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[REVIEW]

Biom mineralization of the Spicules of Sea Urchin Embryos

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ABSTRACT—The formation of calcareous skeletal elements by various echinoderms, especially sea urchins, offers a splendid opportunity to learn more about some processes involved in the formation of biominerals. The spicules of larvae of euechinoids have been the focus of considerable work, including their developmental origins. The spicules are composed of a single optical crystal of high magnesium calcite and variable amounts of amorphous calcium carbonate. Occluded within the spicule is a proteinaceous matrix, most of which is soluble; this matrix constitutes about 0.1% of the mass. The spicules are also enclosed by an extracellular matrix and are almost completely surrounded by cytoplasmic cords. The spicules are deposited by primary mesenchyme cells (PMCs), which accumulate calcium and secrete calcium carbonate. A number of proteins specific, or highly enriched, in PMCs, have been cloned and studied. Recent work supports the hypothesis that proteins found in the extracellular matrix of the spicule are important for biomineralization.

Key words: spicules, sea urchin skeleton, biomineralization

INTRODUCTION

Animals of the echinoderm phylum possess elaborate calcareous endoskeletons. The formation of skeletal elements during development of echinoid larvae and the subsequent construction of the definitive adult endoskeleton during metamorphosis are examples of complex organogenesis. The morphogenesis and differentiation of the skeleton in the sea urchin embryo has received considerable attention from embryologists during the past 120 years, but the central defining process of endoskeleton formation—the actual deposition of the mineralized skeleton—is still not well understood. That should not be surprising. The cellular and molecular processes that govern biomineralization in any instance have remained obscure. On the other hand, there is a considerable amount known about the composition and structure of many biomineralized structures.

There are many different biominerals, and there are many different strategies involved in their formation. The extensive world of biomineralization is discussed in two excellent books, one by Lowenstam and Weiner (1989), the other by Simkiss and Wilbur (1989). Biomineralization in euechinoids takes place in very close association with the mesenchymal cells that form the spicules, spines, tests, and

other mineralized elements.

The developing sea urchin embryo is well suited to study cellularly regulated biomineralization. The embryology of spicule formation, including the cellular lineages, details of morphogenesis, cellular interactions, and characterization of marker genes and proteins, has been well studied. An important advance was made by Okazaki (1975) when she devised a simple method for culturing micromeres, *in vitro*; the micromeres recapitulate almost perfectly their development in the intact embryo, including robust spicule formation. One may consult reviews by Etensohn *et al* (1997), Klein and Brandhorst (2001) and Angerer and Angerer (2002) for the status of current studies on the determination, differentiation, and morphogenesis of the cells forming the skeleton. Reviews by Lennarz and Decker (1987) and Benson and Wilt (1992) summarized information about spicule formation proper, as does a recent review by the author (Wilt, 1999).

I shall concentrate here on recent studies focused on the actual formation of the spicule and related structures with an emphasis on the secretion of the mineral and matrix proteins. The latter may be located occluded within the mineral, or surrounding the spicule in a specialized extracellular envelope.

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Composition and Morphology of the larval spicule

Let us start with the end result, the actual larval spicule. The mineral phase is the calcite form of CaCO_3 , containing about 5% Mg CO_3 , termed magnesian calcite. The calcite contains an occluded organic matrix of glycoproteins that constitutes approximately 0.1% of the mass. This small amount of protein changes the texture, flexibility, hardness and fracture characteristics so that the mineral behaves when fractured more like a hard glass-like material than a crystal. The composite is crystalline, however, showing well defined x-ray diffraction patterns characteristic of calcite. The highly birefringent spicule is composed of micro-domains of calcite whose crystallographic axes are all aligned, albeit imperfectly, in the same direction. Studies of mineral structure of the spicule were reviewed recently (Wilt, 1999) The spicule is not perfectly isotropic; when the broken ends of spicules are examined by scanning electron microscopy, a clear pattern of cylindrical laminae can be discerned. A scanning electron micrograph of the surface of a "clean", broken spicule from the pluteus larva is shown in Fig. 1.

An important recent discovery was made by Beniash (Beniash *et al.*, 1997) and his collaborators at the Weizmann Institute. The crystalline spicule contains substantial amounts of amorphous calcium carbonate (ACC). ACC is not stable and when prepared in the laboratory will quickly crystallize, usually forming calcite. Specialized additives that inhibit crystal nucleation can stabilize ACC to some extent. Even though it might seem unlikely, relatively stable ACC is found in naturally occurring skeletal elements. In addition to previously documented examples (see Lowenstam and

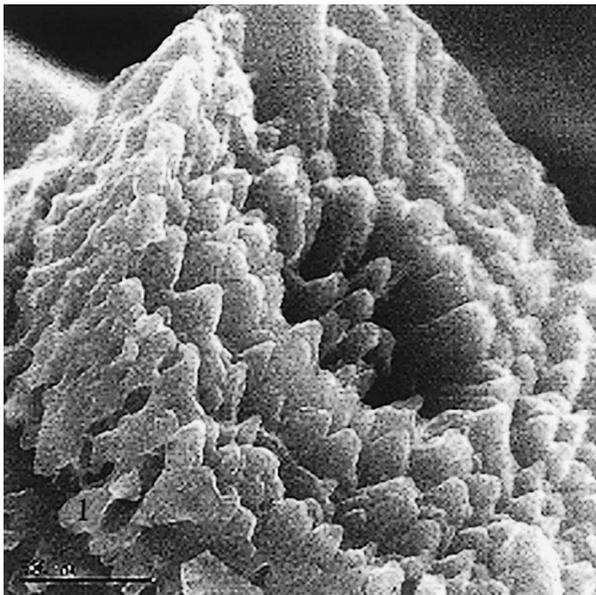


Fig. 1. The surface of a broken spicule was examined by scanning electron microscopy. The calcite has been partially etched during preparation for microscopy. The remaining calcite seems to be arranged in concentric "rings". The center of the spicule shows extensive dissolution of mineral, a common observation seen by others. This micrograph was obtained by Jong Seto of our laboratory. Scale bar= 500 nm.

Weiner, 1989), recent work on skeletal spicules of some sponges and ascidians has revealed stable ACC (Aizenberg *et al.*, 1996). In some cases the stable ACC is found in the same element conjoined with calcite. Beniash *et al.* (1997) used several methods to demonstrate that freshly isolated triradiate spicules from post-gastrula stage embryos of the sea urchin *P. lividus* contained about 2/3 ACC and about 1/3 calcite. Spicules prepared from progressively older larvae contained a progressively lower proportion of ACC. The scenario described by Beniash *et al.* (1997) is that considerable ACC is present in the young, growing spicule, but that as the spicule matures the previously deposited ACC undergoes a regulated transformation to calcite. This finding has implications for the mechanisms by which biomineralized spicules are formed.

The spicules of sea urchin larvae are covered with a cytoplasmic sheath. An electron micrograph (Fig. 2) of the space inhabited by the spicule shows an almost, but not quite, continuous cytoplasmic process surrounding the space. Early work by Okazaki (1960) using light microscopy demonstrated a distinct membrane enclosed space surrounding the tip of the growing spicule. More recently Beniash *et al.* (1999) used freeze fracture methods to examine the interface between spicule and cell membrane of the investing cytoplasmic sheath. The results support the notion that the space between spicule and cell membrane cannot exceed a few nanometers. The data does not illuminate whether there is a thin extracellular matrix between mineral and cell membrane. The freeze fracture results apply only to regions along the shaft of the spicule intersected by the fracture, and not to the growing tip. Whether or not there is a distinct "space" at the tip of the developing spicule, light and electron microscopy both attest to the close and almost con-

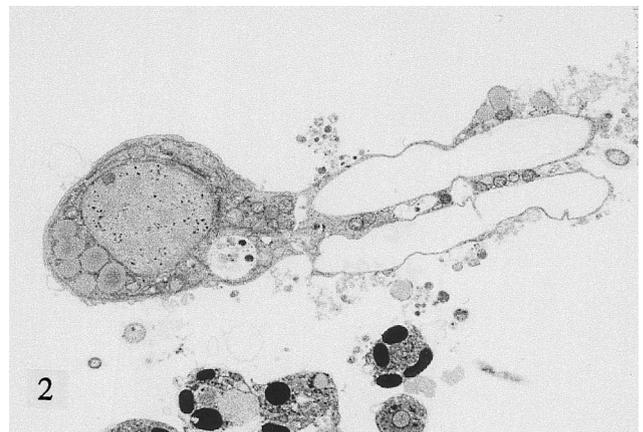


Fig. 2. An *in vitro* culture of PMCs containing spicules was fixed with glutaraldehyde in sea water at pH 6, stained with osmium, sectioned and examined by transmission electron microscopy. The section shows a single PMC with a portion of the cytoplasmic cord that contained two mineralized spicules. The spicules have dissolved during preparation for microscopy, and the space that formerly enclosed the spicule is surrounded by cell membrane. Note the abundant membrane systems and inclusions present in the PMC itself.

tinuous envelopment of the mineralized spicule by the cell membrane of the fused PMCs. Gibbins *et al.* (1969) proposed that the spicule resides in an intracellular vacuole; hence, spicules were thought to be intracellular. However, Decker *et al.* (1987) demonstrated that the spicule present in cultured living cells, *in vitro*, can be dissolved by exposure to slightly acidic pH or by chelating agents. Furthermore, Decker *et al.* (1987), and Ingersoll and Wilt (1997) showed occasional but clear interruptions in the membrane surrounding the spicule. Decker *et al.* (1987) also showed that living PMCs exposed to cell impermeant gold particles attached to conA displayed the gold particles localized on the spicule. It is likely, therefore, that the spicule is deposited vectorially in an extracellular space and that the mineral is enrobed with an extracellular matrix and the cytoplasmic cord of the fused PMCs. Fig. 3 shows this diagrammatically.

The issue of whether there is a distinct extracellular matrix between the cell membrane and the mineral is important for understanding how the spicule is formed. Two spicule matrix proteins (SM50 and PM27), which we will discuss later in more detail, are abundant on the surface of the spicule adjacent to the cell membrane (Harkey *et al.*, 1995; Urry *et al.*, 2000; Kitajima and Urakami, 2000). Decker *et al.*, (1987) clearly demonstrated material staining with ruthenium red surrounding the mineralized spicule. I consider the presence of an extracellular matrix between the cell membrane and mineral very probable; rigorous proof of this will require purification and characterization of such a putative extracellular matrix. For sake of convenience in the rest of this review I shall refer to this matrix as an "envelope".

The mineral of the spicule contains occluded proteins (Benson *et al.*, 1987). SM50, and PM 27, in addition to their presence in the envelope, are also part of the group of proteins that become soluble when spicules cleaned with

NaOCl are demineralized (Killian and Wilt, 1996; Kitajima and Urakami, 2000). When spicules are etched with dilute acid, pits in the mineral react with antibodies against SM50 and PM 27, as well as a third protein called SM30. (Urry *et al.*, 2000). Killian and Wilt (1996) characterized the soluble proteins released from clean, demineralized spicules by 2D gel analysis, and found approximately 45 different proteins. Most of these proteins are acidic and glycosylated; however, SM50 is an exception to this generalization. Table I lists the soluble spicule matrix proteins that have been characterized to some extent. Zhu *et al.* (2001) have recently cloned two other putative spicule matrix proteins from a PMC specific EST library, but they have not been characterized yet. One is SM 50-like, and another is a C-type lectin.

When the spicule is demineralized with chelating agents or dilute acids in the presence of fixatives, a fibrous organic structure with the overall shape of a spicule can be visualized (Benson *et al.*, 1983; Okazaki, 1960). When partially demineralized spicules were sectioned, stained and examined by electron microscopy, concentric sleeves of this same meshwork were observed (Benson *et al.*, 1983). Recent work using synchrotron radiation (Berman *et al.*, 1990) showed that the "single" crystal of the spicule is a collection of small crystallites which are well, but not perfectly, aligned. Beniash *et al.* (1999) concluded that ACC and calcite were interspersed with one another in the developing spicule. Further details on the nature of the distribution and interaction between mineral and matrix in the intact spicule have remained elusive. (See Wilt, 1999)

The characterized proteins of the spicule are also found in mineralized tissues of the adult, as is indicated in Table I. The localization in adult tissues has been demonstrated by identification of the cognate mRNAs, and immunostaining both at the light (Richardson *et al.*, 1989) and electron microscope (Ameye *et al.*, 1999) level. It is interesting that Kitajima (Kitajima *et al.*, 1996) was not able to identify SM 30 protein in the test of adult *H. pulcherimus*, but SM50 is present in spines of this species. Kitajima noted that calcite in the test is organized parallel to the "a" crystallographic axis, while calcite of spines and spicules are organized along the "c" axis, and he proposed that SM30 might have a function in directing axial growth along the "c" direction. There has been some work on the proteins found in teeth and of adults (Weiner, 1985; Veis *et al.*, 1986), but no individual tooth proteins have been characterized yet.

There is little data about whether any of the proteins occluded in spicules of euechinoids are found in mineralized structures of other invertebrates. Lambert (1966) reported that spicules found in the ascidian, *Hermania momus*, stained with antibody against SM50. We have learned using Western blot analysis that proteins isolated from tunic spicules of the ascidian *Pyura pachydermatina* have a protein of 45 kD that reacts with an antibody against SM50 (Seto and Wilt, unpublished).

In sum, the sea urchin larval spicule, a seemingly simple tissue when compared to vertebrate skeletal elements,

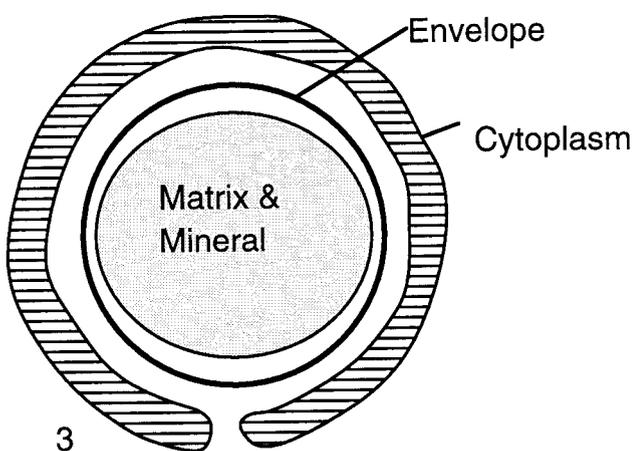


Fig. 3. A diagram of the hypothetical arrangement of membranes and mineral in the spicule, shown in cross section. The cord of syncytial cytoplasm connecting primary mesenchyme cell bodies surrounds a thin extracellular envelope, which in turn surrounds the mineralized spicule in which are occluded various matrix proteins. The spaces between cell membrane, envelope and spicule have been enlarged for diagrammatic clarity.

is really an extraordinarily elaborate composite. It is composed of a high magnesium calcite with an ACC component, which is gradually crystallizing, and an occluded constellation of proteins of unknown function. The spicule is probably enrobed with a specialized extracellular matrix as well as a closely applied cable of cytoplasm extending between the PMC cell bodies.

The Cells that Make the Spicule: PMCs

The cells responsible for forming the spicule are primary mesenchyme cells (PMC). The founder population of cells that form the PMCs arise at the vegetal pole of the embryo at the 5th cell division. The 4 large micromeres divide three more times, and the 32 descendants ingress from the epithelial wall of the blastula to become a mesenchymal cell population migrating within the blastocoel. A review of the mechanisms of this complex morphogenesis may be found in Ettensohn *et al.* (1997). A number of characteristic and specific marker proteins are synthesized by PMCs. First among these is a glycolipid anchored cell surface protein, msp 130. (Anstrom *et al.*, 1987; Farach-Carson, 1989). These workers proposed that msp 130 might function in Ca transport. Antibodies against this protein inhibit spicule elongation (Farach-Carson, 1989). SM50, SM37, PM 27 and SM30 are PMC specific; SM50, SM37 and PM27 accumulate in PMCs many hours before spicule formation (Killian and Wilt, 1989; Lee *et al.*, 1999, Harkey *et al.*, 1995; Urry *et al.*, 2000). A number of PMC specific antigens, mostly uncharacterized, have served as useful markers for this population of cells (see Ettensohn *et al.*, 1997). A number of genes encoding transcription factors are also expressed primarily or exclusively in PMCs (see Brandhorst and Klein, 2001; Angerer and Angerer, 2002). A cDNA library of genes expressed by PMCs has been constructed and the profile of genes analyzed. A number of PMC specific (or enriched) extracellular matrix proteins, cell surface proteins, putative spicule matrix proteins and transcription factors were identified (Zhu *et al.*, 2001).

For our present purposes there are two salient aspects of PMC behavior that deserve mention. First, PMCs of euechinoid species fuse with one another during gastrulation, establishing a syncytial population of cell bodies connected by slender cytoplasmic cables. The formation of spicules takes place in the cytoplasmic cords of this syncytial array. It is not known whether syncytium formation is a precondition for spicule formation. In unpublished work from the authors' laboratory, we have occasionally observed single PMC cells in culture that contain very small spicules. This is exceedingly rare, however. When cell densities of cultured micromeres are sufficient to allow syncytium formation, spicule formation becomes robust, though there are no published studies giving quantitative data on the incidence and vigor of spicule formation as a function of cell density and/or syncytium formation. It is possible that higher cell densities allow the simple culture medium to become "conditioned", or that syncytium formation, per se, is salutary for biomineral-

ization.

Second, PMCs are a cell population dedicated to skeleton formation. PMCs have been transplanted to heterotopic location in the embryo by many investigators over the years, and they never form any tissue but skeleton. In contrast, almost all other cells in the cleavage stage embryo, except small micromeres, have the capacity, given the appropriate stimuli, to form mesenchyme that produces spicules. Animal hemisphere mesomeres and vegetal hemisphere macromeres can both give rise to spicule forming cells (Horstadius, 1973 ; Khaner and Wilt, 1990). Ettensohn and his co-workers have shown clearly that the PMCs themselves produce factor(s) that prevent secondary mesenchyme from following a skeletogenic pathway (Ettensohn *et al.*, 1997).

It is known that factor(s), which are probably released from cells of the blastocoel wall, are crucial for active biomineralization. The chemical identification of the factor(s) has been difficult, and though they can be isolated from blastocoel fluid, their precise nature is still unknown. (Kiyomoto and Tsukuhara, 1991). Micromeres from some species will form spicules, *in vitro*, if a low concentration of horse serum is added to the sea water culture medium. The window of competence for PMCs to respond to horse serum, *in vitro*, is rather long. Page and Benson (1992) studied this issue by adding or withdrawing horse serum at various times from the medium of Okazaki type cultures. They found that the factor(s) present in horse serum needed to be present for at least 5–10 hr any time between the beginning of PMC migration and the end of gastrulation, a period of about 15 hr in *S. purpuratus*, the species used for their study. Spicule formation itself usually starts about 6–8 hr after ingression of mesenchyme (at 15 C); Page and Benson's work shows that even if horse serum is added many hours after the usual time for beginning of spicule formation, the horse serum effectively induces spicule formation. However, by the time control embryos have reached the prism stage PMCs are no longer able to respond to serum. Similar results were found by Kiyomoto and Tsukuhara (1991). The factor(s) involved must not be strictly species specific. Von Ubisch (1939) carried out many transplantation studies forming intergeneric chimeras; PMCs from one genus could construct skeletal elements in another genus. More recent work by Armstrong and McClay (1994) using different species reached the same conclusion.

The embryo constructs its skeleton by elongation of the tips of a nascent spicule within the syncytial array of PMCs established during gastrulation. Throughout larval life, however, congregations of cells at the tips of the spicules participate in biomineralization. These aggregates of mesenchyme cells located at the elongating tip of the spicule are sometimes called "plugs", and their involvement in skeleton formation has been described by many workers, notably Wolpert and Gustafson (1961). The cells in these "plugs" show high level of expression of SM50 and SM30 (Urry, personal communication), and Ettensohn and Malinda (1993) have shown that the ectoderm adjacent to these plugs is

probably secreting factors that stimulate skeleton deposition. The development of mineralized tissues of the post-metamorphic sea urchin have been described by several workers (cf. Gordon, 1928; Kryuchkova, 1979). However, the origins of the cells in the juvenile that are engaged in these many instances of biomineralization are not known. Such cells could conceivably arise from embryonic PMCs, "plug" cells, or originate from cells of the coelom. Formation of mineralized structures in the echinus rudiment (the forming juvenile in its premetamorphic state) is associated morphologically with cells found at the ends of larval spicules (Urry, personal communication).

Finally, the secretory nature of the PMCs should be noted. PMCs are really extraordinary factories engaged in secretion. Not only do they deposit spicules, but other extracellular components as well. Wessel *et al.* (1991) have shown that PMCs secrete copious amounts of collagen and proteoglycan into the medium, and onto the culture dish, of micromere cultures. The electron micrograph shown in Fig. 2 shows a prominent Golgi apparatus and elaborate endomembrane system, both characteristic of cells engaged in high levels of secretion.

Calcium metabolism of PMCs

The source of the calcium (and presumably magnesium) of the mineralized spicule is sea water, as was shown in the early studies of Nakano and Okazaki using ^{45}Ca (Nakano *et al.*, 1963). A variety of studies using inhibitors of Ca transport implicate the PMCs in the deposition of ACC and calcite. In other words, it is unlikely that Ca from the sea water precipitates directly in the "privileged space" in which the spicule elongates, but rather, the Ca must pass through the PMC. Massive amounts of Ca must be imported into the PMC, and massive amounts must somehow be exported during actual spicule deposition. Decker and Lennarz (1988b) showed, using ^{45}Ca , that spicule elongation is not intercalary, but occurs by addition of newly precipitated Ca at the tip of the spicule. Their data did not exclude some addition of Ca along the surface of the shaft, and indeed, there is some increase in girth of the spicule, as well as along its length, during its development. (Ettensohn and Malinda, 1993). Guss and Ettensohn (1997) used the fluorescein derivative, calcein, to follow spicule growth in the embryo. This dye binds to Ca and becomes intensely fluorescent when incorporated into precipitates of calcium. When PMCs were exposed to calcein, dye preferentially localized at growing tips, and lesser amounts were present in the spicule shaft as girth increased. Thus, the spicule is much like a plant: terminal growth at a meristematic tip and cambial growth along the stalk of the plant provide increase in length and girth.

Hwang and Lennarz (1993) used calcium channel blockers to inhibit calcium uptake and spicule elongation. Earlier work by Matsunaga *et al.* (1986) implicated Ca channel activity by use of a large number of different Ca channel antagonists and inhibitors (older literature reviewed by Ben-

son and Wilt, 1992). Unpublished work from our laboratory has used exposure of cultured PMCs to pulses of calcein to follow the path of Ca deposition. After 30 min of exposure to calcein punctate fluorescence is seen in PMCs, and during a subsequent "chase" the calcein becomes primarily localized to growing spicule tips. Beniash *et al.* (1999) prepared quickly frozen (subsequently dehydrated) PMCs attached to spicules; they observed electron dense granules of ACC within the PMCs. I favor the hypothesis that Ca is imported by relatively low affinity, high capacity Ca transporters, since sea water contains millimolar levels of calcium. The calcium would subsequently be precipitated intracellularly in vesicles as ACC in association with protein(s) that encourage ACC stability. Exocytosis of ACC composites into the "privileged space" enclosed by the envelope could then occur, and the ACC could gradually transform to calcite as the spicule develops. We still need a clear indication of exactly what Ca transporter(s) is involved in Ca import, and what are the mechanisms of precipitation and intracellular stabilization of the ACC, the pathway of exocytosis, and mechanism of the incorporation of secreted ACC/protein mixtures into the developing spicule. Benson and Wilt (1986) showed that the soluble spicule matrix proteins could bind Ca with low affinity (10^{-4} M), but which particular proteins of this mixture bind Ca is unknown.

Other aspects of protein modification and mineral modification should be remembered as well. Inhibition of glycosylation interferes with spicule deposition (Kabakoff and Lennarz, 1990). Since the majority of occluded matrix proteins are secreted glycoproteins (Killian and Wilt, 1996), any compromise of glycosylation or the secretory pathways might be expected to affect spicule formation. Alteration of the ratios of Mg and Ca in sea water (Okazaki 1961) influenced the morphology of the spicules formed by embryos. Removal of sulfate ion from sea water interferes with PMC migration and stops spicule formation.

Nucleation of the spicule occurs by different mechanisms than the elongation of the spicule. Okazaki and Inoue (1976) noted that the first deposited granule possessed the rhomboidal shape of pure calcite, and this was clearly shown in their scanning electron micrographs. The "space" surrounding the initial granule is much more evident than the space between envelope and growing tip seen at later stages (Urry, personal communication). Inhibitors of metalloproteases reversibly inhibit spicule elongation (Ingersoll and Wilt, 1987) but not nucleation of the initial calcite granule. This is also true of a specific inhibitor of a collagen C-terminal proteinase (Huggins and Lennarz, 2001), which inhibits collagen fibril formation and spicule elongation, but does not stop granule deposition. Inhibition of SM 50 accumulation by use of an anti-sense oligonucleotide lowers SM 50 to 20% of normal levels and blocks spicule elongation, but does not inhibit granule nucleation (Peled-Kamar *et al.*, 2001). Okazaki (1975) described the appearance of several minute calcite granules in the congregated cells of the syncytium, but only one of these eventually persisted; the other

granules were transient. (Cf. Urry *et al.*, 2000) There is apparently some regulatory mechanism in place that permits the persistence, and subsequent elongation, of only one initial calcite granule in each of the two ventrolateral clusters of PMCs in the gastrula embryo.

Spicule growth has been measured in real time in embryos of *L. variegatus* by Malinda and Etensohn (1993) and Guss and Etensohn (1997). The rates of elongation are independent of the number of PMCs clustered along a given syncytial chain. Rates of elongation, ranging from about 5 to 13 $\mu\text{m/hr}$, are characteristic of different portions of the skeleton. For instance, post-oral rods can elongate at 13 $\mu\text{m/hr}$, but ventral transverse rods only elongate at 5.6 $\mu\text{m/hr}$. How the rate of elongation is controlled is completely unknown, but obviously depends ultimately on intracellular regulation of the rate of secretion. Detailed features of the spicules are known to be species specific. Using interspecific hybrids, Boveri (1895) showed that the curves of the skeletal elements, and hooks, spurs and fenestrations characteristic of different species, are under genetic control. Again, we have no information at the level of cell or molecular biology that bears on how detailed patterns of biomineralization are controlled. An intriguing possibility is that ACC is used as a precursor material, a material which presumably is more deformable and plastic, and hence is more useful for microanatomical modeling than is calcite.

Proteins of the Spicule.

Of the some 45 proteins found occluded in the spicule, there is detailed information about four of them. (Table I). Much of this information has been reviewed recently (Wilt, 1999); hence, I shall only summarize previous studies and emphasize more recent findings. SM30 is acidic and glycosylated and has an amino acid composition typical of mineral matrix proteins. Though it has amino acid sequences similar to certain canonical domains (e.g., C type lectins; lithostatins), its function remains a mystery. The mass (deduced from amino acid sequence) of the secreted protein is 30.6 kD, while its mobility in denaturing acrylamide gels is in the range of 42–43 kD. Exhaustive digestion with N-glycanases only removes an apparent 4 kD of mass, and evidence for O-glycosylation is lacking. The nature of the remaining 8kD of apparent mass is unknown and is the sub-

ject of ongoing investigation. SM30 is a gene family of which two members are known (Akasaka *et al.*, 1994), though one or two additional members are possible. The spicule of *S. purpuratus* contains 2 isoforms of SM30; glycosylation differences are not the basis for this heterogeneity, though it is not known whether they differ in amino acid sequence or in some other post-translational modification. Another puzzle is that mesenchyme cells contain primarily the high molecular weight isoform, whereas the spicule contains either a mixture of two forms, (*S. purpuratus*) or simply the lower mass isoform (*H. pulcherrimus*). SM 30 is either low or absent from the ventral transverse rod, and is also low or absent in tests of *H. pulcherrimus* (Kitajima *et al.* 1996) and *S. purpuratus* (Killian, unpublished). What is needed here is some way to probe the function of SM 30, and other matrix proteins. Thus far use of SM30 antibodies, or injection of possible dominant negatives which might prevent SM30 association with other proteins, have been unrewarding. Current studies are underway using antisense oligonucleotides to study SM30 function.

One thing is clear about SM 30. While it is found in Golgi membranes and intracellular vesicles (Urry *et al.*, 2000; Wilt unpub), it is not associated with the envelope around the mineral. SM30 is embedded in the mineral phase itself. SM30 is found in situations where the axis of the skeletal element follows the "c" crystallographic direction, but not where the "a" direction of growth (e.g., ventral transverse rods, test) is followed, and as mentioned previously this has led Kitajima to propose that SM30 is associated with regulation of direction of crystal growth.

The other three well characterized integral matrix proteins are atypical in that they have isoelectric points above 7 (only 10 of the 45 in the spicule do, and they are all quantitatively minor occluded proteins), and SM50 and PM27 are not glycosylated. SM37 (Lee *et al.*) has an amino acid sequence with some similarities to SM50 and it is closely linked to SM50 in the genome. SM50 and PM 27 (Harkey *et al.*, 1995) both have C lectin type sequence domains and SM 37 has similarity to carbohydrate recognition domains of C-type lectins. It is intriguing that SM50, SM37, PM27, and "SM50-like" and "C-lectin" cloned by Zhu *et al.* (20021) all possess sequences showing some similarities to C-type lectins. Could there be some functional role for such a domain

Table I. Occluded Matrix proteins of the sea urchin spicule

Protein	Mass/Glycosylation/ pI ¹	Presence in ECM*	Presence in Adult	Reference
SM50	46.3/ - /11.3	+	test,spine,t.f.*	Katoh-Fukui <i>et al.</i> (1991)
SM30	30.6/ + / 5.9	-	spine, t.f.	George <i>et al.</i> (1991)
SM37	37 / + /10.63	?	?	Lee <i>et al.</i> (1999)
PM27	27 / - / 7.82	+	spine	Harkey <i>et al.</i> (1995)

¹ Mass: calculated from amino acid composition; Glycosylation: presence of consensus sequences for N-glycosylation in amino acid sequence; pI: calculated from amino acid composition.

* ECM=extracellular matrix around spicule

* t.f.= tube foot

family in the biomineralization process? SM50 possesses a long C terminal repeat (QPGVG) that is known to confer a β spiral configuration. SM 50 is a secreted protein found in Golgi and small vesicles, but in contrast to SM 30 is also found very abundantly in the extracellular matrix on the surface of the spicule. This is also true of PM27. (Urry *et al.*, 2000; Kitajima and Urakami, 2000). Lesser amounts of SM50 are occluded within the mineral. It is not known yet whether either SM30 or 50 are associated with ACC precipitates in the cell, nor is it known whether they are co-localized in the mineral of the spicule. While inhibitors of spicule elongation, like protease inhibitors, lathryogens or lack of serum in micromere cultures severely depress SM 30 accumulation, they have much less effect on SM 50 accumulation.

Peled-Kamar *et al.* (2002) have recently devised a new approach to study the function of SM 50. She injected phosphothiorate linked anti-sense deoxynucleotides into the blastocoel of late cleavage stage *S. purpuratus* embryos. This resulted in a specific block of spicule elongation; though the initial calcite nucleation occurred, spicule elongation was blocked. The antisense treatment lowered the accumulated SM50 protein by 80%. Peled-Kamar was able to show that the oligonucleotide was taken up by the PMCs, and the effect was highly specific and reproducible. The difficulty in interpretation of this kind of experiment is that while it strongly implicates SM50 in biomineralization of elongating spicules, it does not tell us where in the pathway SM50 is acting, or how it acts. It may be a first step, however, in delineating an important component in the biomineralization process. It is noteworthy that the main extracellular location of SM 50 is not within the mineral, but in the envelope surrounding the mineral.

Another approach that we have already mentioned is to use functional inhibitors rather than loss of function of specific proteins. Thus, inhibition of metalloproteases, glycosylation inhibitors specific for various steps in the glycosylation process, or inhibitors of collagen processing and cross linking implicate various steps of the secretion process. Of particular interest is the inhibition of collagen function. Application of antibodies against collagen or inhibitors of collagen cross linking inhibited spicule formation (Wessel *et al.*, 1991), Collagen is not a protein occluded in the mineral. Some form of collagen could conceivably be part of the extracellular matrix envelope, and the integrity of this envelope (which itself is being dynamically formed as spicule formation proceeds) may be important in actual mineralization.

Conclusions

Calcium carbonate based biomineralization is very widespread, especially in invertebrates. Biological mechanisms of calcite or aragonite deposition are surely not the same everywhere. Mollusc shells, composed of both calcite and aragonite, are formed in an extracellular matrix at some distance from the secretory mantle, and the proteins found there (cf. Belcher *et al.*, 1996) are not the same as in echi-

noderns. We do not even know how widespread are the molecules and mechanisms found in sea urchin embryos. Based on the distribution of SM50, SM30 and PM27, adult tissues of urchins may utilize similar matrix molecules, There is almost no information on matrix molecules of other echinoderm classes. Only the ophiuroid embryos among the other echinoderm classes have endoskeletal spicules, but the proteins of their spicules have not been examined yet. Nor do we know if such proteins exist in urochordates, hemichordates, or cephalochordates. An examination of other forms related to euechinoids should be rewarding.

The cell biological underpinnings of the secretion of mineral and matrix has not occupied a central place in the study of biomineralization. There is a good reason for this since only in the last decade has there been considerable progress in understanding the molecular basis of secretion. There are splendid opportunities now to learn more of how spicules, shells, carapaces and the like are deposited by the cells that elaborate them. We shall surely encounter variety in the cell biology of mineral formation. Perhaps there will be some common themes and mechanisms as well. If there are common themes, it is possible that some of these common mechanisms will also be found to apply to the calcium phosphate based minerals prevalent in vertebrates.

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