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COMPARISON OF BIOCALCIFICATION PROCESSES IN THE TWO CORALLINE DEMOSPONGES *ASTROSCLERA WILLEYANA* LISTER 1900 AND *"ACANTHOCHAETETES" WELLSI* HARTMAN AND GOREAU 1975

G. Wörheide[1], J. Reitner[1] and P. Gautret[2]

[1]Institut und Museum für Geologie und Paläontologie, Universität Göttingen, Goldschmidtstr. 3, 37077 Göttingen, Germany
[2]Laboratoire de Pétrologie sédimentaire et Paléontologie. Université Paris-Sud, Centre d`Orsay, Bât. 504, 91405 Orsay Cedex, France

ABSTRACT

The different biomineralization processes of the two "living fossils" *Astrosclera willeyana* and *"Acanthochaetetes" wellsi* were studied by epifluorescence microscopy, SEM, TEM, and biochemical analyses. The basal skeleton of *A. willeyana* is made of aragonitic spherulites. These spherulites are built in the dermal layer in large vesicle cells (LVC) in the early stages. After a releasing event the spherulites fuse together by epitaxial growth. In *"A." wellsi* the basal skeleton is made of high Mg-calcite. It is constructed of bundles of elongated, tangentially orientated crystals. The biomineralization takes place in 3 different places in the skeleton, the active zones are localized in the uppermost parts of the skeleton. In both cases the mineralization is associated with highly acidic mucus substances, rich in aspartic and glutamic acid, which control the biomineralization processes. The understanding of the biomineralization processes in these two cases gives insight into the formation of the skeleton of ancient reef constructors like the stromatoporoids and the chaetetids.

INTRODUCTION

Calcified sponges were dominant reef building organisms at the beginning of the Phanerozoic. The archaeocyaths are thought to be the oldest representatives. Later the very widely developed and diversified stromatoporoids and chaetetids occurred. Since the late Paleozoic they developed a large variety of skeletal morphologies and microstructures. Their role was again very important at different periods of the Triassic, Jurassic, and the Cretaceous. Replaced in their reef-building function by scleractinian corals in modern reefs, the living relatives of these calcified sponges ("coralline sponges") are living in cryptic habitats of almost all Recent coral reefs. Back in the Cambrian sponges were the first metazoans producing a carbonate skeleton. Their microstructural features have remained nearly unchanged over the very long period of time. The biomineralization processes are extremely conservative and still present in extant calcified sponges.

Both studied coralline sponges are ultraconservative taxa with very slow phylogenetic alterations. The oldest representatives of the agelasid taxon Astrosclerida are known from middle to late Permian reefs of Tunisia and Sicily. The diversity of this type of coralline sponge with intracellularly formed spherulites was very high in the late Triassic. Fossil remains of *Astrosclera* are rare during younger earth periods. The extant genus *Astrosclera* is the only relic taxon which occurred first in Cenomanian cryptic reef environments of the Tethyan realm (Reitner 1989). The taxon *"Acanthochaetetes"* occurred first in the Lower Cretaceous. The oldest types are known from the lower Aptian of northern Spain and the Mural Limestone in Arizona (USA). This type of sponge has close phylogenetic affinities to the soft sponge *Spirastrella* (Vacelet 1985; Reitner 1992) and is restricted to dark cryptic niches of tropical reefs of the Tethyan realm. (Reitner 1989; Reitner and Engeser 1987).

Within this group of organisms very little is known about the modalities of skeletal formation. The basal carbonate skeleton is produced independently from the spicular skeleton and combines a mineral phase of calcium carbonate (calcite or aragonite), and a complex organic matrix. The latter reveals two major compounds when extracted during decalcification using EDTA or acetic acid (pH 4): the so called "soluble organic matrix" (SOM) is mobilized with calcium salts while the "insoluble organic matrix" (IOM) can be easily isolated by centrifugation. The organic matrix plays an important role for the nucleation of initial seed crystals as well as for the further growth of the skeleton (Simkiss 1986; Mann et al. 1989).

Biomineralization events have been investigated in two different taxa of coralline sponges from the Great Barrier Reef (Australia). The secondary calcareous skeleton of the agelasid stromatoporoid *Astrosclera* is made of aragonite spherulites (Lister 1900; Ayling 1982; Reitner 1992), the hadromerid chaetetid *"Acanthochaetetes"* produces a high Mg-calcite skeleton beside the spicular skeleton (Hartman and Goreau 1975; Reitner and Engeser 1987). The present study is an attempt to understand the processes of biomineralization in two different coralline sponge taxa and to locate the different sites of mineralization during skeletal formation.

MATERIAL AND METHODS

The investigated specimens of *Astrosclera* and *"Acanthochaetetes"* were collected by SCUBA diving in shallow water reef caves around Lizard Island in the northern part of the Great Barrier Reef (Australia) (Fig. 1).

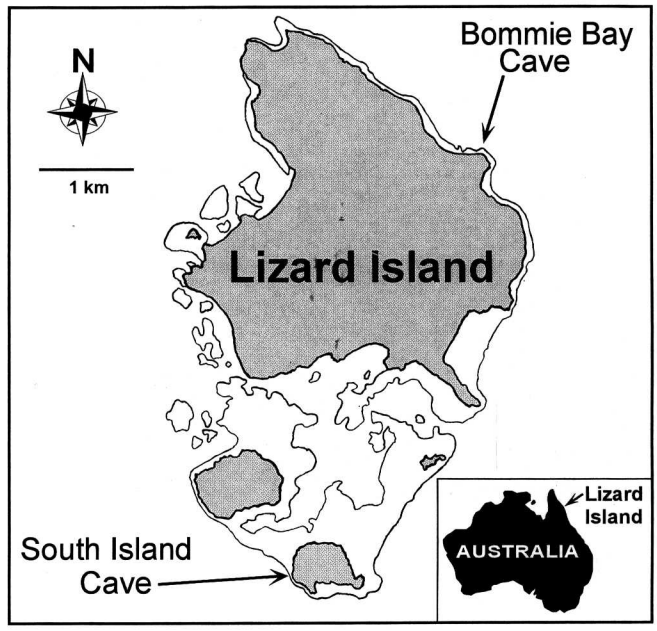

Fig. 1: Location of investigated reef caves at Lizard Island (northern Great Barrier Reef, Australia)

For histological, SEM, and TEM studies some specimens were fixed in a sodium cacodylate-buffered 4% glutaraldehyde solution in seawater immediately after collecting. They were stored at 8-4°C in the refrigerator for 1-2 days according to their size, then washed in running sea water. Postfixation was carried out in 2% osmium tetroxide solution in filtered sea water for 1-3 hours depending on the amount of organic matter. After fixing, the specimens were washed in 30% ethanol, moved in ascending ethanol concentrations and stored at 70%. For TEM, the specimens were first decalcified with EDTA and then desilicified in 5% HF. Samples for SEM were not demineralized, but dehydrated with 100% ethanol, and treated with PELDRI II (Plano), then shock frozen with liquid nitrogen and immediately broken.

Some specimens were selected for *in vivo* investigations using staining techniques. These were stained with Ca^{2+}-chelating fluorochromes (Calcein, Na_2-Calcein, Achromycin-HCl, and Chlorotetracycline-HCl) which allow one to study growth rates and the *loci* of calcification fronts. The sponge was *in situ* covered with a plastic bag filled with fluorescent dye. Because of the toxicity of the tetracyclines (antibiotics) to the symbiotic bacteria of the sponge, we used highly concentrated solutions in seawater and stained the specimens between 15-30 minutes only. Using short staining times with very high concentrations was successful and not lethal for the sponge (Reitner 1993; Gautret et al. 1996).

For examination they were embedded in epoxy resins (araldite and LR White) and cut with a LEITZ hardpart microtome. Thin sections were studied with an epifluorescence microscope ZEISS Axiophot using the UV high-performance narrow-band pass filter 01 (BP 365/12 nm, LP 397 nm, and wide-band pass filters 05 blue violet (BP 395-440, LP 470), 09 blue (BP 450-490, LP 520), and 17 green (BP 485-515, LP 565).

Also non-fluorescent histological staining dyes with the basic red fuchsin, toluidine blue O, and methylene blue were applied to fixed specimens for histological studies using optical microscopy (more details in Reitner 1993).

Freeze-dried specimens were used for biochemical analysis of the intraskeletal organic phase (amino acid and monosaccharide composition). For the biochemical analyses, the intraskeletal organic matrices were extracted by a soft decalcification with acetic acid (pH 4, automatically controlled by a titrimeter) and separated from calcium salts by a desalting gel filtration chromatography with a Sephadex 25 column after a centrifugation aiming to remove insoluble compounds such as collagen fibrils. Half of the purified soluble matrix material was used for the determination of amino acid contents, the other half for the monosaccharide composition.

The amino acid composition was determined after 6N HCl hydrolysis (24 hrs, 1 IO'C, in free air sealed tubes) and phenylisothiocyanate (PITC) derivatisation, by reverse phase chromatography using a HPLC-BECKMAN system (Na-phosphate buffer with a nonlinear gradient of acetonitrile) and a 5gm-C18 NUCLEOSIL column.

Glucidic components were hydrolyzed with 2N TFA (4 hrs, 1 100C). Neutral and amino monosaccharide chromatographic separation was performed with an HPAE (High performance anion exchange)-DIONEX system coupled to a pulsed amperometric detection (PAD) with a sodium hydroxyde gradient (10 to 100 mM).

RESULTS

Astrosclera willeyana Lister 1900

The soft tissue organization of *Astrosclera willeyana* exhibits an easily recognizable architecture in a dermal zone (Fig. 2), a choanosomal part, and a prominent exhalant system (cf. Lister 1900, Pl. XLVI, XLVII). The soft tissue itself occupies only a few millimeters of the youngest portion of the basal skeleton. The inner choanosomal layer is characterized by a more or less dense mesohyle with numerous bacteria (mostly *Vibrio*-types) and small choanocyte chambers (about 10-15 µm). The bacteria may represent 30-50 % of the biomass within this zone. The prominent exhalant canal system ends in astrorhizae patterns often located on superficial mamelons.

The biocalcification processes in *Astrosclera* can be divided into two main processes:

1. The dermal layer and related mesohyle is free of bacteria and enriched in motile cells. Most of them have an archaeocyte character.

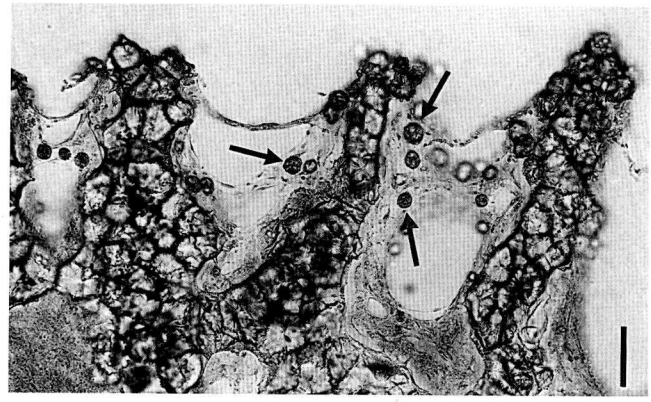

Fig. 2: Light microscope photograph showing the upper part of the basal skeleton (dermal area) of *A. willeyana* with newly formed spherulites (arrows). Scale Bar: 50 µm.

The mesohyle is formed by a dense network of EDTA insoluble fibers where the mobile cells are moving. Within the studied specimens up to 60 % of the mobile cells are spherulite forming (large vesicle cells = LVC). They increase in size as they grow. The smallest of the LVC have a size of 3-5 µm and the largest of about 20 µm. The external shape of the LVC is more or less round to egg-shaped. They possess a large nucleus with a nucleolus, abundant mitochondria, and a lot of small vacuoles with reserve granules and/or phagocytised bacteria. The granules are extremely electron dense (osmophilic) and therefore enriched in lipids. Early ontogenetic stages of the LVC are characterized by one large vacuole which includes a minimum of 50 % of the total volume of the entire cell. In mature cells, shortly before the release of the spherulite from the cell, the volume of the vacuole is more than three times larger than that of the remaining cell (Fig. 3). The vacuole is primarily filled up with a three dimensional network of fibers and sheets which are probably formed under control of the electron dense reserve bodies. Sheets and fibers form small containers (30-50 nm) in which the first seed crystals are formed. The entire vacuole is filled up with Ca^{2+}-binding glycoproteic mucus which exhibits a strong tetracycline and/or calcein induced fluorescence.

The seed crystals are euhedral and in the early, 2-3 µm stage, randomly orientated. In the later stages of development (3-10 µm) the seed crystals become more orientated in the direction of the c-axis of an aragonite crystal. All observed aragonite fiber crystals of the spherulites are compounds of 30-50 nm-sized seed crystals (Reitner 1992). The aragonite fiber crystals are now more or less radially orientated and form aster shaped spherulites with a large remaining space filled with acidic organic mucus. The aster spherulites hatch normally in the 15µm stage. At this

stage the LVC is lysing, the membranes are broken and the aragonite asters are free in the mesohyle. In some sections an enrichment of small amoebocytes was observed which probably transport the asters to certain places.

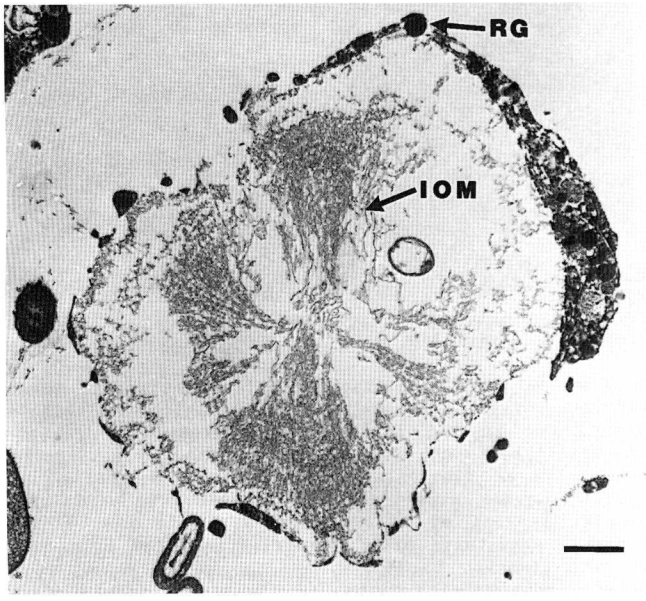

Fig. 3: Large vesicle cell (LVC) of *A. willeyana* with a large vacuole containing a newly formed spherulite. The vacuole is totally filled with the insoluble organic matrix (IOM) of the spherulite, now visible after decalcification. RG = reserve granule. TEM section, decalcified with EDTA. Scale Bar: 1 µm.

2. The isolated asters grow together by epitaxial processes. The spherulite-fibers, no longer embedded in a cell, grow in the direction of the c-axis of the aragonite crystal. When the fibers get in contact with the fibers of other spherulites, they interfinger with them and stop growing (Fig. 4). The fibers not disrupted in growth grow until they interfinger with other spherulites.

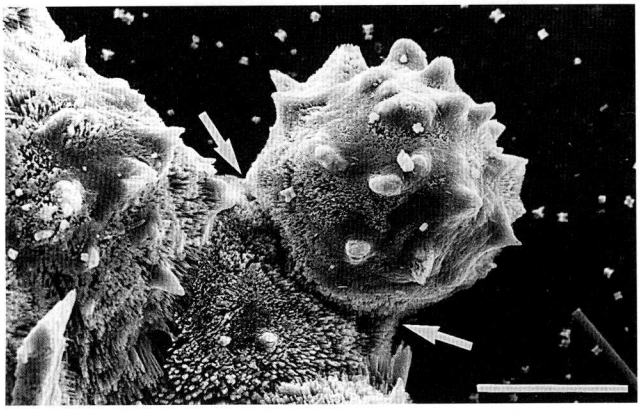

Fig. 4: SEM photograph of *A. willeyana*, showing an aster-shaped spherulite. The aragonite fibers of the spherulite interfinger with fibers of other spherulites, already forming the basal skeleton (arrows). Scale Bar: 20 µm.

Due to this cessation of growth, the spherulites assume an asymmetrical shape in the older part of the basal skeleton. Mostly they show one elongated part and develop an "egg-like" shape (cf. Gautret 1986: 83, Fig.1d). After enzymatic proteolysis, which destroys the organic envelope, distinctive concentric growth lines are visible (cf. Gautret 1986: 106, Pl. III, Fig. 3/4). The fibers are about 0,5-1 µm in diameter and are composed of parallelly arranged smaller fibers of about 50-70 nm in diameter (Gautret 1986). The surface of the growing spherulites is covered by basopinacocytes. The space between the top of the fibers and the basopinacocytes is filled with acidic mucus, which exhibits a strong calcein induced yellow epifluorescence (cf. Wörheide et al. in press, Pl. 1). This mucus functions as a buffer for Ca^{2+}-ions and controls therefore the speed and direction of the epitaxial growth of the aragonite fibers. The process of mucus formation and origin (secreting cell type) is not yet fully understood in detail.

The amino acid and monosaccharide composition were studied on an approximate 400-year-old specimen from Ribbon Reef No. 10 (Lizard Island Section, Great Barrier Reef, Australia) (detailed data in Wörheide et al. in press). Amino acid and monosaccharide composition of the insoluble intracrystalline matrix are very stable in all portions of the skeleton. No strong diagenetic effect on the insoluble organic matrix (IOM) is visible due to the stable composition. The IOM is dominated by proteins and is represented by the intravacuole fibers and sheets forming the containers for the seed crystals. Collagen was not detected in the IOM.

The soluble organic matrix (SOM) is characterized by acidic glycoproteins, high amounts of proline, which is needed for the synthesis of glutamic acid, and high amounts of aminosugars. The glucids are the dominant fraction of the SOM. The character of the SOM is very typical for Ca^{2+}-binding mucus substances. A strong diagenetic effect is visible in the SOM, both in composition of amino acids and monosaccharides and in the quantity (cf. Wörheide et al. in press).

***"Acanthochaetetes" wellsi* Hartman and Goreau 1975**

Only the youngest part of the calicles (0,5-1 mm thick) of the chaetetid-type basal skeleton is occupied by the living soft tissue. Soft tissue and basal skeleton exhibit a vertical anatomy divided in five major zones (Fig. 5).

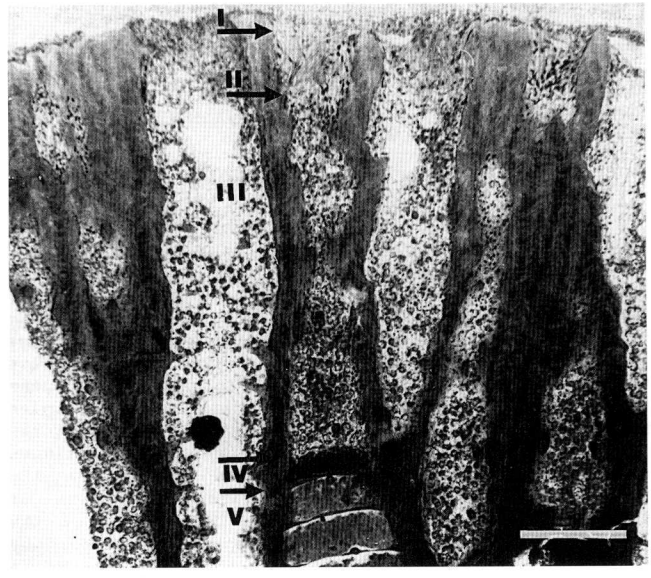

Fig. 5: Vertical section of *"A." wellsi* showing the 5 major zones of the living part of the skeleton: I. outer dermal area with layer of spiraster microscleres, II. internal dermal area with LCG´s, III. choanosome, IV. enrichment of LCG´s upon horizontal tabula, V. closed space between tabulae containing storage cells (Archaeocytes). Scale Bar: 600 µm.

At the uppermost dermal area settles a thick crust layer of spiraster microscleres (Zone I) and tylostyle megascleres which are arranged in clear plumose bundles suggesting a close phylogenetic relationship to *Spirastrella*. Below the outer dermal area, the internal dermal area (Zone II) is formed by mesohyle tissue. It is enriched in mobile cells and devoid of choanocyte chambers. Large inhalant chambers (lacunae) and canals cross this zone, serving the choanosome with water filtered through the ostia. The mesohyle is characterized by large cells (ca. 10 µm) containing numerous inclusions (LCG: large cells with granules) (Reitner 1992). These cells are mobile. LCG cells are not typical spherulous cells as known from *Vaceletia crypta*. Their shape varies often and normally they exhibit a triangular and flat shape. Only in rare cases do they show a spherulous shape.

The biocalcification process can be divided into three main locations and processes:

1. LCG´s are enriched in the upper part of the tubes at the top of the walls. The LCG´s are responsible for the secretion of collagen fibrils (cf. Reitner and Gautret 1996: Pl. 50, Fig. 1) and they probably derive from lophocytes. Collagen fibrils form strong bundles which cross through the basal pinacocyte layer, and anchor into the rigid skeleton (Vacelet and Garrone 1985; Reitner and Gautret 1996)(Fig. 6).

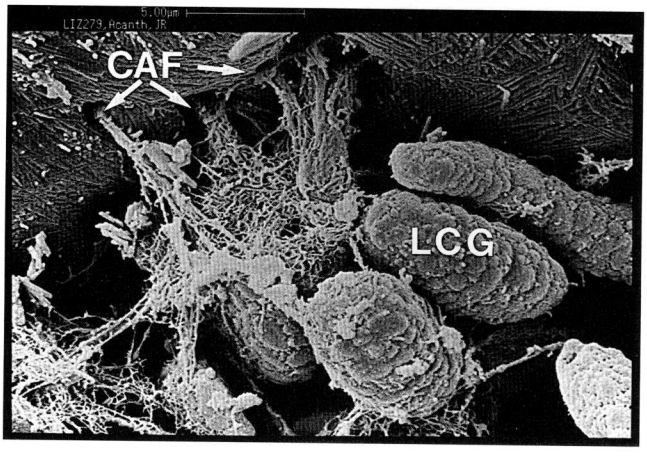

Fig. 6: *"A." wellsi*: LCG´s secreting collagen anchor fibers (CAF), anchoring in the basal skeleton (arrows). SEM photograph. Scale Bar: 5 µm.

The space between the basopinacoderm and the calcareous wall is filled up with acidic mucus substances, probably derived from metabolic processes during the synthesis of the collagen fibrils. This mucus and the aquatic fluids are enriched in Ca^{2+}-ions, detected by a strong epifluorescence behavior in calcein and chlorotetracycline stained specimens. The aquatic fluids exhibit an increased carbonate alkalinity. The source of the carbon is not related to the sponge metabolism but to the ambient seawater (Reitner 1992, 1993). The mucus is acting as the soluble organic matrix and is forming molecular monolayers in the form of ß-sheet structures (cf. Simkiss 1986) on the above described collagen fibrils. The collagen fibrils are acting as the insoluble organic matrix. The first nucleation of irregular Mg-calcite seed crystals takes place on the ß-sheet. The mature high Mg-calcite crystals formed by this process have a size of 0,5-1µm and exhibit often an anhedral shape (Fig. 7).

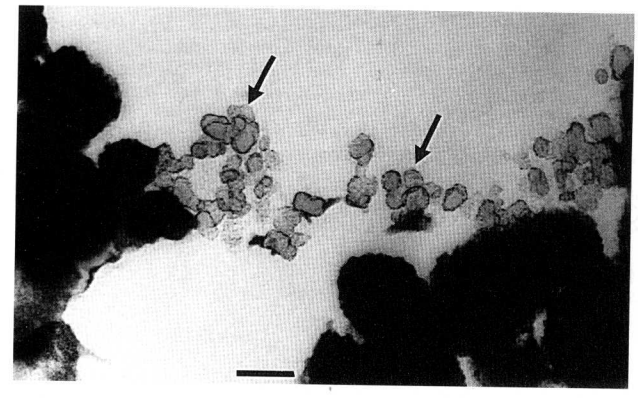

Fig. 7: *"A." wellsi*: TEM photograph of anhedral high Mg-calcite seed crystals (arrows). Decalcified with EDTA. Visible are the remains of the organic matrix (IOM). Scale Bar: 250 nm.

2. A second type of collagen fibrils is present. These fibrils are produced by lophocytes which are widely distributed in the intercellular mesohyle. "*Acanthochaetetes*" bears only few small-sized bacteria, located between these fibrils. At the top of the walls the fibrils become organized into a weak frame-building matrix. Remains of this matrix are entrapped inside skeletal structures after calcification. The main area of calcification is located in the very narrow space between the basopinacoderm and the mineralized surface of basal skeleton. This space is filled up with acidic mucus substances. The basopinacocytes produce soft folded organic strings (Fig. 8) ("cooked spaghetti" *sensu* Reitner and Gautret 1996), which are templates for the acicular high Mg-calcite crystals. The mucus substances become organized on these templates in a molecular monolayer (ß-sheet, SOM, see above). Mineralization starts on the templates in the form of very small seed crystals (50-100 nm). They grow together epitaxically (c-axis orientated) and during this process the folded templates become stretched. Newly formed high Mg-crystals exhibit therefore a strong "knobby" structure (cf. Reitner and Gautret 1996, Pl. 51, Fig. 7). Due to further epitaxial crystal growth the irregular biocrystals become flat as known from the mature basal skeleton. The mature high Mg-calcite crystals have an elongated, acicular shape, an average length of 2-5µm, and a diameter of 200-500 nm (Fig. 9).

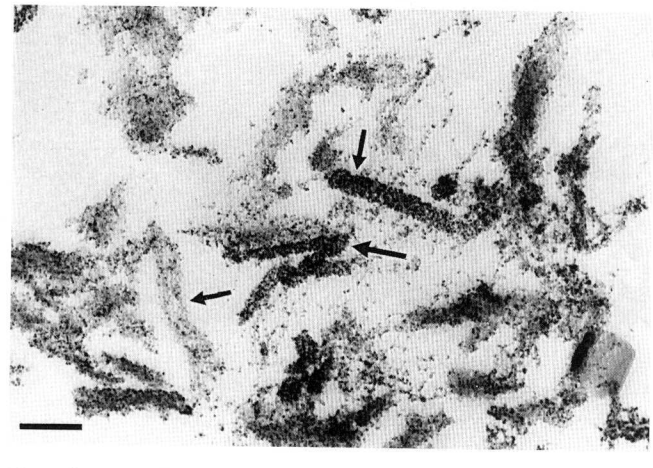

Fig. 8: TEM photograph of *"A." wellsi* showing the soft folded organic strings (arrows), produced by basopinacocytes in the narrow space between the basopinacoderm and the basal skeleton. These organic strings are templates for seed-crystal nucleation. Scale Bar: 500 nm.

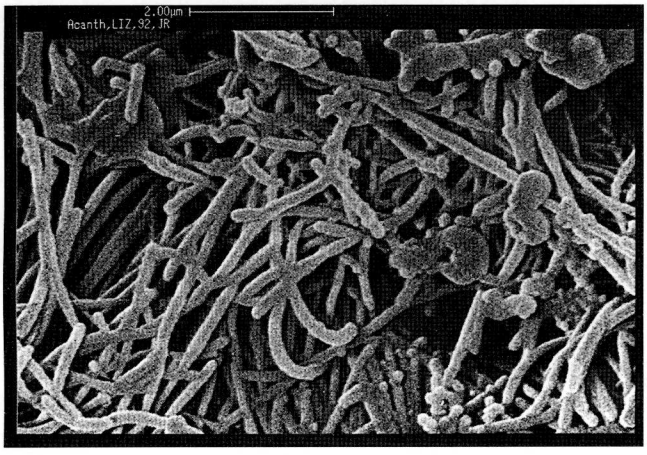

Fig. 9: SEM photograph of *"A." wellsi* showing the soft folded organic strings in a later stage of development. Compare Fig. 8. Scale Bar: 2 µm.

3. The third area where biomineralization happens consists of the horizontal tabulae which are dividing the calicle tubes of the chaetetid skeleton (zone IV). These are formed by the basopinacoderm also, first as a thin organic phragma or sheet. Below the choanosomal zone, LCG cells become sometimes enriched and cause the mineralization of the organic sheet (Fig. 10). Continuous upward moving basopinacoderm is forming a space filled with Ca^{2+}-binding and mineralizing organic mucus as known from the upper portions. This mineralizing process happens only when LCG´s are present (Reitner 1992).

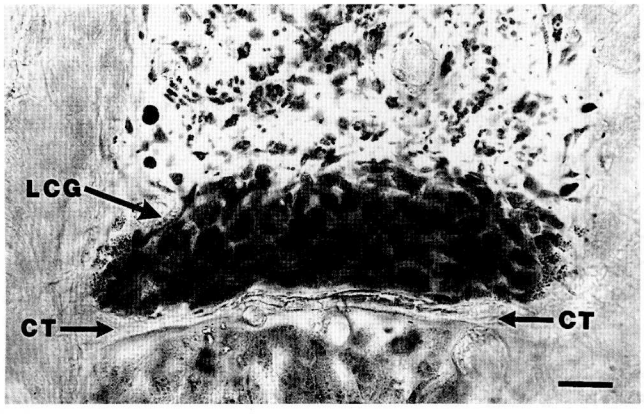

Fig. 10: Light microscope photograph of *"A." wellsi* showing an enrichment of LCG´s directly above a calcified tabula (CT). Scale Bar: 50 µm.

The mineralization of the skeleton in all above described situations is only happening when the LCG´s are present and physiologically active.

The closed spaces between tabulae contain accumulations of modified archaeocytes with numerous storage granules (thesocyte-like cells) and few spiraster microscleres (zone V). These cells play a role in regeneration processes (Vacelet 1985, 1990) enabling the sponge to grow again when it has been drastically damaged.

The soluble matrix extracted from the superficial part of the skeleton contains high amounts of glycine, proline and hydroxyproline-rich compounds (collagenic affinity). Amino sugars are enriched in this zone. The presence of highly concentrated materials with collagenic or glucidic affinities results in the fact that relative amounts of acidic amino acids (Asp and Glu) appear less represented here than in the immediately underlying older part. However, absolute quantities of these two amino acids should be at least 3 to 5 times higher in the uppermost part of the skeleton (Gautret et al. 1996). The transformation starting from the area immediately below the active mineralizing zone exhibits a regular tendency with decreasing acidic amino acids as the most obvious feature, whereas aromatic amino acids (Tyr and Phe), serine and amino sugars also decrease. The increasing constituents are basic and aliphatic amino acids (mainly glycine).

The insoluble matrices exhibit quite similar, collagenic amino acid compositions in all parts of the skeleton. Only the quantity of insoluble matrix changes in an important way, decreasing considerably from the surface to the base. This matrix completely differs from soluble compounds, with much less acidic amino acids, less serine and threonine and almost no amino sugars. It is strongly enriched in all aliphatics (Gly, Ala, Val, Leu), aromatics (Phe, Tyr), proline and hydroxyproline (for detailed data see Reitner and Gautret 1996).

DISCUSSION

Two different *modi* of formation of the rigid calcareous basal skeleton in two different taxa of coralline sponges were described. Leading to a comparable end-product, a rigid calcareous skeleton, each taxon forms the basal skeleton in its highly specialized way using different modifications of calcium carbonate (*Astrosclera* = aragonite; *"Acanthochaetetes"* = High Mg-calcite). In both cases modified lophocytes play an important role in the initiation of the mineralization. In *Astrosclera*, the LVC´s form a template of 3-dimensional fibers inside a large vacuole. The vacuole is filled with highly acidic mucus, rich in Asp and Glu. The fibers and the mucus act as the organic matrix for seed crystallization. In *"Acanthochaetetes"*, the lophocytes secrete collagen fibrils which act sometimes as a framebuilding matrix for the soluble highly acidic organic matrix which initiates the nucleation of calcite seed crystals. At a later stage, modified basopinacocytes control the mineralization process. In *Astrosclera*, basopinacocytes cover the released and fused spherulites. The epitaxial growth of the spherulites is controlled by a highly acidic mucus between the crystal surface and the basopinacocytes. This mucus is EDTA soluble and exhibits a strong calcein induced epifluorescence. In *"Acanthochaetetes"*, the epitaxial crystal growth is also controlled by acidic mucus substances, filling up the space between the basopinacocytes and the mature basal skeleton or the tabulae.

Most important in both cases is the presence of an organic matrix, forming a template for crystal nucleation (Simkiss 1986; Mann et al. 1989). The presence of a soluble organic mucus, rich in Asp and Glu, is controlling the crystal growth in the earliest and latest stages of mineralization. The mineral preference, aragonite or Mg-calcite, is controlled by the structure of the organic macromolecules, forming molecular monolayers (ß-sheets) on an EDTA insoluble template (IOM) and acting as attractors for divalent cations (Ca^{2+}). The distances of the free carboxyl-groups of the macromolecules control the crystallographic base plane of the initial calcite or aragonite crystal (Mann et al. 1989; Reitner 1993).

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