has the same stoichiometry (for more details, see the legends of Figs. 4,5). The observed differences do hint at why the cystoliths are so unstable once extracted from the leaves, whereas the other two phases are stable once extracted from the animals. There is more order in the ions around the calcium in the cystoliths as compared to the other two phases. These ordered centers can thus more

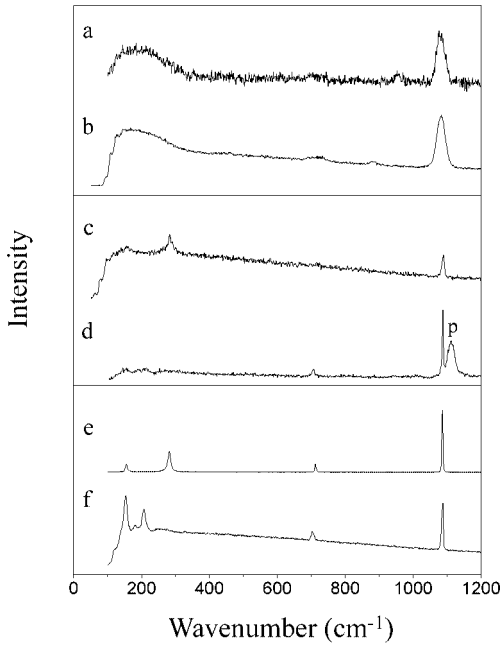

Fig. 3. Raman spectra of: stable biogenic ACC from: a) *Pyura pachydermatina* body spicules; b) cystoliths extracted from the leaves of *Ficus microcarpa.* Transient biogenic ACC from: c) spicules of *Strongylocentrotus purpuratus* extracted after 48 h in culture; d) nine days old larval shell of *Mercenaria mercenaria* (P = periostracum); Synthetic crystalline $CaCO_3$: e) calcite; f) aragonite. The Raman spectra of stable ACC ((a) and (b)) are characterized by a broad peak in the lattice frequency region 150–300 cm^{-1}, that occasionally appears as a continuous baseline rise rather than a discrete peak. The carbonate symmetric stretching ν_1 peak at approximately 1085 cm^{-1} of stable ACC is also substantially broadened, relative to the crystalline counterparts (e and f). The spectra in (a) and (b) are practically identical, although the spicules in (a) were found to contain large amounts of phosphorous atoms. The Raman spectra of calcium phosphates are dominated by a very strong band around 960 cm^{-1} [70]. A broad, weak peak is barely detectable in (a) around this frequency. In agreement with the IR data, this may indicate the presence of small amounts of an amorphous calcium phosphate phase. In the spectra of skeletal parts composed of transient ACC (c and d), all the peaks are sharper than the corresponding peaks in stable ACC, implying that the environment of the carbonate ions is more ordered. In sea urchin larval spicules [33] composed of transient ACC and calcite, the ν_1 peak is broadened, and the intensity of all peaks decreases substantially. In larval mollusk shells, composed of transient ACC and aragonite, the ν_1 peak is as sharp as in non-biogenic aragonite, but the peaks in the lattice mode region disappear almost completely [15].

Table 2. The number of atoms (coordination number (Coord.) and their distances (d [Å]) from the calcium ion in the first, second, and third shells in three stable biogenic ACC phases as determined by EXAFS analysis [24,21].

Source of ACC	First shell Ca–O		Second shell Ca–C		Third Shell Ca–Ca		Best model compound structures
	Coord.	d	Coord.	d	Coord.	d	
Lobster carapace	2	2.23	4	3.47	2	3.79	Monohydrocalcite
	4	2.41					
Plant cystoliths	3	2.22	2	3.46	4	3.79	Monohydrocalcite
	3	2.42					
Ascidian spicule	7.4	2.37	1.5	3.03	—	—	Monohydrocalcite
			3	3.36			

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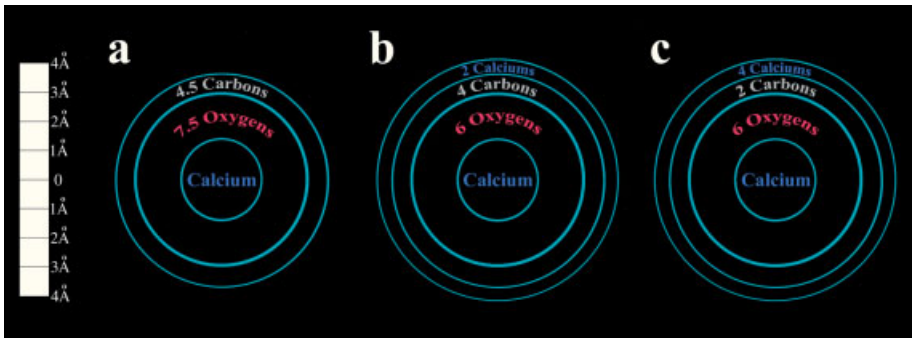

Fig. 4. Schematic representation of the information obtained from EXAFS measurements (Table 2) of: a) *Pyura pachydermatina* body spicules; b) Cuticle of the lobster *Homarus americanus*; c) Cystoliths from the leaves of *Ficus microcarpa*. The blue circles represent the coordination shells, within which the number of atoms in the shell is reported. The accurate distances of the different atoms are listed in Table 2. Average numbers of atoms in each shell and their distances from calcium can be accurately derived, and their distributions are clearly different in the three ACC phases. Actual structural models similar to those in Figure 5 cannot, however, be drawn, because EXAFS analysis does not contain vectorial information, and a range of different local organizations may correspond to the same average distances. The resolution of the data does not allow an accurate analysis beyond 4 Å, even when the structure is ordered over larger distances. The fact that the EXAFS spectrum of *Pyura pachydermatina* body spicules does not provide information beyond the carbon atom shell, indicates that this structure is more disordered than the other two. Note that the analysis of the data is not compatible with other ions, besides carbonate, being present around the calcium ions in substantial amounts. This is because quality of the fitted models would be adversely affected by not taking into account contributions of additional anions, such as phosphate, if they are present in concentrations larger than 10–15 %.

easily nucleate the crystalline phase. Hasse et al.[25] studied the larval shells of the freshwater snail *Biomphalaria* using EXAFS analysis. They showed that the shells are composed of ACC and their short-range order is more similar to aragonite—an intriguing observation.

These observed differences support the notion that ACC is structurally not one mineral phase, but a family of related phases. The differences are determined by the organism producing the phase, and hence are genetically controlled. How this is achieved is not known, but will also be discussed below. The short-range order differences may well be the key to understanding the varying stabilities within the ACC family. The EXAFS results also emphasize the inappropriateness of the term "amorphous" for describing these materials.

It is interesting to note that biogenic amorphous calcium phosphate (ACP) may also be a family of different materials. Lowenstam[7] noted that when stable ACP phases were heated to 500 °C, each transformed into a specific form of crystalline calcium phosphate, and that this was always genus specific.

In general little is known about the macromolecules associated with biogenic ACC. In the crustacean cuticle where stable ACC and/or calcite are present, the major matrix component is α-chitin. It is associated with many proteins, some of which have been characterized.[26] We, however, do not know whether some of these proteins are related to the formation and/or stabilization of the ACC component. More informative are the proteins extracted from crustacean gastroliths, the temporary storage deposits around the intestinal tract, as these are composed entirely of ACC.[27] One of these proteins has been sequenced and the sequence contains characteristic repeat motifs that are rich in glutamic acid and glutamine.[28] Aizenberg et al.[18] extracted the assemblages of proteins from stable ACC phases formed by a sponge and an ascidian—two taxa at opposite ends of the animal phylogenetic tree.[29] They are both rich in glutamic acid and/or glutamine, based on amino acid compositions. Interestingly, both these spicules also have a calcitic crystalline layer separate

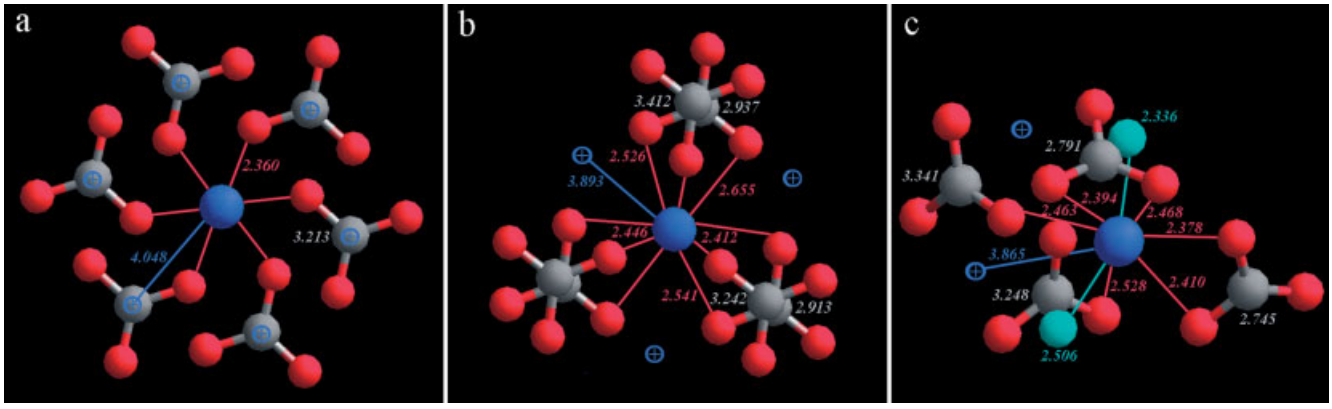

Fig. 5. Atomic structures of the coordination sphere (to approximately 4 Å) around the calcium ion of: a) calcite; b) aragonite; c) monohydrocalcite (Ca1). Ca^{++}: blue (the next neighbor Ca^{++} ions are marked by ⊕); O: red; C: gray; H_2O: cyan. The coordination of the first-shell oxygen atoms to the central calcium ions are marked in red and the distances are expressed in angstroms. The distances of the second-shell carbons are marked in grey, of water in cyan, and of third-shell calcium in blue. Identical distances are shown only once. In (b), the three Ca–Ca distances are slightly different. The models show the differences in symmetry between the structures, which are manifested in their spectroscopic and physical properties. The calcite structure (a) has hexagonal symmetry, and is the most symmetrical. This explains the relative simplicity of both the IR and Raman spectra. Aragonite (b) is orthorhombic; the coordination shell around calcium has mirror symmetry. Monohydrocalcite (c) is trigonal, and has no symmetry around any of the three independent calcium ions. These structures were used as starting models for fitting of the EXAFS data from ACC.

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from the ACC layer. The crystalline layers in both cases have protein assemblages rich in aspartic acid and/or asparagine. Aspartic acid-rich proteins are known to be associated with many crystalline forms of biogenic calcium carbonate.[30] There is thus good reason to believe that the properties of glutamic acid and glutamine are important in the formation of ACC.

2.2. ACC as a Transient Precursor Phase for Calcite or Aragonite

In an unrelated study on the textures of biogenic single crystals,[31] we noted that the diffracting intensity of a calcitic spicule from a sea urchin larva (Fig. 6a) was weak relative to the volume of mineral in the beam. This was most curious as it had been claimed for a long time that each spicule is composed of a single crystal of calcite,[32] and indeed our own X-ray study confirmed this.[31] In fact they diffracted as highly ordered single crystals. We, therefore, investigated this further by comparing the diffracting intensity per unit volume of several spicules that were extracted from 48 h old sea urchin larvae of *Paracentrotus lividus* with those from 96 h old larvae.[14] The diffracting intensity per unit volume of the older spicules was indeed much greater than that of the younger ones. We, therefore, concluded that there must be another mineral phase present, and as the spicule is composed of only calcium carbonate, it had to be ACC. Furthermore, the ACC is not stable, but with time converts into a single crystal of calcite. We confirmed this using infrared spectroscopy[14] by taking advantage of the fact that ACC does not have a sharp and strong absorption at 713 cm^{-1}, whereas calcite does (Fig. 2). Raz et al.[33] also used infrared and X-ray diffraction to study the mineral phases of another species of sea urchin larva (*Strongylocentrotus purpuratus*), and confirmed the observation that growth occurs via a transient ACC phase.

The growth stages of the sea urchin larval spicule involve the initial deposition of a very small single crystal of calcite.[34] The arms or radii grow to form the triradiate spicule, and then one of these turns to grow along the *c*-axis. Except for the initially deposited crystal, which clearly exhibits the rhombohedral faces of calcite, the remainder of the spicule has very smooth rounded surfaces that bear no hint of the fact that the spicule diffracts as a single calcite crystal (Fig. 6a). The results of Beniash et al.[14] can reconcile this paradox in that most of the growing spicule is actually composed of ACC, and being isotropic it can be shaped by the inner surfaces of the confining vesicle. The single crystal subsequently propagates through the ACC phase, without, however, an identifiable crystallization front. It would be most interesting to have a detailed understanding of the manner in which the ACC transforms into calcite.

Another key question is whether or not other crystalline calcium carbonate forming animals also use the same transient precursor phase strategy. Weiss et al.[15] investigated the shell formation process of mollusk bivalve larvae (Fig. 6b),

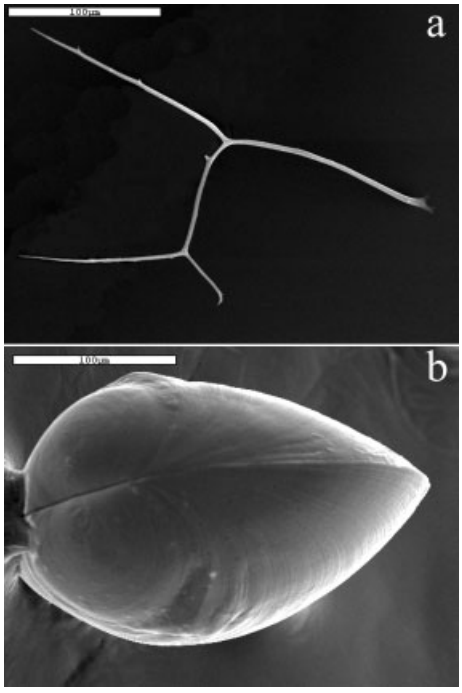

Fig. 6. SEM images of skeletal parts containing transient ACC: a) Spicule of a 72 h old larva of the sea urchin *Litechinus pictus*. b) Shell of a 9 day old larva of the mollusk *Mercenaria mercenaria*. The sea urchin larva has a skeleton composed of two spicules, each comprising transient ACC and calcite. In the mature larva, the whole spicule is a single crystal of calcite [33]. Larval mollusk shells are composed of a prismatic layer and a granular layer. Both layers contain transient ACC and aragonite [15].

that can also be grown in synchronous culture, and mineralize rather rapidly.[35] In a study of two species, *Mercenaria mercenaria* and *Crassostrea gigas*, Weiss et al.[15] used polarized light microscopy, infrared and Raman spectroscopy, and high-resolution SEM to show that in these species as well, ACC is the precursor form of aragonite—the stable crystalline phase of all bivalve larvae.[36] The mollusks and the echinoderms are on two different branches of the phylogenetic tree of the animal kingdom.[29] This could indicate that the strategy of using a transient precursor phase to form crystalline aragonite or calcite is widespread. Obviously more studies, particularly among adult calcium carbonate forming animals, need to be performed in order to determine whether or not this is correct. At this stage, we think it is a serious possibility.

Significantly, the ACC precursor phases both in the larval spicules of the sea urchin *Strongylocentrotus purpuratus* and the larval shells of the bivalve *Mercenaria mercenaria* have little or no structurally bound water, as shown by thermogravimetric analysis.[33] This is in sharp contrast to the stable forms, whose average chemical formula is $CaCO_3 \cdot H_2O$.[21] Weiss et al.[15] noted that the Raman spectrum of the transient precursor phase of ACC in the bivalve larval shell of *Mercenaria* has a relatively sharp 1085 cm^{-1} peak, compared to that of the stable biogenic ACC phases (Fig. 3d). Raz et al.[33] noted the same for the spicules extracted from the 48 h old larvae of the sea urchin *Strongylocentrotus* (Fig. 3c). At this growth stage these larval spicules are comprised mainly of ACC. Calcite

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and aragonite both have sharp 1085 cm^{-1} peaks (Figs. 3e,f). It thus seems reasonable to assume that this sharp peak is related to the absence of lattice water.

We conclude based on the limited available information, that the stable forms of biogenic ACC contain water molecules in their lattice structure, whereas the known examples of transient forms of ACC, do not. Interestingly, the major carbonate infrared absorption peak at around 1450 cm^{-1} in the transient forms of ACC from both mollusk and sea urchin larvae are not split (unpublished observations), in contrast to the stable forms of ACC. All these differences within and between the stable and transient forms of ACC, point to the existence of structural variations within the family of biogenic ACC phases.

3. Synthetically Produced ACC

It has been known since at least 1916 that a hydrated form of calcium carbonate that does not diffract X-rays can be precipitated in vitro from a saturated solution.[37] Several other studies have characterized this inherently unstable phase, and in the last decade many studies have been made in which the phase is partially or completely stabilized with or without the addition of various additives. The insights gained from these studies, together with the information on the biogenic ACC reviewed above, enable us to suggest some rather unexpected mechanisms for understanding the manner in which ACC may be stabilized to a greater or lesser extent.

3.1. Transient ACC

The formation of an ACC transient phase was first identified in systems in which no additives were used. Brecevic and Nielsen[38] characterized an unstable precursor phase of crystalline calcium carbonate that was obtained by rapid mixing of concentrated solutions. The precursor phase comprised spherical microparticles and was amorphous according to its infrared spectrum. Thermogravimetric analysis showed that it contained less than one third of a molecule of water per $CaCO_3$ unit, and its relatively high solubility characterized it as the least stable form of calcium carbonate. The same concept of transient deposition of ACC from supersaturated solutions was recently used to form calcite with constrained morphologies by precipitation at low temperature within the micrometer-sized pores of polycarbonate membranes.[39] Thus, lowering the temperature may stabilize ACC in supersaturated solutions long enough to exploit its isotropic properties and impose the morphology of the unstable phase on the crystalline products.

Additives may retard the precipitation of more stable crystalline phases by adsorption onto the nascent nuclei of crystallization. The relative supersaturation of the solution increases, until it reaches the saturation level of ACC. This unstable ACC phase then precipitates and subsequently transforms into one of the more stable crystalline forms. Thus the additives in effect transiently stabilize the ACC phase.

Magnesium ions,[40,41] triphosphate ions,[42] polymers such as diphosphate-substituted poly(ethylene glycol),[43] and polyaspartate[44] are all able to transiently stabilize the ACC form for relatively long periods (tens of minutes). Inspired by the observation that ACC transforms into calcite in developing sea urchin larval spicules,[14] Xu et al.[45] used polyacrylate to temporarily stabilize a thin film of ACC under a monolayer consisting of amphiphilic carboxylated diporphyrin templates. They indeed found that the transient amorphous phase subsequently transformed into a polycrystalline thin film of calcite. Aizenberg et al.[46] demonstrated that this transformation process can be exquisitely controlled in vitro by incorporating just one nucleation site specific for calcite on the substrate beneath the ACC film. If a patterned substrate is used then a single calcite crystal of millimeter dimensions is obtained.

There are two examples of the formation of a more stable yet transient ACC phase by using the cooperative activity of several additives. These additives usually belong to different categories, for instance, ions and macromolecules. The macromolecules extracted from the alga *Corallina*-stabilized ACC when used in solutions containing magnesium. In this case, the ACC was stable for days. The activity of the proteins could be simulated by polyacrylate or polyaspartate, indicating that it is not very specific.[41]

Raz et al.[33] extracted the proteins from 48 h and 72 h old spicules of *Strongylocentrotus purpuratus*. In this species in culture the 48 h old spicules are composed mainly of ACC, whereas the 72 h old spicules are almost entirely calcitic. When these extracts were added to solutions saturated with respect to calcium carbonate that also contained Mg in a Mg/Ca ratio of 2:1, the 48 h extracts induced the transient formation of ACC, whereas the 72 h extracts induced the formation of calcite. Without Mg in solution, only calcite formed in both cases. This shows that these assemblages of proteins in vitro are capable of inducing ACC or calcite depending upon the stage of development, as occurs in vivo. It would be interesting to know if the same macromolecules are responsible for the two effects, perhaps after some sort of structural alteration, or different macromolecules perform these functions. These in-vitro studies also showed that Mg is essential for ACC formation under these conditions. Whether its presence is necessary to raise the supersaturation level of the solution, and/or to allow the proteins to function properly, has yet to be answered.

The transient ACC phases formed in vitro seem to be very different from the biogenic ones. They presumably contain a substantial amount of incorporated water. Furthermore, the transformation to the stable crystalline forms usually occurs relatively fast and often the subsequent crystallization is not polymorph specific.

Tlili et al.[47] monitored the transformation of ACC into anhydrous crystalline forms of calcium carbonate by in-situ micro-Raman spectroscopy. The material precipitated as a massive gel at 0–3 °C. Its Raman spectrum is characteristic

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of a poorly crystalline material, as the observed peaks are broad (full width half maximum (FWHM) of 18 cm^{-1} rather than 3 cm^{-1} for monohydrocalcite). Slow crystallization at 5 °C resulted in monohydrocalcite being formed, with concurrent sharpening of the peaks. Interestingly, Tlili et al.[47] also observed that dehydration of calcium carbonate hexahydrate proceeded through the formation of the ACC phase. Thus when the hexahydrate form loses water, the Raman peak at 1070 cm^{-1} broadens and shifts to 1080 cm^{-1}. In so doing, the Raman spectrum becomes identical to that of stable ACC.

Another in-vitro experiment in which a transient form of ACC is formed involves the deposition of calcium carbonate in an assemblage comprising β-chitin and silk fibroin.[48] These are the major constituents of the mollusk shell organic matrix. The unique aspect of this system is that when acidic proteins from an aragonitic shell layer are added to the assemblage, aragonite forms, and when proteins from a calcitic layer are added, calcite forms.[49] In both cases, Raman spectroscopy shows that the crystalline product forms via a transient ACC phase.[48] This phase also has a sharp Raman peak at 1085 cm^{-1}. So in this case, the in-vitro transient form may well be anhydrous.

3.2. Stable ACC

There are additives that appear to stabilize ACC indefinitely. These include strong ionic inhibitors that are thought to individually poison crystallization nuclei. The ACC produced may be stabilized for more than several days, and in most cases even when dried. Additives that belong to this category include polyphosphonates,[50] hydrogen-bonding molecular inhibitors such as mono-, di-, and trioligosaccharides, propylene glycol and amino acids.[51] Phosphonates adsorb to ionic crystals much more strongly than other additives. They preferentially interact with kinks on the crystal nucleus surface and thus efficiently inhibit crystal growth. We note that an attractive alternative mechanism of inhibition has been suggested,[52] involving direct interaction between inhibitor and hydrated $CaCO_3$ forms, such as the $CaCO_3$(aq) ion pair.

A different mechanism for inhibiting the formation of calcium carbonate nuclei involves confining the hydrated mineral in a hydrophobic coat, such that the water cannot escape. The small particles are therefore isolated from the aqueous environment and stable ACC forms. Donners et al. produced ACC in this manner by precipitation with poly(propylene imine) dendrimers modified with long aliphatic chain surfactants.[53] ACC particles of radius ~40 Å were also stabilized as dispersions in cyclohexane, by coating with calcium alkylbenzenesulphonate surfactants.[54,55]

Interestingly, synthetic ACC is stable when prepared at > pH 11.2.[56,57] Colloidal particles of 0.05 μm are obtained, that have the molecular composition of calcium carbonate monohydrate. It was suggested that the degree of atomic ordering of ACC increases with decreasing pH and the transformation into calcite upon dehydration occurs more easily.[56] We wonder whether there is an active participation of OH^- ions in the stabilization of the disordered phase.

In contrast to the above examples where kinetic stabilization of ACC is directly induced by additives, the following cases involving protein additives, operates in our opinion, by a mechanism substantially different from individual inhibition of crystallization nuclei. The reason is that the calculated concentration of the additives used is not sufficient to individually poison crystallization nuclei precursors, which may not be larger than a few tens of ions. Aizenberg et al.[58,20] extracted the proteins from the ACC of the ascidian antler spicules that are composed entirely of stable ACC. They then added them back into a saturated solution of calcium carbonate. Stable ACC precipitated out of solution. These macromolecules can, therefore, stabilize ACC. The macromolecules isolated from stable biogenic ACC phases (the body spicules of the ascidian *Pyura pachydermatina* or the amorphous core of the spicules of the sponge *Clathrina* sp.) are present in concentrations that we estimate as $< 7 \times 10^{-6}$ mole/mole $CaCO_3$.[58,19] This is equivalent to as little as one macromolecule per 150 000 molecules of $CaCO_3$, too few to inhibit every nascent nucleus. (Note: this was calculated based on molecular weight 10 KD, a conservative estimate. For comparison, the protein GAMP from the gastroliths of crayfish, also composed of ACC, has a weight-average molecular weight ($\overline{M}_w$) of ~60 KD[28]).

4. Mechanisms of Formation and Stabilization

Despite the paucity of data, it is clear that ACC is a fascinating and potentially important phase. The fact that organisms use such a metastable phase clearly has many disadvantages, but there must be as many or more advantages. One strategic advantage could be that being metastable, it is a far more flexible medium to "work with", in that it can be finely tailored to the specific functions it is required to fulfill.

The ACC types that have already been identified can be divided into two basic categories; the stable hydrated form that contains a mole of water for every mole of calcium carbonate, and the transient precursor form, that is essentially anhydrous. Furthermore, studies of the structure of a variety of stable forms of ACC show that each differs with respect to its short-range order around the calcium ion. Biology therefore clearly "knows" how to exercise exquisite control over the structural properties of ACC, and in so doing presumably fixes the stability properties of each ACC type. How this is achieved at the mechanistic level, is a fascinating question that when understood will provide insights into basic mechanisms of biomineralization, and may well have practical implications for improving materials fabrication. Although there are no definitive answers as yet, we will try to integrate the information from biogenic and synthetic ACC to better understand the mechanisms of formation and stabilization. We will then discuss the broader implications of these observations to the field of biomineralization.

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The first question in trying to understand the basis for the stability of amorphous calcium carbonate formed in vivo is whether or not each state is in thermodynamic equilibrium or trapped kinetically. In other words the amorphous phase might be stabilized by increasing the energy of the crystalline forms (thermodynamic), or by slowing down the ACC transformation, such that it will not occur in a measurable time-scale (kinetic). Even though there is no direct evidence favoring one alternative over the other, various diverse observations tend to point towards mainly kinetic stabilization. There probably are also concomitant thermodynamic components (such as the presence of magnesium or phosphate ions).

In the sea urchin larvae, the ACC of the spicule is apparently not in contact with an aqueous solution, but is enveloped by a tightly adhering membrane.[59] The same is true for the cases where ACC coexists with calcite, in compartments delimited by membranes.[19] This appears to be a requirement for stabilizing a phase that would otherwise crystallize in an aqueous environment in an uncontrolled energy cascade. Furthermore, calcium carbonate is delivered to the sea urchin spicule-forming compartment as pre-deposited ACC, which is also confined in vesicles.[59] Several in-vitro experiments also support a kinetic stabilization scenario. One such experiment shows that colloidal particles of calcium carbonate are stabilized by the presence of a surfactant coat, which essentially functions as a barrier to ion exchange with the surroundings, while concomitantly preventing the water molecules inside the phase from escaping.[54]

Assuming that the stabilization mechanism is mainly kinetic, how is ACC induced to form? The scant available information points to the setting up of a microenvironment disfavoring organization of the nuclei from their inception, rather than the action of additives that actively interfere with the growth of crystallizing nuclei. The latter mechanism is well known in crystallizations in vitro, but in the biological mineralizing environment the amounts of macromolecules present are too low to be effective in such a highly supersaturated medium. As the stable ACC phases investigated to date all have a stoichiometry of $CaCO_3 \cdot H_2O$,[21] water is clearly involved. Its presence in the coordination sphere around calcium ions must prevent the reorganization into one of the stable crystalline anhydrous phases.

The stable biogenic ACC phases are best regarded as having short-range orders resembling to a certain extent that of monohydrocalcite, with very limited or no long-range order. This statement requires some consideration of the meaning of short-range order. All the spectroscopic techniques used, including Raman, IR, and EXAFS, provide information extending for not more, and often less than 8 Å, corresponding to the average distance between two calcium ions on the two sides of a given central calcium ion (Fig. 5). We do not know whether any order extends beyond that. Within a shell of 4 Å diameter, there are notable differences between biogenic ACCs from different organisms (Table 2, Fig. 4), indicating that the short-range order is genetically controlled. How this is achieved we do not know, besides the fact that it is induced mainly through biological macromolecules.

The transient species of ACC are different from stable ACC in structure and composition. They are anhydrous, and we have some indications that even at an early stage they resemble the crystalline form into which they are going to transform. These include spectroscopic data, showing a greater symmetry around the carbonates (sharper peaks in IR and Raman), although some vibrations may be completely missing, or substantially broadened. EXAFS data from Hasse et al,[25] as well as from our own laboratory (Levi-Kalisman, Sagi et al. unpublished), also support this inference. Hasse et al.[25] showed that in the larval shell of a freshwater gastropod, the ACC phase does have short-range order that resembles aragonite. It was not, however, shown that this ACC phase is a transient precursor phase of aragonite in this animal. Sea urchin larval spicules at a stage in which they are composed of >70 % ACC (by X-ray), produced EXAFS spectra very similar to those of calcite, while mollusk larval shells with >50 % ACC composition gave spectra indistinguishable by visual inspection from those of aragonite (unpublished data).

The implication of these observations is that a local molecular structure is imposed on the ACC phase. This molecular structure is ordered at the level of the coordination sphere around the calcium ion as in the stable crystalline form, but is disordered in the intermediate and long ranges. This locally ordered structure functions as the basic unit that, in contrast to stable ACC, reorganizes into the corresponding crystalline form. Such a transformation cannot evidently occur by dissolution and reprecipitation and still have it maintain the effect of the nascent nucleating structure. It conceivably involves a solid–solid state rearrangement, whereby the local centers of short-range order align and coalesce. These then propagate cooperatively to form crystalline microdomains. The absence of a defined macroscopic crystallization front in the sea urchin larval spicule indicates that this process occurs contemporaneously at many locations throughout the solid phase.

The macromolecules introduced inside the amorphous phase from the start, or at an intermediate stage, may fulfill two distinct functions. Those that contribute to the transient stabilization of the amorphous phase must be broken down or otherwise neutralized in an accurately orchestrated series of events, to allow for crystallization. Other macromolecules, which have the ability to modulate crystal growth,[60] are presumably adsorbed at the boundary planes of single crystalline microdomains as the crystal grows. They thus locally influence crystal growth in certain directions rather than others.

The question arises as to whether the presence of a precursor ACC phase necessarily changes other generally accepted concepts regarding nucleation and crystal growth at least in the sea urchin and mollusk larvae. In the former case mineralization takes place in a multicellular vesicle (syncitium),[61] whereas in the latter it is in an extracellular matrix.[62] In the sea urchin larva the ACC is not known to be formed in the syncitium, but in small intracellular vesicles located in the adjacent cells.[59] It is presumably transported into the syncitium. It is conceivable that a similar situation could exist in the larval mollusk, as in the adult ACC containing vesicles are pres-

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ent in the mantle cells adjacent to the sites of new mineral formation.[63] Nucleation of the first crystalline phase in the process of sea urchin larval spicule formation takes place before ACC is present in the syncitium.[34] This occurs at a very specific locality and is oriented;[64] properties that point to template-directed nucleation presumably from solution. The propagation of the crystalline phase through the ACC filled syncitium is enigmatic. Although we have not been able to identify a crystallization front, overgrowth experiments on the spicule surface show that it is essentially a continuous single crystal.[65] Nothing is known about how aragonite nucleation takes place in the larval bivalve shells. As the crystals are oriented, we assume that a template is prepositioned and that the initial nucleus forms on its surface directly from the ACC phase. A similar scenario was proposed by Lowenstam and Weiner[17] for the formation of oriented crystals of carbonated apatite in a preformed amorphous calcium phosphate milieu in the chiton tooth. The resulting crystals were aligned with their long axes in the plane of the matrix sheet surfaces. Aizenberg et al.[46] demonstrated the feasibility of this mechanism in vitro. Thus from the little information available, it appears that in the larval sea urchin and mollusk mineralization processes, template-directed nucleation and modulation of crystal growth in confined spaces do take place.

5. Broader Implications to the Field of Biomineralization

The stable forms of biogenic ACC have been documented for almost a century. Despite this, the list in Table 1 probably underestimates the natural distribution of ACC. The main reason is that it is easy to overlook the presence of ACC especially when it co-exists with one of the crystalline forms of calcium carbonate. Only two cases are known to date[18,19] and both were discovered in the last few years. The crustaceans are among the chief producers of stable ACC both at temporary storage sites, and in the cuticle where it presumably fulfills a mechanical function. In the cuticle it is often reported to be present with calcite.[1] It would be interesting to determine whether this calcite forms de novo or is itself the product of the transformation of ACC.

The two documented examples of ACC functioning as a transient precursor phase for calcite or aragonite, may have far-reaching implications to the field of biomineralization. The examples are from the larvae of echinoderms and the mollusks. These phyla are located on two different branches of the animal kingdom phylogentic tree (irrespective of which scheme is used).[29] Thus the fact that both larval forms use the same basic strategy to form their mature crystalline mineral phases raises the tantalizing possibility that many other calcium carbonate mineralizing animal phyla use this strategy. If this indeed proves to be the case, this would change certain basic concepts in biomineralization. Furthermore, it would necessitate a reevaluation of the possible role of amorphous calcium phosphate (ACP) as a transient precursor phase of carbonated apatite—the mature mineral form in vertebrate skeletons and a few invertebrates as well. Before reaching such widespread conclusions, however, it remains to be shown that adult echinoderms and mollusks use the same strategy and that at least some other phyla also use ACC as a transient precursor phase. The facts that both larval echinoderms and mollusks do use ACC as a precursor phase, and that in both cases this is the anhydrous form of ACC, is a good basis for assuming that among larvae at least, common basic mechanisms are involved.

6. Concluding Remarks

ACC may well have a much broader role to play in the processes of calcium carbonate formation in biology than was suspected to date. There is enough data in hand to make this a serious prospect, that if proved correct will radically change the manner in which we understand biological calcium carbonate formation to date. It may be timely to begin reexamining the possibility that amorphous calcium phosphate may also play a fundamental role in calcium phosphate formation. Even if biology does not widely use these solid-state amorphous precursor phases, the chemistry involved in the known cases is fascinating and when understood may well lead to interesting applications for materials science.

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