Calcification in echinoderms

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CONTENTS

1 Introduction
2 The skeleton-forming cell and its relationships to the mineral phase
  2.1 The skeleton-forming cell of larvae
  2.2 The skeleton-forming cell of postmetamorphic echinoderms
  2.3 Discussion
3 The organic matrix
  3.1 The membrane of the calcifying site
  3.2 The trabecular coat
  3.3 The intrastereomic organic components
  3.4 Discussion
4 Ions
  4.1 Calcium ions
  4.2 Carbonate ions
  4.3 Protons: acid-base balance at the calcifying site
  4.4 Magnesium ions
  4.5 Trace elements
5 Skeletal dynamics
  5.1 Skeletal resorption
  5.2 Skeletal dynamics
6 Conclusion
7 Acknowledgements
8 References

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1 INTRODUCTION

Biominerals are found in all major phyla. They are involved in basic biological functions such as structural and mechanical devices, acid-base buffering systems, ion reservoirs and detoxification (Simkiss 1980, Lowenstam 1981). Biominerals are formed either extracellularly or intracellularly. In triploblastic metazoans, they are built up either by ectodermal or by mesodermal tissues, a difference that could indicate their polyphyletic origin (for discussion of the evolution of biomineralization, see Lowenstam & Weiner 1983).

Biomineralization results from complex physicochemical interactions (for review, see Mann 1983). Two main processes of biomineralization are recognized, viz. the biologically induced process and the biologically controlled process (Lowenstam 1981, Mann 1983). It should be clear, however, that these two processes are the limits of a continuous spectrum in which the organism exercises increasing control over the properties of the mineral to be deposited (Lowenstam & Weiner 1983).

The biologically induced process is the 'primitive stage' of biomineralization (Lowenstam 1981). The mineral is formed under minimal biological control as a result of interactions between the activity of the organism and the surrounding physical environment, i.e. between biologically produced metabolites and cations present in the external environment. The mineral is formed without the elaboration of any organic matrix and displays crystal properties similar to those formed by inorganic processes. Such a process is widespread among the monerans and also occurs in some green and red algae (Lowenstam & Weiner 1983).

The biologically controlled process – or matrix mediated process – of biomineralization is under strict genetic control. An organic matrix always occurs (the organic matrix is defined as 'any localized surface comprising organic constituents such as lipids, proteins, and carbohydrates, which acts as a mediator of mineralization in biological systems'; see Mann 1983). This matrix acts as a mediator in facilitating and regulating both nucleation and crystal growth by offering mechanical support, limiting the volume and constructing an organic framework or mold. Ions are actively introduced into the organic matrix where crystal formation and growth is induced (Lowenstam 1981, Mann 1983). The biologically controlled process results in minerals with well-defined properties, often in disequilibrium with the external physicochemical conditions (Lowenstam & Weiner 1983). It occurs in most eukaryotic phyla.

Three levels of regulation (viz. chemical, spatial and structural) may be defined in the biologically controlled process of biomineralization depending on how the organic matrix is involved (Mann 1983). At the chemically controlled level, the organic matrix is simply a membrane limiting a vesicle.

This allows control of the level of supersaturation of major ions, the concentration of coprecipitating ions and the level of accelerator/inhibitor molecules at the mineralizing sites. There is no need for an organic matrix to be present within the deposited minerals at this level. In contrast, the organic matrix plays a leading role in both the spatially and the structurally controlled levels. At the spatially controlled level, the organic matrix forms a tri-dimensional framework which acts either by limiting volumes in which numerous mineralization sites are promoted, or as a meshwork that orientates growth or aggregation of crystallites. As a consequence, at the spatially controlled level, the organic matrix pervades the whole mineral deposit. At the structurally controlled level, the organic matrix is a two-dimensional net which determines both the crystallographic structure and the orientation of growing minerals through interfacial interactions. At this latter level of control, the organic matrix intimately contacts the mineral phase – eventually through ionic bonds – but can only surround it.

Echinoderms deposit only a few biominerals, namely, high-magnesium calcite (making the endoskeleton), protodolomite (in the consolidated part of echinoid teeth), amorphous hydrous ferric phosphate and opal (in dermal granules of molpadiid holothuroids) (Chave 1952, Schroeder *et al.* 1969, Lowenstam & Rossman 1975); weddelite and ilmenite have also been reported incidentally (Lowenstam & Weiner 1983). Almost all investigations have been devoted to the principal biomineral deposited by echinoderms, i.e. the high-magnesium calcite making the endoskeleton.

The echinoderm endoskeleton is of mesodermal origin, a fact that markedly distinguishes the phylum from most other invertebrate phyla and unites it with the Chordata. The skeleton of postmetamorphic echinoderms basically consists of single elements – the ossicles – bound together by connective and/or muscular fibres and which form the integument skeleton (including the outer appendages and the jaw apparatus). The ossicular structure is highly characteristic in that each ossicle consists of a tridimensional mineral network (the so-called stereom; Plate 1A) which delimits an internal and complementary network filled by connective tissue (the so-called stroma; Plate 1B). Simpler skeletal elements (or spicules) also exist, however. They build up the larval skeleton (e.g. the pluteus skeleton), and are seen in the holothuroid body-wall (most holothuroids lack intra-integumentary calcareous plates), as well as in the connective tissue layer of some organs of postmetamorphic individuals (viz. ambulacral organs, gonads or digestive organs).

The present review is devoted to calcification mechanisms of echinoderms. Calcification will be addressed in regard to the cellular and molecular aspects, such as the relationships between the mineral phase and the forming cell, the question of the organic matrix, the nucleation and growth of calcite, the regulation and dynamics of the incorporation of involved ions, and the fate of calcite after formation.

2 THE SKELETON FORMING-CELL AND ITS RELATIONSHIPS TO THE MINERAL PHASE

2.1 *The skeleton-forming cell of larvae*

The larval skeleton of echinoderms consist of rods which serve supporting functions; these rods are largely resorbed during metamorphosis. Only ophiuroids and echinoids develop an actual larval skeleton. It is well established that the skeleton-forming cells of larvae are the primary mesenchyme cells, i.e. the cells that migrate into the blastocoel just prior to gastrulation (however, in larvae of cidaroid echinoids, the migration of the skeleton-forming cells seems to occur later, i.e. after the mid-gastrula stage; Schroeder 1981). The skeleton-forming cells of echinoderm larvae have been investigated only in echinoid larvae (pre-1960 studies dealing with the formation of the larval skeleton were reviewed by Okazaki 1960). Bevelander & Nakahara (1960) reported that initial spicular growth takes place within the cell body of a single mesenchyme cell. They reported, moreover, that spicular growth goes on extracellularly as soon as the rudiment becomes triradiate. This proposal, however, has been rejected by most authors who agree that spicule formation results from a syncytium in which several primary mesenchyme cells are involved (see, e.g. Okazaki 1960, 1965, 1975a, Wolpert & Gustafson 1961, Gustafson & Wolpert 1961). According to them, primary mesenchyme cells gather together to form two cell aggregates and at least four cell chains (Fig. 1). The cell aggregates lie in the basal part of the embryo on both sides of the archenteron, while the cell chains occur above the aggregates (one longitudinal cell above each aggregate) and between the aggregates (two latitudinal cell chains surrounding the archenteron and located in the spaces separating the two aggregates). As soon as the aggregates are formed their cells send out pseudopodia that progressively fuse together and form two central triangular hyaloplasmic areas (one area in each aggregate) (Fig. 2A-C). The two rudiments of the larval skeleton develop (as a small calcareous granule) within each triangular area (the so-called skeletal envelope; see Okazaki 1960). The granules then increase in size and take a triradiate shape (Fig. 2D-E). The cells grouped in chains behave similarly to those of the aggregates, i.e. they send out pseudopodia that also fuse together forming a pseudopodial strand in which local enlarged hyaloplasmic areas occur (Fig. 2C-E). As soon as the rudiment becomes triradiate, the extremities of the strands adjacent to the aggregates fuse with the closest corner of the triangular areas (Fig. 2E). This results in an increase of the skeletal envelope in which the spicule will extend. The growing spicules thus always lie – at least until the late endotrophic stage of the pluteus – within the cytoplasm of the syncytium formed by the pseudopodia of the primary mesenchyme cells.

When cultured *in vitro*, isolated primary mesenchyme cells or their

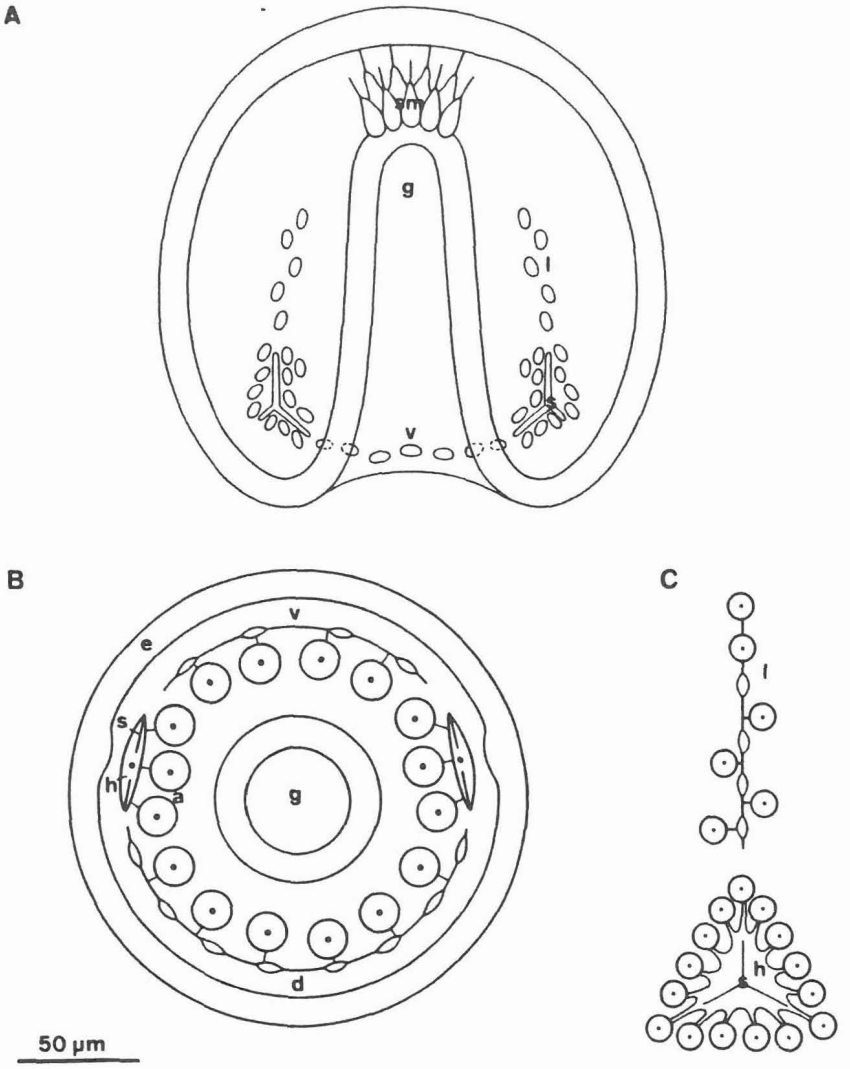


Figure 1. Location of the primary mesenchyme cells forming aggregates and chains in the euechinoid embryo (A, adapted from Hyman 1955; B and C, adapted from Okazaki 1975a). A. Late gastrula stage (ventral view) showing the two aggregates with spicules, the two longitudinal cell chains and the ventral cell chain. B. Late gastrula stage: cross-section showing the location of the aggregates as well as the ventral and dorsal cell chains. C. Late gastrula stage: front view of a mesenchymal cell aggregate with its triangular hyaloplasmic area (enclosing the triradiate spicule) topped by its corresponding cell chain. a: mesenchymal cell aggregate; d: dorsal cell chain; e: ectoderm; g: archenteron; h: hyaloplasmic area of a mesenchymal cell aggregate; l: longitudinal cell chain; s: spicule; sm: secondary mesenchyme; v: ventral cell chain.

precursors (i.e. micromeres) also form larval spicules (Okazaki 1975b, Harkey & Whiteley 1980, Kitajima & Okazaki 1980). As observed *in vivo*, the cells gather together and form aggregates and chains (however, they do not show the definite spatial arrangement observed in whole larvae). The spicule rudiment appears in the hyaloplasmic area formed by some cell aggregates and develops either into a triradiate spicule or into a linear rod. The spicule then extends into the pseudopodial strand(s) formed by the adjacent cell chains (Okazaki 1975b).

TEM observations have shown that the spicule growing *in vivo* is enclosed in a membrane-bound vacuole (i.e. the calcifying vacuole) which is itself located within the cytoplasm of the pseudopodial syncytium (i.e. skeletal envelope) (Gibbins *et al.* 1969, Millonig 1970). As previously reported by Okazaki (1960), it is noteworthy that growing spicules always occur in enlarged pseudopodial extensions, and not in the cell bodies themselves, the latter being in contact with the former through thin pseudopodial processes. Although Kerkis & Isaeva (1984) reported that, in some *in vitro* cultured primary mesenchyme cells, spicules lay directly within the cell body, Decker *et al.* (1987) clearly showed that *in vitro* spiculogenesis also occurs within a pseudopodial syncytium built by cultured cells. Contrary to what was observed on *in vivo* growing spicules, the pseudopodial syncytium surrounding the *in vitro* growing spicules seems to be sometimes discontinuous (Decker *et al.* 1987). If this is true, *in vitro* growing spicules are thus topographically extracellular. In support of that, Decker *et al.* (1987) showed that the membrane impermeant conjugate of ConA-gold binds to the internal membrane of the calcifying vacuole whereas it is not seen within the cytoplasm of the primary mesenchyme cells.

The inner surface of the calcifying vacuole is covered by an electron-dense material whose aspect depends on the fixation method (when using the Millonig's method, it seems to be structured in a coherent layer) (Gibbins *et al.* 1969, Millonig 1970, Decker *et al.* 1987). As for the cytoplasm of the pseudopodial syncytium, it includes mitochondria, double vesicles, coated vesicles, microtubules (parallel to the pseudopodial axis) and free ribosomes (Gibbins *et al.* 1969, Millonig 1970). The coated vesicles that are located close to the calcifying vacuole contain material similar to that found in the calcifying vacuole. The main organelles of the cell bodies are a well-developed juxtanuclear Golgi complex and short segments of rough endoplasmic reticulum (RER) (Gibbins *et al.* 1969). Cultured primary mesenchyme cells prepared by freeze substitution methods show electron-dense deposits in their perinuclear region, mitochondria, RER cisternae, and 'smooth-surface' vesicles. X-ray probe analysis demonstrated that these deposits contain high levels of calcium (Decker *et al.* 1987).

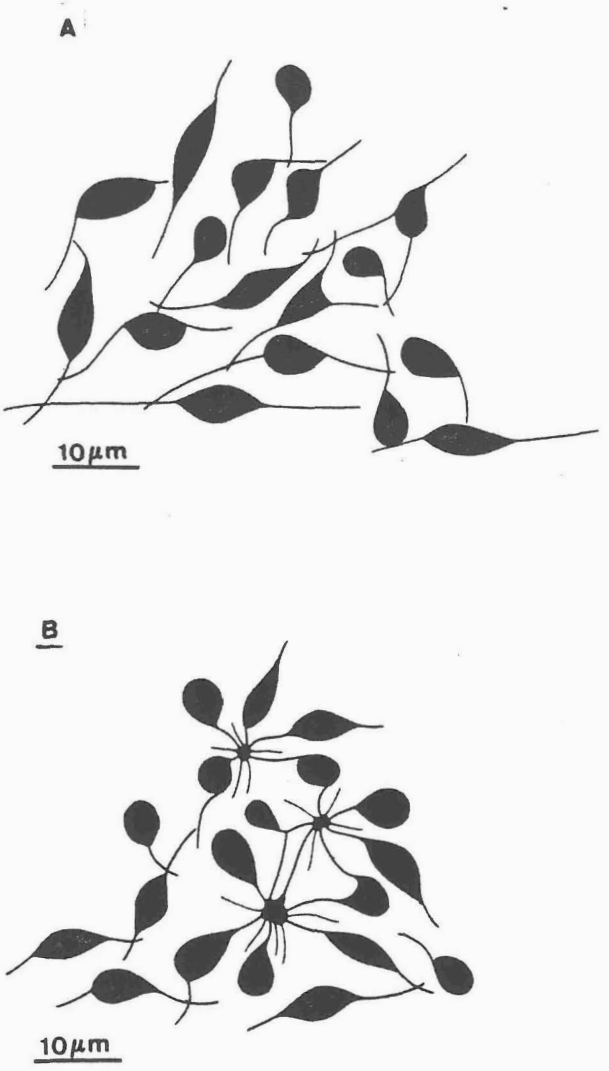


Figure 2. Formation of a cell aggregate and its corresponding longitudinal cell chain (adapted from Okazaki 1975a). A. The primary mesenchyme cells gather together in an aggregate. Note the numerous thin pseudopodia. B. Some of the pseudopodia of the aggregating cells fuse together and form enlarged hyaloplasmic masses within the pseudopodia.


C
D
10µm

E

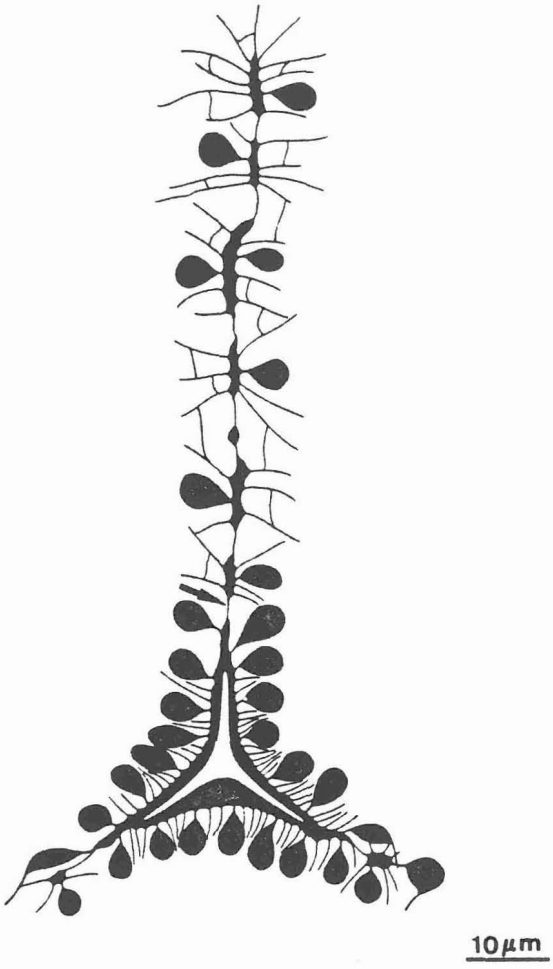


Figure 2 (cont.). C. The hyaloplasmic masses of the aggregate fuse together and form a triangular hyaloplasmic area. Other mesenchyme cells align themselves in a longitudinal cell chain and begin to form hyaloplasmic masses. D. The granular rudiment of the primary larval spicule appears at the center of the triangular hyaloplasmic area. The primary mesenchyme cells of the longitudinal cell chain increase the number of connections. E. The spicular rudiment develops into a triradiate spicule. The longitudinal cell chain develops a pseudopodial strand with locally enlarged hyaloplasmic areas. Note the connection of the hyaloplasmic area surrounding the spicule with the longitudinal pseudopodial strand (arrow).

2.2 *The skeleton-forming cell of postmetamorphic echinoderms*

The skeleton-forming cell of postmetamorphic echinoderms has been investigated in newly formed ossicles, regenerating ossicles, and fully developed ossicles (Table 1).

2.2.1 *Newly formed ossicles*

The formation and growth of new postmetamorphic ossicles were studied by Woodland (1906, 1907a, b) in all classes of echinoderms except asteroids. Woodland's observations were performed on non-decalcified material, viz. thick histological sections containing whole developing ossicles or *in toto* preparations. He reported that the ossicle rudiment appears either in a single cell (crinoids, echinoids and ophiuroids) or in a syncytium (holothuroids). When appearing within a single cell (the scleroblast), the rudiment has a granular shape and progressively develops into a triradiate spicule. At the triradiate stage the slceroblast nucleus undergoes its first division and the cytoplasm increases in size without subsequent cell division. The spicule then grows by successive bifurcations and anastomosis giving a typical stereomic unit. This unit develops into a syncytial mass resulting from numerous nuclear divisions, the nuclei being located at the periphery of the syncytium. On the contrary, the first rudiment of holothuroids appears in a preformed syncytium (Woodland 1906, 1907a, b). The granular rudiment grows within the syncytium to form first a rod with two bifurcated extremities. The rod then develops into a stereomic unit by successive bifurcations and anastomosis. There is almost no nuclear division in the syncytium during the development of holothuroid spicule.

TEM studies show that some holothuroid spicules are surrounded by a seemingly continuous cytoplasmic sheath while others are only surrounded by small cytoplasmic vesicles, leaving the spicules in contact with the extracellular matrix (Stricker 1985, 1986). According to Stricker, the latter are fully developed spicules (see Part 2.2.3) while the former are developing spicules. The cytoplasmic sheath of developing spicules is part of the syncytial pseudopodia produced by adjacent sclerocytes. The spicule would thus be enclosed in a vacuole within the syncytial pseudopodia, and its early development would be, as a consequence, intracellular (Stricker 1985, 1986). The cytoplasmic sheath is separated from the spicule by a thin layer of flocculent material (Stricker 1985). It contains only mitochondria while the sclerocyte cell body has numerous mitochondria, free ribosomes, a few Golgi bodies and cisternae of RER. An electron-dense boundary layer surrounds the membrane of the sclerocyte cell body (Stricker 1985, 1986).

Woodland's descriptions of developing holothuroid spicules probably corresponds to Stricker's observations, the pseudopodia being inconspicuous with the low resolution methods used by Woodland. The morphological

Table 1. Studies on the skeleton-forming cells of postmetamorphic echinoderms

A. NEWLY FORMED OSSICLES	
Crinoidea	
Antedon bifida (ossicle)	Woodland 1907a
Echinoidea	
Echinus esculentus (ossicle)	Woodland 1907a
Eucidaris tribuloides (tooth)	Märkel *et al.* 1986
Lytechinus variegatus (tooth)	Chen & Lawrence 1986a, Veis *et al.* 1986
Paracentrotus lividus (tooth)	Prenant 1926, Kniprath 1974
Ophiuroidea	
Amphipholis squamata (as *Amphiura elegans*) (ossicle)	Woodland 1907a
Ophiothrix fragilis (ossicle)	Woodland 1907a
Holothuroidea	
Cucumaria miniata (spicule)	Stricker 1986
Cucumaria sp. (spicule)	Woodland 1906
Eupentacta quinquesimita (spicule)	Stricker 1986
Leptosynapta clarki (spicule)	Stricker 1985, 1986
Leptosynapta inhaerens (spicule)	Woodland 1907b
Leptosynapta makrankyra (as *Synapta hispida*) (spicule)	Woodland 1907a
Labidoplax digitata (spicule)	Woodland 1907a, b
Ocnus lactea (as *Cucumaria brunnea*) (spicule)	Woodland 1906
Thyone fusus (spicule)	Woodland 1906
B. REGENERATING OSSICLES	
Echinoidea	
Eucidaris tribuloides (spine)	Märkel & Röser 1983a
Strongylocentrotus droebachiensis (test)	Kindred 1924, Vocisano 1971
Strongylocentrotus intermedius (test and spine)	Shimizu & Yamada 1976, 1980
Strongylocentrotus purpuratus (spine)	Heatfield & Travis 1975a
C. FULLY DEVELOPED OSSICLES	
Crinoidea	
Comanthus japonica (stalk ossicle)	Grimmer *et al.* 1984a
Democrinus conifer (stalk ossicle)	Grimmer *et al.* 1984b
Florometra serratissima (arm ossicle)	Holland & Grimmer 1981
Metacrinus rotundus (stalk ossicle)	Grimmer *et al.* 1985
Echinoidea	
Echinus esculentus (spine)	Pilkington 1969
Eucidaris tribuloides (spine)	Märkel & Röser 1983a
Ophiuroidea	
Ophioderma longicauda (scale)	Märkel & Röser 1985
Holothuroidea	
Eupentacta quinquesimita (spicule)	Stricker 1986
Leptosynapta clarki (spicule)	Stricker 1985
Pentamera sp. (spicule)	Stricker 1986
Psolidium bullatum (spicule)	Stricker 1986
Stichopus californicus (spicule)	Stricker 1986

relationships between the stereom and the associated cells are indeed very difficult to distinguish in histological preparations (see, e.g. Vocisano 1971). The same interpretation could be valid for ossicles of other classes investigated by Woodland.

The echinoid tooth grows continuously, being abraded at its chewing tip and formed at its aboral tip (the plumula). It is made of many homologous plates (the primary plates) arising in the plumula as separate units and producing several extensions during their formation (viz. the lamellae, needles and secondary plates) (see, e.g. Märkel 1970). The primary plates are solid and do not show the classical stereomic structure of other echinoderm ossicles. The plates (as well as their extensions) are bridged and cemented together during a second stage of calcification by the so-called calcareous discs. From this process results the functional rigid structure of the mature echinoid tooth. The precursors of the skeleton-forming cells (i.e. the preodontoblasts) are densely packed in the very end of the plumula where they actively proliferate (Holland 1965). The preodontoblasts produce thin pseudopodia filled with longitudinal microtubules. They bear a cilium provided with a complex ciliary apparatus. Golgi complexes and lipoid membrane-bound bodies are the more conspicuous organelles (Märkel *et al.* 1986). When developed into odontoblasts, the skeleton-forming cells align in parallel rows and form pseudopodial syncytia that probably arise from the fusion of the preodontoblast pseudopodia. The cell bodies remain distinct and the calcifying vacuoles are formed within the fused pseudopodia, being surrounded by a very thin cytoplasmic sheath (Fig. 3a) (Prenant 1926, Kniprath 1974, Chen & Lawrence 1986a, Märkel *et al.* 1986). Before mineralization starts, a fibrillar reticular structure partly fills the calcifying vacuole (Kniprath 1974, Märkel *et al.* 1986). The formation of the very first crystallites have not been described but the cytoplasmic sheath that surrounds the youngest plates is separated from the calcite by a vacuolar space (Fig. 3a). This indicates that mineralization has begun in the center of the vacuole and is thus centrifugal (Märkel *et al.* 1986). On the contrary, Kniprath (1974) claimed that the first crystallites appear in close contact with the vacuolar membrane and that calcification is centripetal. (Kniprath's TEM micrographs showed a row of crystallites closely apposed to the membrane of an otherwise empty vacuole.) These interpretations will be discussed in Part 3.2.

The calcite of the plate is sheathed by granular to amorphous material up to 45 nm thick (the matrix coat) (Kniprath 1974, Märkel *et al.* 1986). The matrix coat (and the calcifying vacuole) extends beyond the previously deposited calcite, indicating that it is synthesized before calcite formation. In these areas, the matrix coat is loose and irregularly shaped (Märkel *et al.* 1986). It is noteworthy that fibrillar reticular structure no longer occurs in the calcifying vacuole as soon as mineralization is initiated (Kniprath 1974, Märkel *et al.*

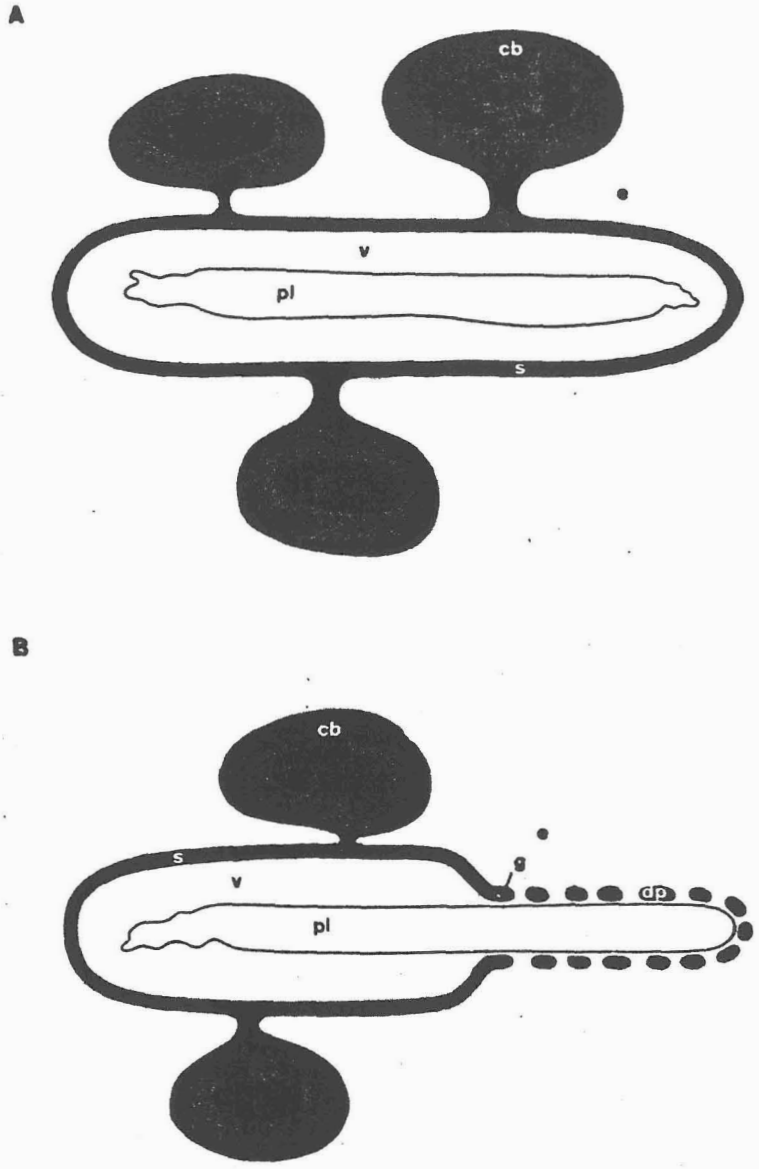


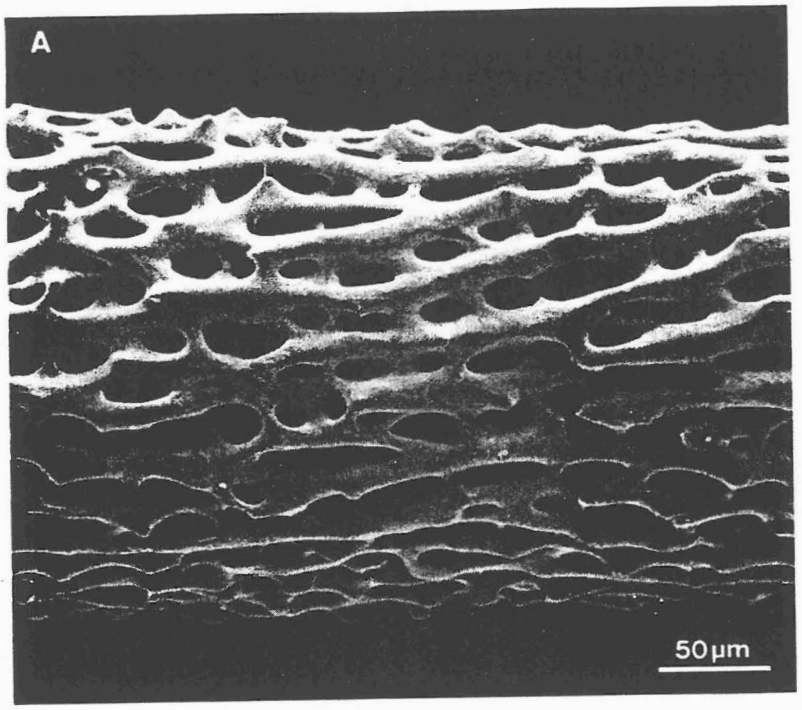
Figure 3. Schematic representation of the sclerocytes associated with primary plates of an echinoid tooth (A, adapted from Kniprath 1974; B, adapted from Märkel *et al.* 1986). Not to scale. A. Growing plate: intracellular stage. B. Plate with an extracellular fully developed part and a still growing part separated from the extracellular matrix by gaskets. cb: cell body of the sclerocyte; dp: distal process; e: extracellular matrix; g: gasket; pl: primary plate; s: pseudopodial sheath; v: vacuolar space.

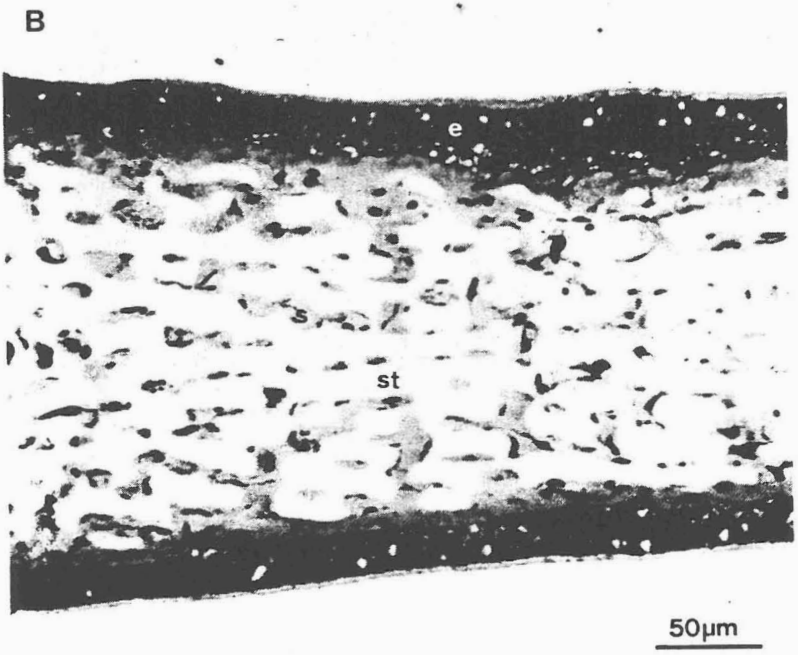
1986). The only structures in the vacuolar space are 'thin-walled' vesicles (membrane-bound?) which become detached from the vacuolar membrane (Märkel *et al.* 1986). Their actual function remains unknown. The pseudopodial cytoplasmic sheath contains glycogen α-particles, microtubules and Golgi-like vesicles (Kniprath 1974, Märkel *et al.* 1986). The latter are formed at the growing front of the vacuole and are supposed to fuse with it (Kniprath 1974). Cell bodies show perinuclear Golgi complexes, numerous vesicles, mitochondria, cisternae of RER, homogenous granules (lipid droplets?) and glycogen α-particles (Kniprath 1974, Chen & Lawrence 1986a, Märkel *et al.* 1986, Veis *et al.* 1986). The odontoblasts no longer have cilia and the ciliary apparatus is converted to centrioles. During the calcification process the calcifying vacuoles increase and ramify, producing the characteristic projections of the primary plates (viz. the lamellae, needles and secondary plates). Increase in size of the calcifying vacuoles occurs together with a progressive incorporation of new odontoblasts in the pseudopodial syncytium (Kniprath 1974, Chen & Lawrence 1986a, Märkel *et al.* 1986). The not yet incorporated odontoblasts are aligned with those already engaged in the syncytium, prefiguring the shape of the future calcifying vacuole (Märkel *et al.* 1986). Formation of plates and plate projections is thus a wholly intracellular process (Kniprath 1974, Märkel *et al.* 1986). However, fully developed parts of the plates are no longer covered by a continuous cytoplasmic sheath (see Kniprath 1974, Figs 5 and 7, and Veis *et al.* 1986*, Fig. 3; Märkel *et al.* 1986). The latter has seemingly split (by fusion of the vacuolar membrane with the cytoplasmic membrane?) leaving the so-called distal processes at the calcite surface (see Part 2.2.3) (Fig. 3b). As a consequence, the skeletal element becomes extracellular. On the other hand, the integrity of the calcifying vacuole in still growing areas of the same plates is maintained by 'gaskets' which closely attach the pseudopodia to the calcite (Märkel *et al.* 1986).

The calcareous discs which cement the plates together seem to be produced by the same odontoblasts that are involved in the first stage of calcification. These odontoblasts would restore (at least partially) the continuity of the cytoplasmic sheath before reinitiating calcification (Märkel *et al.* 1986). According to the micrographs of Märkel *et al.*, the plates remain extracellular, the calcifying sites alone being preserved by a continuous sheath and tightened by gaskets. Otherwise, the process of disc formation is similar to the formation of the tooth elements (Märkel *et al.* 1986).

*Veis *et al.* (1986) mistook the matrix coat for a vacuolar membrane.

Plate 1. Stereom and stroma of the adambulacral spine of *Asterias rubens* (original). A. SEM-view of a proteinase-cleaned spine showing the tridimensional arrangement of the stereom. B. Longitudinal semi-thin section through a decalcified spine. e: epidermis; s: stroma; st: white areas corresponding to the decalcified stereom.


A
50µm


B
e
st
50µm

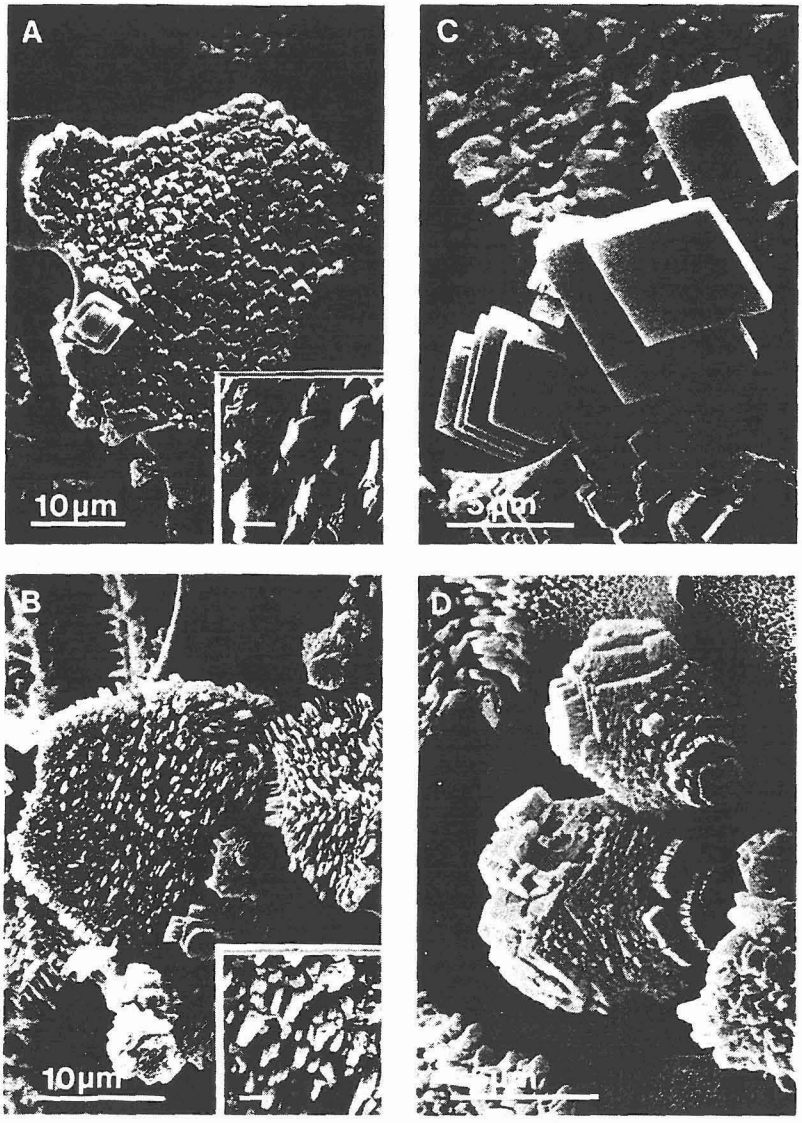


Plate 2. Effect of intrastereomic organic material of *Asterias rubens* (EDTA-soluble fraction) on crystal decoration of fractured adambulacral spines of the same species (SEM micrographs). A, B. Calcite crystals epitaxially grown on broken trabeculae, in the absence of organic material (A), and in the presence of 100 µg/ml of intrastereomic organic material (B). Insets: high magnification of the epitaxially grown crystals, Scale bar 1 µm. C, D. Calcite crystals nucleated in the decorating solution (and loosely adsorbed on the stereomic element, see Dillaman & Hart 1981), in the absence of organic material (C), and in the presence of 100 µg/ml of intrastereomic organic material (D).

2.2.2 *Regenerating ossicles*

As reported by several authors, regeneration of the echinoid test occurs within a membrane – the so-called healing membrane – built up by syncytial leucocytes (viz. the so-called bladder amoebocytes). Stereom formation occurs in a scattered way within the syncytium, leading to the formation of several platelets (Kindred 1924, Vocisano 1971, Shimizu & Yamada 1976). Calcification occurs in vacuoles which include small crystallites, granular or amorphous material, and several small Golgi-like vesicles. After partial decalcification, the crystallites sometimes leave membranous structures in parallel array (Shimizu & Yamada 1976). Besides calcifying vacuoles, the cytoplasm contains numerous secondary lysosomes, mitochondria with dense granules, and well-developed perinuclear Golgi complexes having numerous associated vesicles. Numerous free ribosomes and glycogen α-particles are seen during the initial stages of calcification (Shimizu & Yamada 1976).

Spherulous cells also occur in the healing membrane (Kindred 1924, Vocisano 1971, Shimizu & Yamada 1976). According to Vocisano (1971) many of these cells are degenerating and in process of being absorbed by leucocytes, especially in areas actively engaged in calcite deposition. He noted, moreover, that crystallites arise in vacuoles that result from the phagocytosis of spherulous cells by leucocytes. This phenomenon, however, was not reported by subsequent authors.

Further growth of the crystallites is thought to take place within the syncytium, at least until the initial stage of formation of trabeculae. According to Shimizu & Yamada (1980), early trabeculae are surrounded only by thin cytoplasmic extensions of the syncytium. They reported that in some places these extensions are discontinuous and that the mineral phase thus becomes extracellular. They suggested that this is caused by the fusion of both the vacuolar and the plasma membranes so that only the growing tips of the trabeculae remain covered by cytoplasmic processes. This is, however, wholly speculative as no electron micrographs of these important events were published in the case of the regeneration echinoid test (Vocisano 1971, Shimizu & Yamada 1980).

In regenerating echinoid spines, the deposition of calcite begins on the fractured surface of the initial trabeculae (Heatfield 1971a). Initial calcite deposition consists of microspines that rapidly form thin trabeculae. The sclerocytes (or calcoblasts) are the only cells which closely contact the growing trabeculae. They produce thin pseudopodia that tightly surround the trabeculae, forming the so-called skeletal sheath (Heatfield & Travis 1975a, Märkel & Röser 1983a) (Fig. 4). Two cell bodies may be connected with the same skeletal sheath, which may be thus syncytial. It should be said, moreover, that the skeletal sheath surrounds only the growing tip of the trabeculae. As a consequence, no cytoplasmic structure separates the under-

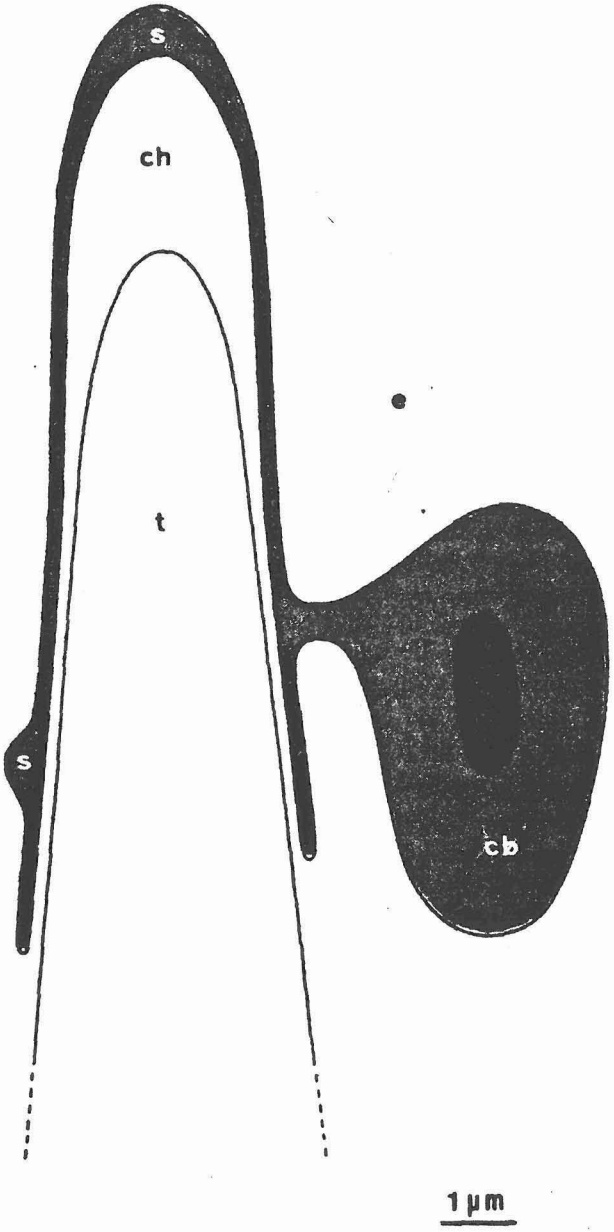


Figure 4. Schematic representation of the sclerocyte associated with the growing tip of a trabecula in a regenerating echinoid spine (adapted from a micrograph of Heatfield & Travis 1975a). cb: cell body of the sclerocyte; ch: tubular extracellular channel; e: extracellular matrix; s: skeletal sheath; t: growing tip of the trabecula.

lying calcite from the extracellular matrix along most of the trabecular length. Calcification in regenerating echinoid spines is thus topographically extracelluar (Heatfield & Travis 1975a, Märkel & Röser 1983a). To the contrary, Shimizu & Yamada (1980) reported that the first crystallites appear in vacuoles within the sclerocytes. According to them, growth of the crystallites (and of the calcifying vacuoles) induces in places the fusion of the vacuole and of the plasma membrane. As a result, these early trabeculae (more than 10 μm long) become outside the calcifying vacuoles and thus can fuse with the stub. Subsequent growth would take place by the extracellular processes described by Heatfield & Travis (1975a). This interpretation, however, is totally inconsistent with Heatfield's (1971a) SEM observations of the regenerating spine. It is clear from Heatfield's micrographs that the initial minute microspines grow directly from the trabeculae of the stub.

Longitudinal sections through regenerating spines show that the skeletal sheath occurs several microns ahead of the trabecular tip, enclosing a dead-end tubular channel beyond the trabecular tip (Heatfield & Travis 1975a) (Fig. 4). This tubular (extracellular) channel is not calcified. It contains a homogeneous non-electron dense material, and sometimes filamentous material located near the mineral surface. Moreover, the deposited calcite is covered by a continuous electron-dense material, called the trabecular coat (Heatfield & Travis 1975a, Märkel & Röser 1983a). The cytoplasm of the skeletal sheath contains free ribosomes, mitochondria and vesicles, while the cell body contains a well-developed and dilated Golgi complex with associated vesicles (similar to that of the sheath) as well as cisternae of RER, mitochondria, numerous free ribosomes and lipid droplets (Heatfield & Trvis 1975a, Märkel & Röser 1983a). An electron-dense boundary layer – which is thought to be characteristic of the skeleton-forming cell – surrounds the sclerocyte outside the plasma membrane (Märkel & Röser 1983a).

Heatfield & Travis (1975a) and Shimizu & Yamada (1980) reported the occurrence of presclerocytes in regenerating spines. These cells are similar to the sclerocytes but do not closely contact the growing trabeculae and have numerous convoluted pseudopodia. They are often aligned near the active growing tips of the regenerating spines, a location suggesting they are precursors of sclerocytes.

2.2.3 *Fully developed ossicles*

The relationships between the sclerocytes and the fully developed stereom have been investigated in echinoids, ophiuroids and holothuroids (Pilkington 1969, Märkel & Röser 1983a, 1985, Stricker 1985, 1986) as well as, more incidentally, in crinoids (Holland & Grimmer 1981, Grimmer *et al.* 1984a, b, 1985). The situation is very similar in all the investigated classes.

The sclerocyte is the only cell which contacts the stereom. The cell body

sends a few enlarged pseudopodia (the so-called 'branches of cytoplasm'; see Märkel & Röser 1983a) which ramify into filiform and convoluted distal pseudopodia (the distal processes) closely appressed to the trabeculae (Märkel & Röser 1983a, 1985, Grimmer *et al.* 1984a). Because of their convoluted pathway, distal pseudopodia are generally seen in transverse section and appear as small vesicles of about 100 mm in diameter. These vesicles correspond to Pilkington's 'second order spherical bodies' (the 'first order spherical bodies' of Pilkington being transverse sections of the enlarged part of the pseudopodia; see Märkel & Röser 1983a).

In TEM sections of fully developed ossicles, sclerocyte cell bodies are very scarce. Transversally sectioned distal pseudopodia are sometimes the only signs of sclerocyte occurrence (Grimmer *et al.* 1984b, 1985, Stricker 1985, 1986). Fully developed ossicles thus have very broad contact areas with the extracellular matrix and are typically extracellular. However, in all studied species the stereom is always separated from the extracellular matrix by a trabecular coat which was mistakenly considered by Pilkington (1969) as a plasma mkembrane (see Märkel & Röser 1983a). The trabecular coat is hypothesized to be secreted by the sclerocytes and to be involved in the maintenance of the flawless condition of the mineral, which is essential to avoid stress concentration leading to a reduction in skeleton strength (Nichols & Currey 1968, Heatfield & Travis 1975a).

Organelles are similar to those described in the sclerocytes of regenerating spines. No organelles, however, are found in the distal pseudopodia. An electron-dense boundary layer surrounds the cell body and the enlarged part of the pseudopodia but does not occur along the distal pseudopodia (Märkel & Röser 1983a, 1985, Stricker 1985, 1986). That layer contains mucopolysaccharides, at least in ophiuroids (Märkel & Röser 1985). A similar layer also surrounds the fibrocyte-like cell type described in the ophiuroid stroma (Märkel & Röser 1985). These two cell types share a very similar ultrastructure – except for the pseudopodia – a fact that suggests that sclerocytes and fibrocytes could have a common origin (Grimmer *et al.* 1984a, Märkel & Röser 1985).

2.3 *Discussion*

Both echinoid larval spicules and echinoid tooth plates clearly develop intracellularly in a syncytium (at least in the early stage). Intrasyncytial development seemingly occurs also in newly formed postmetamorphic ossicles as well as in the regenerating test of echinoids. In contrast, skeletal growth proceeds extracellularly during both regeneration of echinoid spines and formation of tooth calcareous discs. It is noteworthy that larval spicules, tooth plates, postmetamorphic ossicles and test regenerating plates are ossicles which appear *de novo*. On the contrary, spine regeneration and

calcareous disc formation start from a pre-existing skeleton. Furthermore, the fully developed areas of the tooth plates as well as the stereom of fully developed ossicles are typically extracellular. Considering the different relationships that occur between sclerocytes and skeleton, the following hypothesis can be proposed (expanded from the proposals of Shimizu & Yamada 1980, Stricker 1985 and Märkel *et al.* 1986). The rudiments of new ossicles appear intrasyncytially (within a vacuole) and trabecular growth proceeds inside the vacuole until a definite size. Extension of the calcifying vacuole progressively reduces the size of the surrounding cytoplasmic sheath, the vacuolar and plasma membranes becoming very close. Local fusion of these two membranes causes the splitting of the cytoplasmic sheath resulting in the formation of the distal processes (see 2.2.3) which surround the now extracellular trabecula. Sclerocytes involved in growth will remain at the edges of the ossicle, eventually preserving a locally continuous cytoplasmic sheath, whereas those involved in ossicle maintenance will be retained within the ossicle (i.e. in the stroma). Further growth at the edge of the ossicle will be either continuously performed or easily resumed by the sclerocytes that have preserved a locally continuous cytoplasmic sheath. For secondary growth (e.g. trabecular thickening) or stereomic repair, sclerocytes involved in maintenance either will resume their activity (as shown in the echinoid tooth; Märkel *et al.* 1986) or will multiply (as demonstrated in regenerating echinoid spine; Heatfield 1971b). They will delimit an enclosed space around the intact or broken trabecula in which mineralization takes place. Whatever the case, an intracellular calcifying vacuole is no longer needed because the pre-existing trabecula acts as a nucleus, favouring crystallization. This also avoids the necessity of a subsequent fusion between the newly deposited mineral and the existing trabecula.

The skeleton-forming cells of echinoderms share some general characteristics. Whether the location of the calcifying site is intra- or extracellular, it always appears to be a confined space (vacuole or tubular channel) originating from syncytial pseudopodia. Moreover, this space is always separated from the surrounding plasma or vacuolar membranes by an electron-dense material. In postmetamorphic ossicles, that electron-dense material is sometimes complemented by filamentous or membranous structures. Most authors interpreted these materials as an organic matrix for crystallization; this will be discussed in the next section.

The ultrastructural uniformity of the skeleton-forming cell must be stressed. Besides the already quoted morphology of the calcifying space, the following organelles occur in all skeleton-forming cells investigated:

- A well-developed juxtanuclear Golgi complex. That complex is surrounded by vesicles which also occur close to the calcifying space. These vesicles sometimes contain material similar to the electron-dense material found within the calcifying space.

- RER cisternae. They are always present in the cell body, being sometimes plentiful and dilated.
- Mitochondria and free ribosomes. These organelles are always numerous, being typically present in pseudopodia.
- Microtubules. They are often aligned in the pseudopodial axis.

The constant occurrence of such organelles led some authors to speculate about the subcellular mechanisms of calcification (Heatfield & Travis 1975a, Okazaki & Inoué 1976, Shimizu & Yamada 1976). They suggested that molecules are first synthesized in the RER and then translocated to the Golgi complex. Vesicles would bud off the Golgi cisternae and would migrate in the pseudopodia near the calcifying space. Their contents would then be secreted or incorporated into the calcifying space. This material would be basically the organic matrix (Gibbins *et al.* 1969, Shimizu & Yamada 1976). These presumptions received some confirmation as, according to Benson *et al.* (1987), components of the spicule organic matrix were detected in the endoplasmic reticulum and the Golgi complexes of primary mesenchyme cells. Furthermore, a calcium-binding protein whose synthesis is correlated with spiculogenesis, was detected in intracellular granules of the mesenchyme cells (Iwata & Nakano 1986). Finally, mitochondria, RER, and 'smooth-surface' vesicles were also suggested to be involved in calcium transport within the skeleton-forming cells (Shimizu & Yamada 1976, Decker *et al.* 1987).

Although the knowledge of organelles which occur in the skeleton-forming cell is rather satisfactory, the relationships between sclerocytes and the stereom during active mineralization remain poorly known. There is a need for further studies on (1) the early stages of calcite deposition in both intra- and extracellular growing skeleton, (2) the fate of the larval spicule during the exotrophic stage of the pluteus (does it remain intrasyncytial?), (3) the production and fate of the first rudiment of postmetamorphic ossicles (this may be done either during metamorphic events or when new ossicles develop in postmetamorphic individuals), and (4) the three-dimensional relationships between sclerocytes and the stereom in both fully developed ossicles and in regenerating ossicles.

3 THE ORGANIC MATRIX

Mann (1983) defined the organic matrix as any localized surface comprising constituents such as lipids, proteins and carbohydrates which acts as a mediator of mineralization in biological systems. Three structures fit this definition in the calcification system of echinoderms, viz. the plasma or vacuolar membrane of the calcifying site, the organic material surrounding the mineral phase (i.e. the matrix or trabecular coat), and the intrastereomic organic components.

3.1 *The membrane of the calcifying site*

The membrane surrounding the calcifying site in echinoderms mediates calcification by limiting the liquid volume at the mineralization site, regulating the supersaturation levels by active ion transport, and framing the shape of the calcareous elements (namely the three most important functions of the membrane matrix, Mann 1983).

Morphological evidence (see part 2.3) shows that calcification in echinoderms always occurs in a confined space limited by either a plasma or a vacuolar membrane that is generated by syncytial pseudopodia. The membrane closely surrounds the calcifying site and must be necessarily crossed by the ions involved in the mineralizing process (viz. calcium and carbonate ions, mainly). These ions should also cross the cytoplasm of the skeleton-forming cell or at least its pseudopodial component (this is obvious in the case of intracellular calcification). The transport of calcium and carbonate ions through primary mesenchyme cells of echinoid larvae would involve several transport systems (viz. calcium channels in the plasma membrane, a mitochondrial calcium pump, and a Cl^-/HCO_3^- ATPase in the vacuolar membrane; see part 4). In echinoid teeth, carbonate ions are supposedly transported through the vacuolar membrane of odontoblasts as carbon dioxide, and then converted into bicarbonate ions by carbonic anhydrase at the calcifying site (Chen 1985). Autoradiographic studies suggest that carbonic anhydrase is located on or in the vacuolar membrane of odontoblasts (Chen & Lawrence 1986b).

The development and orientation of the calcifying space by the plasma or vacuolar membrane precede mineral deposition in most echinoderm calcifying systems. The shape of the so-defined space prefigures that of the calcareous element and the membrane of the calcifying space is suggested to mold the ossicle. In regenerating echinoid spines, the plasma membrane of the skeletal sheath limits a microspine-shaped tubular channel which extends ahead of the mineralizing front (Heatfield & Travis 1975a). Similarly, calcification of echinoid teeth occurs in pseudopodial vacuoles whose shape and ramifications correspond to those of the plates, lamellae, and needles (Kniprath 1974, Märkel *et al.* 1986). In echinoid embryos, the relationships between the calcifying vacuole and the shape of the spicule have been rather intensively studied. According to Okazaki (1960, 1962) and Wolpert & Gustafson (1961) the growth of the pseudopodial syncytia made by the primary mesenchyme cells – as well as that of the associated calcifying vacuoles – precedes and determines the growth of the spicule. Experimental modification of the shape of pseudopodial syncytia induces corresponding modifications in the shape of the spicule (Okazaki 1962). Moreover, when micromeres (i.e. the potential mesenchyme cells) were exchanged between embryos of different echinoid species, the spicules formed were always

characteristic of the species from which the cells originated (von Ubisch 1937, 1939). According to Harkey & Whiteley (1980), the migration, differentiation, and distribution pattern of primary mesenchyme cells in aggregates and chains are determined by both intrinsic and extrinsic factors. Among the extrinsic factors are interactions with the blastocoel wall (see Harkey & Whiteley 1980, for review) and with components of the extracellular matrix of the blastocoel (Blankenship & Benson 1984, Miyachi *et al.* 1984, Katow & Hayashi 1985, Iwata & Nakano 1985a, Katow 1986).

3.2 *The trabecular coat*

The trabecular coat consists of an electron-dense material lying between the mineral component and the plasma or vacuolar membrane (see part 2.3). The occurrence of such material has been demonstrated in almost all echinoderm skeletal tissues. It is particularly conspicuous around larval spicules (Gibbins *et al.* 1969, Millonig 1970, Decker *et al.* 1987) but also occurs as a thin layer in echinoid teeth (Kniprath 1974, Märkel *et al.* 1986), in regenerating echinoid spines (Märkel & Röser 1983a), in developing holothuroid spicules (Stricker 1985), and in fully developed ossicles (Märkel & Röser 1983a, 1985, Holland & Grimmer 1981, Grimmer *et al.* 1984a, b, 1985). The coating material may be amorphous, granular or fibrous. Gibbins *et al.* (1969) and Millonig (1970) suggested that this material may represent, at least partly, the organic matrix of the spicule. (Millonig proposed that the electron-dense material surrounding the spicule is involved in a stimulation/inhibition process of the spicular growth.) Kniprath (1974) claimed that the coating material is 'obviously' an organic matrix for the tooth ossicle as the first crystallites appeared in close contact with that material. He concluded that mineral growth in the echinoid tooth is a process of epitaxial growth inside a compartment (i.e. one of the structurally controlled processes of mineralization). However, the epitaxy theory implies that a substrate acts as a lattice template for the mineral overgrowth, controlling both the orientation and the structure of the crystal (Mann 1983). Thus, epitaxial growth applied to biomineralization supposes an atomic matching between the organic matrix (the substrate) and the mineral phase. Actually nothing is known about the atomic structure of the coating material, a knowledge that is essential to decide whether or not epitaxial growth is possible. Furthermore, Kniprath (1974) did not resolve the objection of Towe (1972; see also Mann 1983), namely that when epitaxial growth occurs within a compartment some parts of the wall of the compartment have to be active and some other parts inactive in order to avoid incoherent development of the mineral nuclei. Actually the crystallites shown by Kniprath grow over the entire wall of the calcifying vacuoles (a fact that led him to suggest a centripetal growth process). Märkel

et al. (1986) also suggested a matrix role for the coating material. They pointed out (1) that the coating material is always formed prior to calcite deposition, and (2) that it does not contact the vacuolar membrane surrounding developing ossicles while it contacts the vacuolar membrane in apparently fully developed ossicles. From these observations, Märkel *et al.* (1986) suggested that the coating material acts as an organic matrix, initiating crystal growth at the center of the calcifying vacuole, and that its activity is controlled by the cytoplasmic sheath, calcite deposition stopping when the coating material touches the vacuolar membrane.

Ultrastructural studies of echinoid larval spicules (Benson *et al.* 1983) questioned the very significance of the coating material. Benson *et al.* showed that the whole spicule is permeated by a fibrous organic material in the form of interconnected concentric sleeves. As a consequence, the coating material appears as the most external sleeve of this system and thus does not seem to represent an entity of its own. It would belong rather to a more extensive structure which occurs within and around the spicule (this is certainly true in larval spicule and is probable in ossicles of adult echinoderms, see part 3.4). Moreover, the presence of a three-dimensional organic structure within the stereom is a major argument against the use of the epitaxial growth theory as an explanation for echinoderm mineralization. Actually, that theory rests basically on surface interactions (i.e. two-dimensional interactions) between the substrate – the organic matrix – and its mineral overgrowth (Mann 1983).

3.3 *The intrastereomic organic components*

The presence of organic material in stereomic units of echinoderms was demonstrated both by chemical analyses of adult and larval skeletal elements cleaned of their associated soft tissues (Pilkington 1969, Currey & Nichols 1969, Nauen & Böhm 1979, Weiner 1985, Benson *et al.* 1986) and by morphological observations of cleaned and partially demineralized larval spicules (Okazaki 1960, Benson *et al.* 1983). The occurrence of intrastereomic organic material is also indicated by mechanical and crystallographic evidence, namely, (1) the larval spicules of echinoids have the characteristics of composite materials (Emlet 1982), and (2) the columnal ossicles of the crinoid *Neocrinus blakei* observed in TEM ion-thinned sections show a mottled contrast 'due to the existence of an apparently non-crystalline substance incorporated into calcite' (Blake & Peacor 1981). (Moreover, STEM microanalysis of the same sections reveal phosphorus and sulfur in minor quantities.) The organic material detected within the stereom of echinoderms is formed either by pigments and proteins or by proteins only.

3.3.1 *Pigments*

Most pigments reported for skeletal parts of echinoderms are naphthoquinone pigments (i.e. echinochrome or spinochromes). They have been mainly identified in echinoid tissues, being reported only incidentally in other echinoderm classes.

The test and spines of most echinoids contain several types of polyhydroxynaphthoquinones (for review, see Fox & Hopkins 1966, Goodwin 1969, Anderson *et al.* 1969). According to Goodwin & Srisukh (1950) and Lederer (1952), part of these pigments are associated with the skeleton. Goodwin & Srisukh reported, moreover, that the pigments form salts with the calcium of the stereom. One should note that all the reported extractions of naphthoquinone pigments have been carried out on whole echinoid body walls, the ossicles being not cleaned from their associated soft tissues. It is well known, however, that the test of numerous species of echinoids remains coloured after having been cleaned with sodium hypochlorite. Furthermore, pigmented growth bands were reported within the test plates of some echinoids (Moore 1935, Sime 1982, Nichols *et al.* 1985). These bands were revealed by grinding cleaned ossicles (without other treatment like charring, see Jensen 1969) and occur in areas without stereomic banding (Smith 1980a, Sime 1982, Nichols *et al.* 1985). According to Smith (1980a), these bands result probably from an actual incorporation of pigments in the calcite. Moreover, Goodwin & Srisukh (1950) reported that colours of cleaned tests are similar to those of the calcium salts of some spinochromes (the natural colour of the pigments being different from that of their calcium salts). In non-echinoid echinoderms, naphthoquinone pigments have been demonstrated in *Antedon bifida* (Crinoidea; Dimelow 1958), *Polycheira rufescens* (Holothuroidea; Mukai 1958, 1960), *Acanthaster planci* (Asteroidea; Singh *et al.* 1967), and *Ophiocoma erinaceus* and *Ophiocoma insularia* (Ophiuroidea; Singh *et al.* 1967). It seems obvious that the pigment of *P. rufescens* must be principally located in the soft parts of the integument. This is also the case with *A. bifida* where the pigment is located in granules within the superficial part of the dermal tissue (Dimelow 1958). In the investigated asteroid and ophiuroids, naphthoquinones were extracted from roughly cleaned spines (Moore *et al.* 1966, Singh *et al.* 1967). It is consequently possible that they also originate from associated uncalcified tissues. This agrees with the fact that asteroid and ophiuroid ossicles are totally colourless when their soft tissues have been digested either by sodium hypochlorite or by protease (Dubois unpubl.). Naphthoquinone pigments would thus be present only in the mineral phase of echinoid ossicles.

Non-naphthoquinones pigments were reported in a single case, namely in the test of *Arbacia lixula*, where two types of tetrapyrrol pigments were detected (Kennedy & Vevers 1972). The most concentrated one (i.e. chlorin e6) supposedly occurs within the stereom as a calcium salt. Kennedy &

Vevers suggested that chlorin e6 may be derived from chlorophyll a in the diet of the echinoid.

As a rule, pigments trapped in skeletal phases are not considered to be a part of organic matrix (Towe 1972). This seems to be true also in echinoderms where pigments have been recognized only in echinoid ossicles, being absent from those of the remaining classes. The fact that both calcification processes and ossicular stereomic structures are very similar in the whole phylum also indicates that the intrastereomic pigments of echinoids cannot be considered as part of the organic matrix. It should be added, moreover, that no morphological evidence suggests that the red spherulous cells (i.e. the naphthoquinone secreting cells) associated with the echinoid ossicles play any role in calcification processes (see, e.g. Heatfield and Travis 1975b).

3.3.2 *Proteins.* Proteins form the bulk of the organic material detected within the stereom. Intrastereomic proteins have been successively thought to be either collagen or glycoproteins.

3.3.2.1 *Postmetamorphic individuals.* Collagen was first reported from the stereom of echinoid test plates that had been cleaned with pronase before being demineralized by EDTA (Travis *et al.* 1967). TEM observations of the remaining material showed fibrils having the typical banding pattern of collagen. Biochemical analyses of that material indicated an amino acid composition equally characteristic of collagen. Travis (1970) reported, moreover, that collagen fibrils with electron-dense spots in their so-called 'hole' regions occur in asteroid, echinoid and ophiuroid ossicles 'which have not fully calcified'. Travis interpreted these spots as primary stages of calcite crystal formation. She proposed furthermore that during the secondary stage of calcification the collagen fibrils would become completely mineralized inducing the obliteration of the banding pattern of the collagen fibrils. According to Travis, this obliteration would explain the aspect of the stereom she observed in non-decalcified TEM sections of completely mineralized regions. (In such regions, the stereom is reported to be made of closely packed crystals organized into sheet-like layers that are embedded in an apparently unbanded organic material.) However, Travis does not show any micrographic evidence of this secondary stage of calcification at work. Pucci-Minafra *et al.* (1978) and Veis *et al.* (1986) reported that collagen occurs in the plates and teeth of the Aristotle's lantern of two echinoids. However, no attempt was made to remove the soft tissue (i.e. the stroma and the connective tissue of the joints) associated with the plates (it is well-known that collagen is a usual component of these tissues; see, e.g. Kniprath 1974 and Chen & Lawrence 1986a).

The collagen reported by Travis and co-workers (1967, 1970) as being associated with the mineral phase of postmetamorphic ossicles was demon-

Table 2. Composition of the intrastereomic organic material of *Paracentrotus lividus*,[1] *Lytechinus variegatus*,[2] and *Arbacia punctulata*.[2]

	Whole material		Soluble material		Insoluble material	
	EDTA[1]	Acetic acid[2]	EDTA	Acetic acid	EDTA	Acetic acid
%[3] of organic material in mineral	0.15-0.2	0.092	0.05	0.02[4]	0.1-0.15	0.07[4]
Proteins						
% in the considered material	nd[5]	84.9	nd	92.8	2-3	88.1
% of whole proteins	–	–	>90	17-24	<10	76-83
Non-protein material						
carbohydrates						
% in the considered material	nd	14.0	nd	3.5	nd	11.6
phosphate						
% in the considered material	nd	0.31	nd	3.9	nd	0.3

1. EDTA-extraction performed on ossicles of *Paracentrotus lividus* by Weiner (1985).
2. Acetic acid-extraction performed on ossicles of *Lytechinus variegatus* and *Arbacia punctulata* by Swift *et al.* (1986).
3. All percentages are w/w (dry weight).
4. Data calculated from published results.
5. nd: not determined.

strated to result from contamination either from the stroma or from the sutural areas between ossicles (Currey & Nichols 1969, Klein & Currey 1970). When reproducing the amino acid analysis of Travis *et al.* (1967) 'using a slightly different technique', Currey & Nichols (1969) were unable to discover any trace of hydroxyproline, the more characteristic amino acid of collagen. Moreover, amino acid analysis of the organic material from ossicles of adult echinoids preceded by collagenase digestion of the stroma before demineralization reveal also the virtual absence of hydroxyproline (Klein & Currey 1970, Weiner 1985). As noted by Klein & Currey, if one assumes that calcified collagen is protected from collagenase digestion – as is the case in calcified mammalian tissues – then the collagen showed by Travis and co-workers (1967, 1970) should necessarily originate from the stroma. This is supported by all ultrastructural studies of echinoderm calcified tissues (see part 2.2) as collagen fibrils were never observed within the demineralized calcified spaces. Moreover, the organic material which sometimes remains in the calcified spaces after demineralization shows a fibrillar reticular structure without any evidence of banding pattern. (Such intrastereomic material was reported from the echinoid tooth by Kniprath 1974 and Märkel *et al.* 1986, from the regenerating test of echinoids by Shimizu & Yamada 1976, and from the fully developed ossicles of a crinoid by Holland & Grimmer 1981; see part 2.2.).

The actual intrastereomic organic material was extracted from echinoid ossicles cleaned with either crude collagenase solution or sodium hypochlorite and sodium hydroxide (Weiner 1985, Swift *et al.* 1986). Its concentration ranges from 0.1 to 0.2% (w/w) and it is composed mostly of proteins although non-protein material is also detected (Table 2). The non-protein material of EDTA-insoluble fraction was suggested by Weiner (1985) to be pigments. Noteworthy is the carbohydrate fraction averaging 14% of the total organic material. These carbohydrates could be linked to proteins as demonstrated for the intraspicular organic material of larvae (see part 3.3.2.2). Polyacrylamide gel electrophoresis of acetic acid-soluble fraction reveals a single band at 160-180 kD (Swift *et al.* 1986). However, results of gel electrophoresis may be misleading as most soluble components of intraskeletal organic material do not bind commonly used dyes (Weiner *et al.* 1983, Benson *et al.* 1986, Venkatesan & Simpson 1986). According to the extraction agent, the protein distribution between soluble and insoluble material differs (Table 2). The proteins of the EDTA-soluble fraction can be divided into two broad classes, viz. those rich in glycine and aspartic acid/asparagine, and those poor in aspartic acid/asparagine but rich in serine, glycine, and glutamic acid/glutamine (Weiner 1985).

The putative organic matrix role of the intrastereomic organic components was investigated by Swift *et al.* (1986), Berman *et al.* (1988), and Dubois (1988). Swift *et al.* (1986) showed that the whole organic material extracted by acetic acid from echinoid stereom had no effect on the incorporation of dissolved inorganic ^{14}C into *larval* spicules whereas the acetic acid-soluble material significantly decreased $Ca^{14}CO_3$ formation at a concentration precluding a quantitative binding of calcium ions in solution ($1.2\ 10^{-8}$M). Moreover, the soluble material (at $0.24\ 10^{-8}$M) can also delay the onset of crystal formation and reduce the maximal crystal growth rate during *in vitro* crystallization of inorganic $CaCO_3$ (Swift *et al.* 1986). Berman *et al.* (1988) investigated *in vitro* calcite crystallization in presence of the EDTA-soluble material extracted from echinoid stereom. Calcite crystals grown in such conditions develop new faces as compared to control crystals (effective concentrations of extracted material range from 2.0 to 8.0 µg/ml, which corresponds to 1.1 to $4.5\ 10^{-8}$M using Swift *et al.*'s assumption of the molecular weight of intrastereomic organic components). Use of fluorescent-labelled intrastereomic material demonstrated that part of it was occluded inside the crystal. Such crystals with occluded material cleave 'with difficulty' : they fracture surfaces have a curved glassy appearance reminiscent of the conchoidal fracture of echinoderm stereom whereas crystals grown in the absence of intrastereomic material – i.e., pure calcite crystals – cleaved along the classical rhomboedron cleavage (Berman *et al.* 1988). Dubois (1988) studied the effect of EDTA-soluble intrastereomic material from *Asterias rubens* on crystal decoration of fractured asteroid spines. He

showed that the shape of both the crystals nucleated in the decorating solution and these that have grown epitaxially on the broken trabeculae is strongly modified when crystals grow in presence of 10 or 100 µg/ml of instrastereomic material (i.e., 5.6 and 56 10^{-8}M using Swift *et al.*'s assumption) (Plate 2). Particularly, the epitaxial crystals grown in presence of intrastereomic material take a whiskered appearance reminiscent of the tapered prismatic structures observed in the trabeculae of echinoderm sterom (see part 3.4) whereas crystals grown in absence of intrastereomic material show the classical rhomboedron morphology (Plate 2A, B).

Echinoid lanterns and teeth were also investigated regarding skeleton-associated organic material (Minafra *et al.* 1980, Weiner *et al.* 1983, Weiner 1985, Veis *et al.* 1986). However, the studied skeletal elements were not cleaned of their soft tissues prior to analysis. As a consequence, these results reflect more the composition of the stroma than that of the intrastereomic material.

3.3.2.2 *Larvae.* Collagen was also reported from echinoid larval spicules. Ellis & Winter (1967) and Pucci-Minafra *et al.* (1972) noted that larvae incorporate ^{3}H-proline as protein-bound ^{3}H-hydroxyproline into spicules (the labelled molecules were demonstrated within spicules cleaned from their associated cells and tissues). This was confirmed by Pucci-Minafra *et al.* (1980) who observed small amounts of collagen fibrils in demineralized cleaned spicules. It should be said, however, that the banding pattern of collagen was observed only after acetic acid extraction followed by *in vitro* repolymerization of the fibrils. Moreover, Gould & Benson (1978) and Mintz *et al.* (1981) reported that inhibition of the metabolism of collagen-like proteins prevented spicule growth. Actually, this strongly suggests that collagen play a leading role – such as an organic matrix role – during spiculogenesis. Selective inhibition of collagen synthesis (probably at the transcriptional level) by a low concentration of actinomycin D generates pluteus without spicules (Gould & Benson 1978) and inhibits *in vitro* spiculogenesis by cultured primary mesenchyme cells (Mintz *et al.* 1981). Similarly, the inhibition of enzymes involved in post-translation modifications of amino acid residues in collagen-like proteins (viz. prolyl-hydroxylase, lysyl-hydroxylase, and lysyl-oxidase) blocks almost completely spicule formation *in vitro* (Mintz *et al.* 1981). (One may note that this larval collagen-like protein differs from typical collagen as it does not contain pepsin-resistant domains and is not degraded by collagenase; Mintz *et al.* 1981.)

Blankenship & Benson (1984) found that 'when utilizing the technique of Pucci-Minafra *et al.* (1972), it is very difficult to purify spicules free of blastocoelic collagen and basement membrane components'. Moreover, Benson *et al.* (1983) reported that ultrathin sections through partially demine-

ralized spicules showed that the spicules contain fibrous organic material which forms interconnected concentric sleeves (see part 3.2). The fiber component of this material lacks any cross-banding, a fact that suggests that this material is not collagen. The finding of such intraspicular material confirms Okazaki's (1960) observations that spicules cleaned with sodium hydroxide leave an organic cord of spicular shape when they are decalcified. This organic material lacks hydroxyproline, confirming that it is not collagen (Benson *et al.* 1986). Consequently, the collagen-like protein detected in association with the larval spicule and whose metabolism is said to be necessary for spicular growth (see e.g., Mintz *et al.* 1981) appears to be only a component of the extracellular matrix of the blastocoel. According to Blankenship & Benson (1984), that collagen-like protein would be synthesized by the micromeres and would interfere in the migration and organization of the micromeres into aggregates and strands. This is confirmed by observations showing that inhibitors of collagen cross-linking caused unsuccessful gathering and arrangement of primary mesenchyme cells, resulting in failure of spicule formation or in spicule deformation (Uto *et al.* 1984, Uto & Muranaka 1985). The role of collagen, together with fibronectin and dermatan sulfate, in the migration of primary mesenchyme cells was, furthermore, emphasized by *in vitro* studies using artificial extracellular matrixes (Katow 1986).

Extraction of the actual intraspicular organic material revealed that it is about 0.1% (w/w) of the spicule, is mainly protein in nature, and shows calcium binding activity ($Kd = 1.5\ 10^{-4}M$) (Benson *et al.* 1986). Contrary to the organic material extracted from postmetamorphic ossicles, acetic acid- and EDTA-extractions led to the same results. The insoluble fraction is reduced and was not investigated. The soluble fraction shows a protein fraction rich in aspartic acid/asparagine, glutamic acid/glutamine, glycine, serine, and alanine or proline (Benson *et al.* 1986 and Venkatesan & Simpson 1986, respectively). Reduced sugars are present in the soluble fraction as 4% of the amount of protein material, one-fourth being amino sugars (Benson *et al.* 1986). SDS polyacrylamide gel electrophoresis showed that the intraspicular organic material is a mixture of several components. (Apparent molecular weights of major components are 117, 64, 57, 50 and 47 kD according to Benson *et al.* 1986, and 117, 67, 48, 37, 24.5 and 19.5 kD according to Venkatesan & Simpson 1986.) Treatment of the soluble proteins with endoglycosidase F or N-glycanase (enzymes that cleave N-glycosidic bonds) induces a pronounced electrophoretic shift of several components (Benson *et al.* 1986, Venkatesan & Simpson 1986). On the contrary, treatment with endoglycosidase H (hydrolyzing N-glycosidic bonds of the high mannose type), neuraminidase or mild alkaline hydrolysis (cleaving O-linked oligosaccharides) does not alter the electrophoretic mobility of the soluble proteins. According to Benson *et al.* (1986), these results indicate that several

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                                  Leu      Ile      Gln Val
Trp Val Gly Asp Asn Gln Ala  Val      Asn
                                  Trp      Asp      Pro Glu
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Figure 5. Primary structure of the repeated sequence of the 50kD intraspicular glycoprotein of *Strongylocentrotus purpuratus* (from Sucov *et al.* 1987).

intraspicular proteins are N-glycosylated glycoproteins with oligosaccharides of the complex type. The gene that encodes of the intraspicular glycoprotein (viz. the 50kD protein) of *Strongylocentrotus purpuratus* larvae have been isolated and sequenced (Benson *et al.* 1987, Sucov *et al* 1987). The characteristics of the derived protein sequence were studied by Succov *et al.* (1987). As a whole, it is an acidic protein (pI = 5.2) of about 435 amino acids long; it displays an alternating pattern of hydrophobic and hydrophilic regions. The amino terminal half is characterized by a proline rich domain (i.e. 17 proline residues in a sequence of 55 amino acids). The carboxy terminal half contains a domain of 193 amino acids formed by the imperfect repetition of a 13-amino-acid sequence. This sequence consists itself in alternating hydrophobic and hydrophilic groups of amino acids (Fig. 5). The C-terminal end shows a very basic character (pI = 12.7) and contains the glycosylation signal. According to Sucov *et al.* (1987), the repetitive domain shows some similarities (but no significant homologies) with the calcium-binding domains of proteins such as troponin C and parvalbumin. They alternatively suggest that the repeating structure of this domain may also be involved in the ordering of other intraspicular proteins. The C-terminal basic domain is suggested to be involved in electrostatic interactions with negatively charged regions of other intraspicular proteins (Sucov *et al.* 1987).

Consistent with the glycoprotein nature of the intraspicular organic material, the specific inhibition of N-glycosylation of proteins by tunicamycin strongly affects the spiculogenesis of echinoid embryos. If embryos are incubated in tunicamycin prior to gastrulation, the primary mesenchyme cells do not arrange themselves into aggregates and strands (Schneider *et al.* 1978, Heifetz & Lennarz 1979, Akasaka *et al.* 1980, Mizoguchi *et al.* 1981). The spicules which developed in these conditions showed abnormal shapes and had no extinction position in polarized light indicating that the microcrystals of calcite were randomly arranged within the spicule (Mizoguchi *et al.* 1981). If whole larvae or cultured primary mesenchyme cells are incubated with tunicamycin after gastrulation, the spicule formation is stopped or delayed (Schneider *et al.* 1978, Mintz *et al.* 1981, respectively). Blocking spiculogenesis is correlated with a partial inhibition of both the calcium uptake by larvae and the concomitant ^{3}H-glucosamine incorporation into glycoproteins (Schneider *et al.* 1978, Mintz *et al.* 1981). Schneider *et al.* (1978) reported that unpublished observations suggested that a large fraction

of the ^{3}H-glucosamine labeled protein is associated with isolated spicules. However, tunicamycin also acts on embryological events unrelated to spicular growth. In echinoid embryos, it blocks the invagination of the vegetal plate, probably by preventing the formation of the secondary mesenchyme (Schneider *et al.* 1978, Heifetz & Lennarz 1979, Akasaka *et al.* 1980, Mizoguchi *et al.* 1981). In asteroid larvae tunicamycin blocks the bulging of the archenteron tip, the migration of mesenchyme cells, the formation of coelomic pouches, and the formation of the mouth (Dan-Sohkawa *et al.* 1980). These observations suggest that tunicamycin acts principally on the migratory capability of mesenchyme cells when mediated by filipodial movements (indeed, the formation of the primary mesenchyme cells is not inhibited; Mizoguchi *et al.* 1981). The abnormality or the inhibition of the spicular growth induced by tunicamycin could thus result either from affecting the ability of the primary mesenchyme cells to migrate and/or from directly inhibiting the synthesis of a glycoprotein forming the organic matrix of the spicule.

3.4 *Discussion*

The membrane of the calcifying site definitely acts as an organic matrix in both limiting the liquid volume at the mineralizing site and framing the shape of the larval spicule; it is probably also involved in the transport of the precipitating ions. The membrane governs the supersaturation level of the precipitating ions and regulates calcification mostly at the chemically controlled level.

Organic material occurs both around (i.e. the trabecular coat) and within (i.e. the intrastereomic organic material) the stereom. In larval spicules, the coating material surrounding the spicule is in continuity with the intraspicular organic material and the former can thus no more be considered as an organic matrix *per se*. The demonstration of intrastereomic organic material ends the long-time controversy as to whether echinoderms are the only group of animals having a one-phase skeleton (see e.g. Currey & Nichols 1967, 1969). The intrastereomic organic material is mostly made of proteins. It consists of glycoproteins in echinoid larvae (the relationships between proteins and carbohydrates within the stereom of postmetamorphic ossicles have not been investigated). The intrastereomic organic material of partly demineralized sections shows a fibrillar reticular structure. In larval spicules, it clearly forms a three-dimensional net of interconnected concentric sleeves permeating the whole spicule. This arrangement recalls the concentric inorganic layers made of 'small sub-units' observed in echinoid spicules (Millonig 1970, Okazaki & Inoué 1976). These authors reported that echinoid spicules would consist of an 'accumulation of crystallites' embedded in organic material. This observation strongly suggests that echinoid spicules

are made of concentric mineral layers separated by organic laminae resulting in an actual two-phase system. Moreover, inorganic concentric layers made of tapered prismatic structures were also observed in ossicles of postmetamorphic echinoids and asteroids (Nissen 1969, Pearse & Pearse 1975, Bignot 1976, O'Neill 1981, Dubois & Jangoux 1985). Although its significance is not totally understood (see Dubois & Jangoux 1985), this arrangement suggests that the stereom of post-metamorphic echinoderms is composed of mineral layers separated by organic laminae such as in larval spicules. As noted by Towe (1972), the occurrence of a coherent organic matrix within a mineral phase proves the discontinuity of the crystalline network and demonstrates the polycrystalline nature of the entity. Actually, Blake *et al.* (1984) showed by high resolution lattice fringe image of crinoid stereom that echinoderm calcite possesses a mosaic structure, i.e. it is composed of sub-μm-sized crystallites which are slightly misoriented one to the other. The crystallographic discontinuities produced by the mosaic structure were not detected by high voltage electron microscopy (Blake *et al.* 1984). From this point of view, Travis' (1970) observations showing highly ordered discrete crystals in the stereom of several postmetamorphic echinoderms, using conventional TEM, are difficult to interpret. All skeletal elements of echinoderms are thus actually polycrystalline aggregates made of highly oriented microcrystals that are probably arranged in mineral concentric layers alternating with organic laminae. The high orientation level of the microcrystals explains why in most cases echinoderm ossicles and spicules behave like single crystals of calcite either in polarized light, X-ray diffraction or crystal decoration studies (Raup 1959 and Okazaki 1975a; Donnay & Pawson 1969; Okazaki & Inoué 1976 and Okazaki *et al.* 1981, respectively) (slight crystal misalignments were detected in X-ray diffraction spectra of echinoid plates by Garrido & Blanco, 1947, and Nissen, 1963).

The macromolecular components of recognized organic matrixes of several phyla are classically separated into insoluble and soluble fractions in EDTA solutions (see Wheeler & Sikes 1984 for discussion of the validity of this dichotomy). The insoluble constituents are the hydrophobic 'framework' constituents which are thought to be a substrate for the soluble constituents and to contribute to the mechanical properties of the skeleton, whereas the soluble constituents are the acidic hydrophilic 'surface' constituents which are closely associated with the growing mineral (Degens 1976, Krampitz 1982, Crenshaw 1982, Weiner *et al.* 1983, Weiner 1984). Surface acidic constituents are present in all calcified tissues for which data are available (including echinoderms), whereas framework constituents are not always present (Weiner *et al.* 1983). Moreover, the biochemical nature of framework constituents varies substantially according to the animal group (Weiner 1984). On the contrary, surface acidic constituents show a more uniform composition, probably reflecting their calcium-binding activity (Weiner

1984). Two classes of soluble acidic proteins – one rich in aspartic acid, the other rich in serine and glutamic acid but poor in aspartic acid and linked with polysaccharides – have been reported from the skeleton of several phyla (Weiner 1984). These two classes have been demonstrated in the stereom of a postmetamorphic echinoid (Weiner 1985). They seemingly also occur in echinoid larvae where similar characteristic amino acids compose the glycoproteins occurring within the spicules (Benson *et al.* 1986, Venkatesan & Simpson 1986). It should be added, moreover, that the acidic proteins rich in serine and glutamic acid of the echinoid stereom resemble the acidic enamelins of vertebrate enamel (enamelins form part of the organic matrix of enamel; Eastoe 1968, Weiner 1984). That a limited amount of organic intrastereomic material (about 0.1-0.2% w/w) was found in ossicles of postmetamorphic echinoderms could be linked to a maturation process of the organic matrix. Actually this was demonstrated with enamel which loses 75 to 90% of its initial protein content during growth (Eastoe 1968, Degens 1976).

The evidence which indicates that the intrastereomic organic material of echinoderms may act as an organic matrix is (1) the glycoproteins extracted from larval spicules show a calcium-binding activity whose dissociation constant is similar to those of EDTA-soluble matriçes from several invertebrate skeletons (Benson *et al.* 1986); (2) the acetic-acid-soluble material extracted from echinoid stereom partly inhibits both larval calcification and *in vitro* crystallization of inorganic $CaCO_3$ (Swift *et al.* 1986); (3) the EDTA-soluble material extracted from both echinoid and asteroid stereom modify the morphology and cleavage properties of calcite crystals grown in its presence, cleavage and morphology of these crystals are reminiscent of the corresponding characteristics of echinoderm stereom (Berman *et al.* 1988, Dubois 1988). (The signification of such *in vitro* crystallization studies has been discussed by Wheeler & Sikes, 1984; briefly, they consider that *in vitro* studies gain some legitimacy if the formed crystals appear to mimic the morphology of the natural crystalline material.) Calcium-binding activity occurs at the anionic sites of a matrix, viz. acidic amino acid residues (found to be quantitatively important in the soluble protein fraction of both larval spicules and postmetamorphic stereom) or inorganic groups like phosphate (detected with the organic components of the echinoid stereom) and sulfate (Sikes & Wheeler 1983). Calcium-binding components are generally thought to promote nucleation but could also inhibit crystal growth by binding to $CaCO_3$ crystals (Wheeler & Sikes 1984). Indeed, partial inhibition of calcification processes are performed by echinoderm intrastereomic components. According to Wheeler & Sikes (1984), the main role of inhibiting components is probably to regulate crystallization. They would act at concentrations that do not block crystal growth or nucleation. At such concentrations, these components may markedly modify the crystal morphology as

demonstrated with oyster shell soluble matrix (Wheeler & Sikes 1984). As shown by Berman *et al.* (1988) and Dubois (1988), the echinoderm organic intrastereomic components can actually modify the calcite crystal morphology. Such a mechanism could account partly for the unique characteristics of echinoderm stereom. In this context, it is noteworthy that the inhibition of N-glycosylation of proteins (and of the putative organic matrix) causes random arrangement of the calcite microcrystals of larval spicules (Mizoguchi *et al.* 1981). This means that organic components must be involved in the ordering of the microcrystals. Furthermore, the possibility was raised that similar matrix components may sequentially both initiate and inhibit crystal growth (see Wheeler & Sikes 1984). The arrangement in concentric layers of the intrastereomic organic material, sandwiched between mineral layers, could be the result of such process.

The available evidence indicates that the high-magnesium calcite of echinoderms precipitates as highly ordered microcrystals on a putative organic matrix organized in a three-dimensional structure. This matches the occurrence of a spatial control of biomineralization in which the organic matrix controls the vectorial properties of the mineral formation (see Mann 1983). In such a spatially controlled process of biomineralization, the orientation of the microcrystals is directed by thermodynamic relationships between crystal faces and the organic matrix components (e.g. by atomic matching between the organic substrate and the mineral overgrowth or through the presence of active sites on the surface of the organic substrate). The size of the microcrystals is determined by both temporal and spatial regulation of the synthesis of the organic matrix (Mann 1983). One may note here that the calcitic otoconia of mammals share similarities with echinoderm ossicles (otoconia are crystalline ellipsoidal bodies of 5 to 10 μm length which occur in the gravity receptors of the inner ear of most vertebrates). Mammalian otoconia show X-ray diffraction patterns of single crystals although they are composed of microcrystals 50 to 100 nm in diameter which are arranged in sheets of highly ordered domains (Mann *et al.* 1983, Ross & Pote 1984). When partially etched, otoconia appear to be formed by microcrystals and look like etched trabeculae of echinoderms (see Mann *et al.* 1983, Fig. 14b, and compare with Dubois & Jangoux 1985). Otoconia contain protein material. Some crystallographic and morphological properties of their microcrystals strongly suggest that these proteins affect the seeding and growth of the crystals (Ross & Pote 1984). Mann (1983) suggested that the organic material within otoconia controls their formation at the spatial level.

The similarities between the intrastereomic organic material of echinoderms and the organic matrixes of mineralized tissues from other phyla, together with the above-mentioned preliminary evidence, strongly suggest that the intrastereomic material is an organic matrix. This material would

control nucleation and growth of highly ordered microcrystals at the spatially controlled level. Future investigation of the organic matrix of echinoderms should (1) characterize the morphology of the organic matrix of ossicles of postmetamorphic echinoderms, (2) identify the different constituents of the organic matrix of both larval spicules and postmetamorphic ossicles and test their matrix function, (3) precisely locate each constituent of the organic matrix within the mineral phase, (4) determine the steric organization of both polypeptide and polysaccharide chains that constitute the organic matrix.

4 IONS

4.1 *Calcium ions*

The relationships between calcium uptake and skeleton formation have been studied in whole echinoid larvae, in *in vitro* cultured primary mesenchyme cells of echinoid larvae, and in growing and regenerating postmetamorphic ossicles of asteroids, echinoids, and ophiuroids.

In both growing and regenerating ossicles, $^{45}Ca^{++}$ was incorporated from the outer medium into the stereom a few hours after the beginning of the incubation period (Heatfield 1970, Nauen & Böhm 1979, Dafni & Erez 1982, Dafni 1985, Donachy & Watabe 1986). Incorporation may start in less than one hour in regenerating echinoid spines (Heatfield 1970). During both the slow growth stage of *Asterias rubens* (the so-called waiting stage) and the arm regeneration of *Ophiothrix angulata*, net deposition of $^{45}Ca^{++}$ in ossicles is preceded by a short period (a few hours) of saturation of exchangeable calcium pools (respectively, Nauen & Böhm 1979, Donachy & Watabe 1986). Kaneko *et al.* (1981a, b) showed that postmetamorphic echinoids take up $^{45}Ca^{++}$ from the digestive tract and the body-wall surface, especially the tube feet. Labelled calcium ions were rapidly accumulated within the coelomic fluid which reached equilibrium with the medium after 24 to 36 hours of incubation (Heatfield 1970, Kaneko *et al.* 1981b). However, *in vitro* regenerating spines of echinoids show the same rate of calcium deposition as *in vivo* regenerating spines (Heatfield 1970). This demonstrates that calcium ions needed for skeletal growth can be directly incorporated through the epidermis.

In echinoid larvae, the level and rate of calcium uptake increase rapidly during gastrulation (viz. when spicule formation initiates; Nakano *et al.* 1963, Schneider *et al.* 1978, Fujino *et al.* 1985a) (at the late gastrula stage, the rate of calcium uptake is over ten times higher than that of the blastula stage). A similar increase in calcium uptake occurs at the time when spicules begin to be produced by *in vitro* cultured primary mesenchyme cells (Mintz *et al.* 1981). Both cultured mesenchyme cells and whole larvae deposit 70% (at the

Table 3. Inhibition of calcium transport systems in echinoid larvae.[1]

Transport systems	Inhibitors	Inhibited processes						References
		Ca uptake		Ca deposition		Spicular formation		
		L	C	L	C	L	C	
Calcium channels	Diltiazem	+	+	+		+		Mitsunaga *et al.* 1984, Fujino *et al.* 1985a, Iwata & Nakano 1985b, Yasumasu *et al.* 1985a, b
	Verapamil	+	+	+	+	+	+	Mitsunaga *et al.* 1984, Fujino *et al.* 1985a, Iwata & Nakano 1985b, Makihara *et al.* 1985, Yasumasu *et al.* 1985a, b
	Verapamil		–					Mintz *et al.* 1982
	D-600		–					Mintz *et al.* 1982
	Nitrendipine		–					Mintz *et al.* 1982
	Nicardipine		+					Iwata & Nakano 1985b
	Nifedipine		+		+	+	+	Makihara *et al.* 1985, Mitsunaga *et al.* 1986a
Anion transport	DIDS	+	+	+		+		Fujino *et al.* 1985a, Yasumasu *et al.* 1985a, b
Chloride transport	Furosemide	+/–[2]	+	+	+	+	+	Fujino *et al.* 1985a, Yasumasu *et al.* 1985a,
	Ethacrynic acid	+/–[2]	+	+	+	+	+	b, Mitsunaga *et al.* 1986a, c
Respiration-dependant Ca^{++} transport in mitochondria	Ruthenium red	+	+	+	+	+	+	Fujino *et al.* 1985a, Makihara *et al.* 1985
Ca^{++}-ATPase of the plasma membrane	Ruthenium red	+		+		+		Fujino *et al.* 1985a
	R24571		+		+			Mintz *et al.* 1982

1. L: experiments carried on larvae; C: experiments carried on *in vitro* cultured primary mesenchyme cells; +: inhibition; –: no inhibition.
2. For explanation, see text.

prism stage) to 40% (at the pluteus stage) of the incorporated calcium ions into their spicules (Mintz *et al.* 1981, Fujino *et al.* 1985a). Furthermore, in whole larvae, the rate of calcium uptake begins to increase during the mesenchyme blastula stage, before the onset of spiculogenesis (Nakano *et al.* 1963). Autoradiographic studies showed that this early accumulation occurs within the blastocoelian extracellular matrix and not within the primary mesenchyme cells (Nakano *et al.* 1963).

In larvae, calcium ions are taken up from the external medium directly through the ectoderm (Okazaki 1956, 1975a, Yasumasu *et al.* 1985b). Both the intracellular location of the larval spicule (see part 2.1) and the kinetics of incorporation of calcium ions into *in vitro* cultured primary mesenchyme cells indicate that calcium ions are taken up by primary mesenchyme cells before being deposited into the spicules. (Mintz *et al.* 1981, Decker & Lennarz 1988). Moreover, Decker *et al.* (1987) reported, from TEM studies of *in vitro* cultured primary mesenchyme cells, that calcium ions are sequestered during calcification in mitochondria as well as, to a lesser extent, in RER and in 'smooth surface' vesicles.

The mechanisms which control calcium uptake and deposition have been studied only in echinoid larvae. The involvement of calcium-transport systems, cAMP, and extracellular components was suggested.

Studies using inhibitors indicated that several transport systems are involved in calcium uptake and deposition in echinoid larvae (Table 3). Calcium antagonists (i.e. calcium channel inhibitors) were reported by most authors to inhibit calcium uptake and deposition both in whole larvae and in *in vitro* cultured primary mesenchyme cells. An anion transport inhibitor – DIDS (4,4'-diisothiocyano-2,2'-disulfonic acid stilbene) – showed the same effects as calcium antagonists while chloride transport inhibitors – furosemide and ethacrynic acid – showed a different pattern of inhibition. At the swimming blastula stage, calcium uptake in whole embryos was hardly blocked by the chloride transport inhibitors. At the late gastrula stage, they completely inhibited calcium deposition into spicules but only reduced at 50% the level of calcium uptake in whole larvae (Fujino *et al.* 1985a, Yasumasu *et al.* 1985a, b). Indeed, the uptake of calcium in cells not associated with the spicules (i.e. ectodermal and endodermal cells) was only slightly reduced by chloride transport inhibitors. These inhibitors thus inhibit calcium uptake only in skeletogenic cells. Experiments carried out on *in vitro* cultured primary mesenchyme cells showed that calcium uptake in skeletogenic cells is only partly blocked by chloride transport inhibitors whereas calcium deposition in spicules is totally prevented (Mitsunaga *et al.* 1986a). Moreover, chloride transport inhibitors also inhibit the release of CO_2 (or of its associated ions HCO_3^- and CO_3^{--}) from the primary mesenchyme cells (both toward the calcifying vacuole and to the culture medium) probably through blockage of a membrane Cl^-/HCO_3^- ATPase (Mitsunaga *et al.*

1986a; see part 4.2). The inhibition of calcium uptake and deposition by ruthenium red and R 24571 suggests that both the Ca^{++}-ATPase of the plasma membrane and the respiration-dependent calcium transport in mitochondria (see Table 3) are also related to spicule formation (Fujino *et al.* 1985a, Makihara *et al.* 1985). Furthermore, colchicine and podophyllotoxine inhibit calcium uptake and deposition by cultured primary mesenchyme cells at concentrations which do not prevent the elongation of the pseudopodial syncytium (Fujino *et al.* 1985b). This suggests that tubulin assembly is implicated in calcium transport within the calcifying cells.

Inhibition experiments with *in vitro* cultured primary mesenchyme cells showed that some transport systems certainly occur in the calcifying cells themselves (see Table 3) and suggest a transport pathway for calcium ions. Calcium ions would enter the primary mesenchyme cells through calcium channels in the plasma membrane. This transport is probably coupled with the co-transport of chloride ions, neutralizing the increase in positive charge caused by the calcium influx (Yasumasu *et al.* 1985b, Mitsunaga *et al.* 1986a). The decrease in intracellular calcium concentration caused by mitochondrial calcium uptake could make calcium transport through calcium channels easier. TEM studies by Decker *et al.* (1987) confirmed that mitochondria sequester large amounts of calcium during spicule formation. Tubulin could contribute to calcium transport through the pseudopodial syncytium to the calcifying vacuole (Fujino *et al.* 1985b). The mechanism of calcium secretion into the calcifying vacuole is still unknown. Calcium ions could be driven into the vacuole by the release of HCO_3^- by the Cl^-/HCO_3^- ATPase (Mitsunaga *et al.* 1986a). In this context, a 130kD protein involved in spicular deposition has been detected on the surface of primary mesenchyme cells and, to a lesser extent, on their associated extracellular matrix (Carson *et al.* 1985). The monoclonal antibody to this protein inhibits calcium deposition and blocks spicular growth in cultured primary mesenchyme cells already attached to their culture dishes (Carson *et al.* 1985, Grant *et al.* 1985). Experiments uncoupling calcium deposition from calcium uptake showed that the antibody actually inhibits calcium uptake in the primary mesenchyme cells (Grant *et al.* 1985). Three other antibodies (two monoclonal and one polyclonal) were demonstrated to recognize the 130kD protein (Anstrom *et al.* 1987, Leaf *et al.* 1987). It was confirmed that it is a surface protein specifically expressed by primary mesenchyme cells after the mid-gastrula stage and whose expression is related to the spiculogenic ability of the cells (Farah *et al.* 1987, Leaf *et al.* 1987). Moreover, the 130kD protein was localized in the Golgi complexes of primary mesenchyme cells (Decker & Valdizan 1987). It is noteworthy that a 180kD calcium-binding protein whose synthesis is correlated with spiculogenesis had already been detected in intracellular granules of the primary mesenchyme cells (Iwata & Nakano 1986).

The intracellular cAMP level was reported to control the uptake of calcium ions (Mintz & Lennarz 1982, Mintz *et al.* 1982). High cAMP levels (induced by cAMP phosphodiesterase inhibitors, cAMP analogues, or stimulation of adenyl cyclase) block spicule formation and calcium uptake in cultured primary mesenchyme cells engaged in spicule formation. In contrast, calcium uptake is not affected by high levels of cAMP prior to spicule formation (Mintz & Lennarz 1982, Mintz *et al.* 1982). No mechanism has been proposed for the role of cAMP in the mineralization process of echinoderms. One may notice, however, that cAMP is known to exert its effects by activating cAMP-dependent protein kinases and that these enzymes may control the activity of calcium channels or pumps by phosphorylation (Alberts *et al.* 1983).

Iwata & Nakano (1985b) suggested that calcium uptake in cultured primary mesenchyme cells is controlled by a fibronectin-binding acid polysaccharide (FAPS). Indeed, they observed that a monoclonal antibody to FAPS (anti-FAPS) increased both the number of spicules produced *in vitro* by cultured primary mesenchyme cells and the calcium uptake by these cells. The spicules produced under these conditions were, however, smaller than those which develop in control experiments. In whole larvae, FAPS occurs in the extracellular matrix surrounding the primary mesenchyme cells. When anti-FAPS was injected into the blastocoel, more than one pair of triradiate spicules developed (Iwata & Nakano 1985a). Moreover, these spicules had an abnormal morphology. Okazaki (1960) demonstrated that multispiculation results from a failure of the triangular hyaloplasmic areas and the pseudopodial strands to fuse. Moreover, she also demonstrated that spicules of abnormal morphology were produced when the shape of the pseudopodial syncytia was modified (Okazaki 1962). This strongly suggests that FAPS is one of the so-called 'extrinsic factors' which control differentiation and migration of the primary mesenchyme cells (see part 3.1). As a consequence, FAPS is probably not directly involved in calcium uptake contrary to the proposal of Iwata & Nakano (1985b).

When reviewing biochemical and histochemical studies about alkaline phosphatase activity during echinoid larval development, Giudice (1973) pointed out that 'the enzymatic activity starts to increase at gastrulation to become particularly high at pluteus stage'. Alkaline phosphatase activity is principally located within both the cells of the archenteron and the primary mesenchyme cells (Evola-Maltese 1957a, Hsiao & Fujii 1963). Moreover, beryllium – an alkaline phosphatase inhibitor – induces defects in spicular formation (Evola-Maltese 1957b). Because of these observations, both Evola-Maltese (1957a, b) and Hsiao & Fujii (1963) suggested that alkaline phosphatase was involved in spicule formation. However, beryllium is known to compete with divalent metals such as Mg^{++} and Ca^{++} whose role in general physiological processes and in spicular formation have been stressed

Table 4. Carbonic anhydrase in calcified tissues of echinoderms.

Species	Tissue	Activity[1]	References
Echinoidea (larvae)			
Anthocidaris crassispina	whole larva	m; inh. acetazolamide	Mitsunaga *et al.* 1986b
Arbacia aequituberculata	whole larva	inh. benzothiazolesulfonamide	Stolkowsky 1948
Paracentrotus lividus	whole larva	inh. benzothiazolesulfonamide	Stolkowsky 1948
Strongylocentrotus purpuratus	whole larva	m; inh. acetazolamide	Chow & Benson 1979
	cultured primary mesenchyme cells	inh. acetazolamide	Carson *et al.* 1985, Grant *et al.* 1985
Hemicentrotus pulcherrimus	whole larva	m; inh. acetazolamide	Mitsunaga *et al.* 1984, 1986b
	cultured primary mesenchyme cells	inh. acetazolamide	Mitsunaga *et al.* 1986b
Unidentified species	whole larva	+	Mintz *et al.* 1982
	cultured primary mesenchyme cells	inh. acetazolamide	Mintz *et al.* 1982
Echinoidea (postmetamorphic individuals)			
Strongylocentrotus purpuratus	regenerating spine	inh. acetazolamide	Heatfield 1970, Heatfield & Wilbur 1972
Strongylocentrotus droebachiensis	body wall, regenerating test and spine	+	Vocisano 1971
Lytechinus variegatus	plumula of the tooth	m; inh. acetazolamide	Chen 1985, Chen & Lawrence 1986b, 1987

1. m: activity measured; +: activity detected but not measured; inh.: presence demonstrated by effects of specific inhibitors.

(Okazaki 1956, 1961, 1962). This led Giudice (1973) to question the actual role of alkaline phosphatase in spiculogenesis. One may yet notice that the activity of that enzyme is closely correlated to Ca^{++}-ATPase in rat tissue (Russel *et al.* 1972). Moreover, alkaline phosphatase has been suggested to be involved in calcium transport into matrix vesicles (and not to function as a hydrase) in chicken epiphyseal cartilage (Wuthier *et al.* 1981).

4.2 *Carbonate ions*

Metabolic CO_2 is the major source for carbon in the echinoid larval spicule. The ratio between the $^{45}Ca^{++}$ and the dissolved inorganic ^{14}C incorporated in the spicule indicates that, while respiration produces more than enough carbon to support all calcification, metabolic CO_2 supplies only 63.2% of the carbon needed for spicule deposition, the remaining 36.8% being supplied by dissolved inorganic carbon (Sikes *et al.* 1981). There are no direct data about the source of carbon used in skeleton formation of postmetamorphic echinoderms.

Two transport systems of carbonate ions (or their associated species bicarbonate ions and carbon dioxide) were investigated in echinoderms, viz. carbonic anhydrase and a Cl^-/HCO_3^- ATPase. Carbonic anhydrase catalyzes the hydration of CO_2 and dehydration of HCO_3^-. This enzyme is involved in several carbonating biomineralization systems (Degens 1976, Tashian & Hewett-Emmett 1984) although its exact role in most of these systems is not yet clear (Sikes & Wheeler 1983). Carbonic anhydrase activity has been demonstrated in the calcified tissues of both larval and postmetamorphic echinoids (Table 4). The use of specific inhibitors demonstrated that carbonic anhydrase interferes in the calcification processes of echinoderms. Spiculogenesis and calcium deposition are inhibited or reduced within whole larvae and cultured primary mesenchyme cells by both benzothiazolesulfonamide and acetazolamide, i.e. two specific inhibitors of carbonic anhydrase (Stolkowsky 1948; Mintz *et al.* 1982, Mitsunaga *et al.* 1984, 1986b, Carson *et al.* 1985, and Grant *et al.* 1985, respectively). In regenerating spines of postmetamorphic echinoids, $^{45}Ca^{++}$ deposition decreased by 50 to 60% when the spines were incubated *in vitro* with acetazolamide (Heatfield 1970). The same experiment performed *in vivo* showed that the rate of spine regeneration decreased by 88% and that the regenerating stereom of inhibitor-incubated spines was denser than that of control regenerating spine (Heatfield & Wilbur 1972). Production of denser stereom is probably related to slower growth rate (see Pearse & Pearse 1975).

Carbonic anhydrase was localized in the plumula of the echinoid tooth by autoradiography using labelled inhibitors (Chen & Lawrence 1986b). Occurrence of carbonic anhydrase was demonstrated in several places, namely (1) in the mesothelium of the tooth, (2) in odontoblasts not yet engaged in

calcification, (3) in calcifying odontoblasts, (4) in fibroblasts, and (5) in the extracellular matrix. Most of the carbonic anhydrase activity of the odontoblasts is located on or in the plasma membrane and in the membrane of the calcifying vacuole. The function of carbonic anhydrase of the plumula was studied by incubating the teeth *in vitro* with ^{14}C-$NaHCO_3$ as external source of inorganic carbon (Chen 1985, Chen & Lawrence 1987). The kinetics of entry of ^{14}C into the soft tissues in the presence or absence of acetazolamide indicates that carbon enters the tooth as CO_2 and that both mesothelial carbonic anhydrase and passive diffusion are involved in CO_2 transport into the tooth. At high concentrations of external dissolved inorganic carbon, the entry of ^{14}C in the soft tissues of the control group was lower than that of the acetazolamide-treated group. This suggests that epithelial anhydrase can also facilitate the movement of CO_2 out of the tooth to regulate the CO_2 content within the latter. Acetazolamide decreases the incorporation of ^{14}C into the calcareous elements of the plumula showing that carbonic anhydrase of odontoblasts facilitates the movement of CO_2 from the soft tissues through the membrane of the calcifying vacuole. Chen & Lawrence (1987) suggested that membrane-bound carbonic anhydrase would form an ion-channel which would separate H^+ and HCO_3^-, the H^+ ions moving from the vacuolar membrane into extracellular spaces while the HCO_3^- ions would move through the vacuolar membrane to the biomineralizing site.

Involvement of a Cl^-/HCO_3^- ATPase activity was suggested by use of chloride transport inhibitors (viz. ethacrynic acid and furosemide) on cultured primary mesenchyme cells. These inhibitors block both $^{45}Ca^{++}$ and ^{14}C deposition into the larval spicule (see part 4.1) and the release of CO_2 from the calcifying cells (as well to the calcifying vacuole as to the culture medium) (Mitsunaga *et al.* 1986a). They inhibit also the ouabain-insensitive ATPase activity dependent on Cl^- and HCO_3^- which is found in the homogenates and the microsome fraction of cultured primary mesenchyme cells (Mitsunaga *et al.* 1986a, c). Furthermore, the activity of Cl^-/HCO_3^- ATPase in embryos and cultured primary mesenchyme cells increases during development, in close correlation with the rate of calcium deposition in spicules. Finally, after initiation of spiculogenesis, the activity of that enzyme is mostly detected in the spicule vacuole and plasma membrane fractions of cultured primary mesenchyme cells (Mitsunaga *et al.* 1987a). (It should be said, however, that, according to the authors themselves, the spicule vacuole fraction was not sufficiently characterized.) These results led Mitsunaga *et al.* (1986a, 1987a) to suggest that a Cl^-/HCO_3^- ATPase, located in the plasma membrane and in the membrane of the calcifying vacuole, is involved in the release of HCO_3^- to the calcifying vacuole. According to them, carbonic anhydrase would not be involved in HCO_3^- or CO_2 transport through the membrane of the calcifying vacuole that later enzyme would only catalyzes the production of HCO_3^- in the cytosol).

Overall, carbonate deposition is an acid-producing process. *In vitro* observations show that the crystal growth of carbonate is proportional to the quantity of base-titrant required to maintain a constant pH during the carbonate deposition reaction (Wheeler & Sikes 1984). In echinoid larvae, the magnitude of the pH decrease during calcification depends on the source of carbon, being greater when the carbon source is CO_2 (Sikes *et al.* 1981). An alkaline environment favors and an acid environment hampers the formation of the skeleton. For example, larvae of *Paracentrotus lividus* reared in acidified sea water show a very slow spicular development below pH 7.2 and a regression of the spicules below pH 6.4 (Bouxin 1926). Although low pH could affect many physiological processes, one may presume that the acid-base balance has to be maintained at a certain level for the formation of the skeleton. Carbonic anhydrase functions in an unsaturated condition at the mineralizing site of the echinoid tooth, suggesting that the mineralizing site is alkaline (Chen 1985, Chen & Lawrence 1987).

The mechanisms of acid-base balance related to calcification processes were mainly studied in echinoid larvae. Allylisothiocyanate – an inhibitor of H^+/K^+ ATPase – inhibits calcium deposition and spicule formation both in larvae and in cultured primary mesenchyme cells (Mitsunaga *et al.* 1987b). Furthermore, the H^+/K^+ ATPase activity becomes detectable as soon as spicule formation initiates. An H^+/K^+ ATPase activity was principally found in the plasma membrane and the microsome fractions whereas almost no activity was detected in the spicule vacuole fraction (Mitsunaga *et al.* 1987a). As a consequence Mitsunuga *et al.* (1987a) suggested that the H^+/K^+ ATPase, while releasing the protons out of the primary mesenchyme cells, is not involved in the deacidification of the calcifying vacuole. According to them, the CO_2 produced by calcite deposition would passively diffuse into the cytosol and would then be converted by carbonic anhydrase into HCO_3^- and H^+ (the bicarbonate ions being transported into the calcifying vacuole by the Cl^-/HCO_3^- ATPase, see part 4.2). In postmetamorphic echinoderms, Chen & Lawrence (1987) suggested that an alkaline environment could be maintained at the mineralizing site of the echinoid tooth by carbonic anhydrase acting as an ion-channel (see part 4.2). Urease, which converts urea into carbon dioxide and ammonia (which in turn may be subject to protonation to form NH_4^+), may elevate pH during calcium carbonate deposition. Loest (1979) purified an urease from whole echinoids with properties similar to those from other animals with a calcium carbonate skeleton. He suggested that this enzyme is possibly significant in carbonate deposition in echinoderms (as it was suggested to be in snails and corals). However, the urease activity of the echinoid embryos declines by about 30% from the unfertilized eggs to the blastula stage, after which it remains relatively constant (Brook-

bank & Whiteley 1954). Moreover, the uptake of ^{14}C-labelled urea by the soft tissues and its incorporation as carbon source into the spicules of echinoid larvae are very low (Sikes *et al.* 1981). According to Sikes *et al.*, these observations suggest that neither urease nor urea are involved in the calcification process of echinoderms.

4.4 *Magnesium ions*

Magnesium is present in the echinoderm skeleton in solid solution within the calcite and consequently affects the unit cell parameters of the biomineral (Chave 1952, Goldsmith *et al.* 1955). Magnesium concentration within echinoderm calcite ranges from 2.5 to 39.4% $MgCO_3$ (w/w) (Schroeder *et al.* 1969). Despite the quantitative importance of magnesium and its possible involvement in nucleation processes, the only evidence about its origin and role in the echinoderm skeleton come from Okazaki's (1962) experiments on echinoid larvae. According to her, the triradiate spicules of larvae cultured in magnesium-free sea water either did not develop or stopped to elongate and thickened. This thickening caused the tips of the spicule to become cubical. This indicates that magnesium is directly taken up by the larvae from the surrounding sea water. In larvae cultured in sea water containing one-tenth of the normal calcium and magnesium concentrations ('0.1 Ca-Mg' sea water), the rudiment of the spicule grew into a cube. If the transfer from normal sea water to 0.1 Ca-Mg sea water was done at the triradiate spicule stage the arms of the spicule thickened without elongating, each arm ending in a cube (Okazaki 1962). SEM observations show that the faces of such cube correspond to the cleavage faces of a calcite rhomb (Okazaki & Inoué 1976). On the other hand, when larvae were cultured in 0.1 Ca sea water (i.e. 0.1 Ca-1 Mg sea water), the rudiment developed as a spherical mass. If the larvae were transferred from normal sea water to 0.1 Ca sea water at the triradiate stage, the spicule failed to lengthen and thicken, ending in a globular bulge (Okazaki 1956, 1962). These experiments show that magnesium could be involved in concealing the crystalline faces of echinoderm high-magnesium calcite* (this property is obviously related to the solid solution state of magnesium within calcite, a suggestion that had been made for the stereom of postmetamorphic asteroids by O'Neill 1981). That suggestion is supported by SEM observations of synthesized crystals of magnesium calcite with increased magnesium levels: the higher the magnesium level, the rounder

*As quoted by Okazaki (1962), calcium and/or magnesium deficiency also causes deformations of the skeletal envelope. These deformations prevent the lengthening of the spicule but, as noted by Okazaki herself, do not explain the fact that spicules of different shapes are sometimes formed by envelopes of seemingly like shapes (e.g. in 0.1 Ca and 0.1 Ca-Mg sea water).

the shape of the crystal (crystals of pure calcite have a rhombic shape) (Koch, pers. comm.).

Particular attention has been paid to the environmental factors which govern the concentration of skeletal magnesium. Clarke & Wheeler (1922) first reported the occurrence of a relationship between the magnesium concentration of echinoderm skeleton and the temperature of the sea water in which the organisms lived. They observed that within each of the four classes investigated (viz. crinoids, echinoids, asteroids, and ophiuroids) the skeleton of the species from warmer waters showed a higher magnesium concentration than those from colder waters (e.g. the $MgCO_3$ concentration of crinoid species ranged from 7.28% w/w of ash residue at 1.5°C to 13.74% at 28°C). According to Clarke & Wheeler (1922) and subsequent pre-1950 authors (see Vinogradov 1953, for review), the relationship between sea water temperature and magnesium concentration of the skeleton is more obvious in crinoids and echinoids than in asteroid and ophuroids. Chave (1954) calculated the regression lines of skeletal magnesium concentration versus sea water temperature in each class of echinoderms (except holothuroids) using new data from X-ray diffraction analysis (a method which avoids contamination from the soft tissues). He showed that a definite positive correlation occurred between the two parameters in the four classes although the data did not fit the regression lines as closely as in other phyla (this is particularly obvious in echinoids and crinoids for which numerous data have been collected). A more detailed statistic analysis was performed by Weber (1969, 1973) who demonstrated that the relationship between sea water temperature and skeletal magnesium concentration of different species from the same class is real although poorly defined, the data often being widely scattered even within one genus. Moreover, he showed that in both echinoids and asteroids the relationships are significantly different from one taxonomic order to another. In some cases, the difference was significant even at the generic level. The variability of the temperature-magnesium relationship between orders and genera of the same class becomes obvious when analyzing the skeletal magnesium concentration of different species from the same community. Significant differences which occur between species of the same locality are rather low in asteroids and ophiuroids but may be high in echinoids, viz. up to 6.5% $MgCO_3$ (Pilkey & Hower 1960, Raup 1966, Weber 1969). The skeletal magnesium concentration is thus not only controlled by environmental factors but also by genetic factors (Weber 1969).

In order to assess the relative influence of genetic and environmental effects, variations of the skeletal magnesium concentration were studied among individuals from the same species and among ossicles from the same individual.

Weber (1969) reported that magnesium distribution in the stereom of echinoid ossicles is not homogenous at the 10 µm level provided by electron-

microprobe analysis. Yet, this inhomogeneity is limited in most ossicles except teeth whose different regions show differences in $MgCO_3$ concentrations ranging from 2.5 to 39.4% (w/w) (Weber 1969, Schroeder *et al.* 1969). On the contrary, Blake & Peacor (1981) and Blake *et al.* (1984) reported that the magnesium distribution in the stereom of a crinoid ossicle is homogenous at the 100 nm level provided by STEM microanalysis. As a consequence, magnesium ion distribution cannot be related to the general rounded morphology of echinoderm stereom, as hypothesized by O'Neill (1981) (Blake *et al.* 1984).

In crinoids, asteroids, and ophiuroids no significant difference was detected in the magnesium concentration of different ossicles from the same individual (Chave 1954, Weber 1969, 1973). However, different ossicles of the same echinoid individual do not have the same magnesium concentration (Clarke & Wheeler 1922, Vinogradov 1953, Chave 1954, Weber 1969, 1973). According to Weber (1969), the difference in $MgCO_3$ concentration between spines and coronal plates may reach 8.7% w/w in *Echinometra mathaei*. Furthermore, a limited but significant variability occurs (the standard deviation ranging from 0.12 to 1.02% $MgCO_3$ w/w) in homologous ossicles from crinoid, asteroid, ophiuroid or echinoid individuals of the same population (Pilkey & Hower 1960, Weber 1969). The skeletal magnesium concentration thus appears also to be controlled by individual biological factors.

Sumich & McCauley (1972) reported that the variations of skeletal magnesium concentration in individual *Allocentrotus fragilis* from the same population are negatively correlated with the age of the echinoids (range: about 1 to 1.5% $MgCO_3$ w/w). They suggested that differences between young and older echinoids would result from a lower magnesium incorporation into the new plates of older individuals that grow more slowly (see below for discussion about the growth rate effect). On the contrary, Pilkey & Hower (1960) found no relation between the skeletal magnesium concentration and the size (and presumably the age) in *Dendraster excentricus*.

Few authors have compared individuals coming from different populations with contrasting environmental parameters. This is partly caused by the paucity of species living in a wide range of temperature, a fact preventing suitable statistic analysis (Weber 1969). Pilkey & Hower (1960; *Dendraster excentricus*) reported the occurrence of a correlation ($r = 0.63$ to 0.84) between skeletal magnesium concentration and sea water temperature over a temperature range of 10°C representing 24 localities. However, the $MgCO_3$ concentration changed by only 1% over the temperature range and the authors did not test the significance of their correlation coefficients. The same authors also suggested a salinity effect on the skeletal magnesium concentration but their data are too few to draw any conclusion. Sumich & McCauley (1972; *Allocentrotus fragilis*) found no correlation between skeletal magne-

sium concentration and sea water temperature in three stations at different depths. Laboratory experiments by Davies *et al.* (1972; *Arbacia punctulata*) on regenerating broken primary spines under different temperatures indicated that the rate of spine regeneration is constant at a given temperature. Davies *et al.* showed moreover that the magnesium concentration of the regenerate is correlated only with the maturity of the stereom (i.e. the stage of regeneration), the oldest parts of the regenerate have the magnesium concentration closest to that of the stub. The correlation which is apparent between magnesium concentration of the regenerate and temperature at any given time is thus only due to the stage of maturity reached by the stereom (which depends on the growth rate). At advanced stages of regeneration, the chemical compositions of the stub and of the oldest part of the regenerate become equal. These observations fit well with Weber's (1973) studies on spines in various degrees of regeneration of echinoids collected from the field. Thus, during growth of the spine regenerate, the calcite deposited at any specific site gradually changes from an initial low-magnesium calcite to a high-magnesium calcite as in the stub. Temperature does not control the final magnesium concentration of the ossicle. Davies *et al.* (1972) proposed two mechanisms that could explain this change, namely (1) a gradual increase in the magnesium content of the calcite being deposited producing a 'zoned' crystal, or (2) a process of solution and redeposition, or of exchange between the solid phase and the stroma. STEM microanalysis of crinoid stereom showed that the distribution of magnesium ions is homogenous at a subtrabecular level (see above). However, occurrence of concentric inorganic layers in trabeculae of several species (see part 3.4) could suggest a combination of the two mechanisms.

In conclusion, the skeletal magnesium concentration of a given species appears to be governed mainly by genetic factors, the variation between homologous ossicles of different individuals remaining limited always to about 1% $MgCO_3$ (w/w). At higher taxonomic levels, the temperature effect on skeletal magnesium concentration is less obvious than supposed by early authors. Furthermore, the relationship between sea water temperature and skeletal magnesium concentration differs according to the orders and genera investigated. These observations led Weber (1973) to conclude that 'biological factors are enormously more important in controlling the skeletal chemistry of echinoderms than are environmental parameters'. Weber (1969, 1973) proposed that growth rate may account for both temperature and biological effects. (For this purpose, Weber postulated that temperature and growth rate are positively correlated, a fact that is not certain; see Moore 1966.) Weber (1969, 1973) suggested that magnesium is an impurity accidentally complexed by the organic matrix. If the crystallization rate is low, the magnesium ions have a high probability of being exchanged with calcium ions whereas at high crystallization rates, the time available for exchange will

be reduced and more magnesium ions will remain within the calcite. In Weber's hypothesis, the skeletal magnesium concentration is thus dependent on three different parameters: (1) the rate of crystallization, (2) the concentration of magnesium ions at the crystallization site, and (3) the properties of the organic matrix. However, studies by Davies *et al.* (1972) do not support the growth rate effect proposed by Weber, although the experiments of the former involved a single species (i.e. the genetic variability being greatly reduced). Both Heatfield (1971a) and Davies *et al.* (1972) distinguished two patterns of skeletal growth during the regeneration of echinoid spines, viz. a longitudinal growth producing thin trabeculae and a lateral growth during which trabeculae thicken. During the longitudinal growth, the trabeculae increase primarily in length, so that the growth rate (expressed as the lengthening of the regenerate) and the crystallization rate are proportional. During the longitudinal growth, the same concentrations of magnesium are found in regenerating spines whatever their growth rates (and thus whatever the crystallization rates). Thus, in that case, the skeletal magnesium concentration did not seem to be related to the crystallization rate and one may consequently suggest that the organic matrix must play the leading role in determining the magnesium concentration in echinoderms. Furthermore, the maturation of the organic matrix could explain the change in magnesium concentration undergone by the calcite of the regenerate stereom during its maturation.

4.5 *Trace elements*

In addition to calcium and magnesium, the stereom of echinoderms contains several trace elements, the most important ones being sodium and strontium (Table 5). Occurrence of sodium presumably results from contamination as Donnay & Pawson (1969) showed that, when immersed alive in alcohol, echinoid spines had no trace of NaCl in their skeleton. On the contrary, spines which have been dried in air gave the X-ray diffraction pattern of NaCl.

Studies about the origin, transport, and factors influencing the precipitation of the trace elements in the echinoderm skeleton are too few to allow any generalization. Binyon (1978) demonstrated that an increase of strontium concentration in sea water produces a similar increase of the strontium concentration in the soft tissues of asteroids. He also reported that the duration of experiment (ten days) was, however, too short to eventually reflect the strontium increase in the stereom. Pagett (1985) showed, furthermore, that there was no significant difference in skeletal strontium concentration between ophiuroids living in waters which differ in strontium concentration (from 4.3 to 7.9 mg/l). Salinity was reported to be unrelated with skeletal strontium concentration in *Dendraster excentricus* (Pilkey & Hower 1960). Thompson & Chow (1955) found no significant difference in the Sr/Ca ratio

.Table 5. Concentration range of the most important minor elements of echinoderm skeleton (ppm in dried skeleton).[1]

Elements	Concentration ranges	References of extreme values	References of intermediate values
Na[2]	3800-12200	Harris & Pilkey 1966[3]	Riley & Segar 1970[3]
Sr	246-2600	Mauchline & Templeton 1966, Davies *et al.* 1972	Thompson & Chow 1955[3], Pilkey & Hower 1960, Riley & Segar 1970[3], Gunatilaka 1975[3], Binyon 1978, Pagett 1985
K	330-650	Riley & Segar 1970[3]	
Al	120-160	Riley & Segar 1970[3]	
Ba	97	Mauchline & Templeton 1966	
Zn	12-110	Riley & Segar 1970[3]	
Mn	4.5-94	Riley & Segar 1970[3], Harris & Pilkey 1966[3]	Stevenson & Ufret 1966[3]
Fe	1.6-620	Riley & Segar 1970[3], Harris & Pilkey 1966[3]	Stevenson & Ufret 1966[3]
Ni	0.12-78	Riley & Segar 1970[3], Stevenson & Ufret 1966[3]	
Cu	0.42-1.8	Riley & Segar 1970[3]	

1. Post-1950 data have been listed only for methodological reasons; heavy metals from pollution origin have not been considered.
2. See text.
3. Samples of these authors can be contaminated by elements from the peri-ossicular soft tissues. These data are, however, taken into account because most part of the sample (> 90%) is made of skeleton.

of different species of echinoderms (belonging to all present classes) originating from habitats of different depths. On the contrary, Pilkey & Hower (1960) observed variations in the Sr/Ca ratio of tests of *Dendraster excentricus* from different localities and found a negative correlation between sea water temperature and the Sr/Ca ratio. The effect of temperature was, however, not significant in determining the strontium concentration in regenerating echinoid spines (Davies *et al.* 1972). On the other hand, Davies *et al.* found an inverse relationship between the maturity of the stereom and its strontium concentration. This could be explained either by the faster crystallization rate of the longitudinally growing regenerate (following a similar hypothesis to Weber's for magnesium) or by an organic matrix effect. A faster crystallization rate was also suggested by Pagett (1985) to explain the negative correlation between skeletal strontium concentration and individual dry weight (thought to be an approximate indication of the age) in *Ophioco-*

mina nigra. However, this relationship did not occur in the three other species of ophiuroids he investigated.

Skeletal concentration of manganese, iron and nickel differ significantly between two echinoid species from the same community (Stevenson & Ufret 1966). In *Dendraster excentricus*, manganese concentration of the test exhibits a poorly defined negative correlation with temperature (Harris & Pilkey 1966). Stevenson & Ufret (1966) suggested that the significant differences in the skeletal ion concentration exhibited by *Tripneustes esculentus* from two localities are due to similar differences in the iron concentration of their food.

5 RESORPTION AND SKELETAL DYNAMICS

5.1 *Skeletal resorption*

Skeletal elements of echinoderms are not necessarily permanent structures, and resorption processes may occur in both larvae and postmetamorphic individuals.

5.1.1 *Resorption of larval spicules*

The spicules of starved echinopluteus decrease in size (Runnström 1912, Bonar 1978, De Greef & Jangoux 1985). In larvae of *Paracentrotus lividus*, the anterolateral and postoral rods are resorbed during starvation whereas the body rods (i.e. the rods which grow during the endotrophic phase of development) are maintained almost until larval death (De Greef & Jangoux 1985) (for rod terminology, see Okazaki 1975a).

Some echinoid larval spicules are also resorbed during normal growth of well-fed pluteus. The distal part of the body rods (which is generally thickened) is resorbed in several species, the tip of the rods generally fracturing before being totally resorbed (Théel 1894, Mortensen 1931, 1937, De Greef pers. comm.). According to Théel (1894), resorption of body rods is performed by cells which are morphologically similar to skeletogenic cells. He reported that these cells are provided with numerous active pseudopodia that surround the part of the spicule to be resorbed. Then, the pseudopodia-surrounded part begins to dissolve, and the rod becomes constricted and breaks. In the end, the severed fragment is completely dissolved by the resorbing cell. Théel supposed that the dissolution is intracellular and that the dissolving agent is an acid secreted by the resorbing cell. Other larval rods reported to be partly or completely resorbed during echinopluteus development are the ventral transverse rods and the anterolateral rods of *Diadema setosum* (see Mortensen 1931), the recurrent rods of *Nudechinus gravieri* (see Mortensen 1937), and the anal rods and dorsoventral rods of *Paracen-*

trotus lividus (De Greef, pers. comm.). According to Théel (1894), Mortensen (1931, 1937) and De Greef (pers. comm.), rod resorbtion starts when the larval skeleton becomes unsuitable for further larval development (e.g. when the rods either hinder the growth of the digestive tract or that of the echinoid rudiment).

The fate of echinopluteus spicules during metamorphosis is still debated. According to Théel (1892), Mortensen (1937), Okazaki (1975a) and Emlet (1985), some rods are partly resorbed – the unresorbed parts being cast away – whereas some others are either incorporated into postmetamorphic ossicles or cast away by the individuals. On the contrary, Cameron & Hinegardner (1978) and Chia & Burke (1978) reported that the whole larval rods are shed. Contrary to what occurs in echinoplutei, all larval rods of ophioplutei are resorbed during metamorphosis except the posterolateral rods of some species which are cast away (Mortensen 1931, Hendler 1975, Mladenov 1985). Moreover, the embryo of some viviparous species have provisional spicules which are resorbed early during their development (Hendler 1975).

5.1.2 *Resorption of postmetamorphic ossicles*

The described resorption processes of postmetamorphic ossicles may be classified into four categories, namely, negative growth, ossicle remodelling, spicule change, and autotomy-related resorption (Table 6).

Negative growth. Negative growth consists of a decrease of the echinoid test diameter which occurs under natural conditions (Ebert 1967, 1968, Régis 1979). As the test is made of abutting ossicles and as test growth shows only minor deviations from isometry (Märkel 1975, 1981), shrinking of the individuals strongly suggests calcite resorption.

Ossicle remodelling. Resorption processes affecting the corona and the perignathic girdle of growing echinoids were discussed and clarified by Märkel (1975, 1976a, 1981). Such processes occur only where remodelling of the skeleton is necessary for further growth.

The specificity and intensity of resorption processes affecting the perignathic girdle differ for cidaroids and non-cidaroids (see Table 6). During cidaroid growth, the unpaired interambulacral primordials are totally resorbed and the subsequent pair of basicoronal interambulacral plates are partly resorbed at their peristomial edge (Märkel 1981). Moreover, at the border of the peristome, the ambulacral plates detach from the corona. They transform into ambulacral scales embedded in the peristomial membrane, the aporiferous part and tubercles of scales being progressively resorbed (Märkel 1981). In non-cidaroids, no plates detach from the corona. The interambulacral primordials – viz. the plates that form the ridges of the perignathic girdle – resorb only partly. Resorption occurs only inside the bow of the apophyses, at

Table 6. Resorption processes in postmetamorphic ossicles.

Processes	Affected ossicles	References
A. *Negative growth* (regular echinoids)	whole test	Ebert 1967, 1968, Régis 1979
B. *Ossicle remodelling* (regular echinoids)	*perignathic girdle*	
	interambulacral primordials (cidaroids)	Märkel 1981
	basicoronal interambulacral plates (cidaroids)	Märkel 1981
	ambulacral scales (cidaroids)	Märkel 1981
	bow of the apophyses (inner face) (non-cidaroids)	Märkel 1975, 1976a, 1981
	ridge (front face) (non-cidaroids)	Märkel 1975, 1976a, 1981
	gill notches (non-cidaroids)	Märkel 1975, 1976a, 1981
	corona of the test	
	walls of the ambulacral pores	Smith 1980a, Märkel 1981
	tubercles	Jensen 1972, Smith 1980a
	Aristotle's lantern	
	epiphyses (back side of the articular surfaces)	Märkel 1976b, 1979
	rotulae (back side of the articular surfaces)	Märkel 1976b, 1979
C. *Spicule change (holothuroids)*		
change of the type of calcareous spicules (*Neopentadactyla mixta*)	tables with two columns	Féral 1980
loss of spicules in mature individuals (*Caudina chilensis*)	spicules of the anal projections	Hozawa 1928
substitution of calcareous spicules by iron-rich phosphatic granules (molpadiids)	species specific calcareous spicules	Clark 1907, Donnay 1975, Lowenstam & Rossman 1975
D. *Autotomy-related resorption*		
stalk resorption after autotomy of the head in globiferous pedicellariae (regular echinoids)	stalk rod	Holland & Holland 1975
arm autotomy (comatulid crinoid)	brachial ossicles	Holland & Grimmer 1981
serial replacement of ambital spines by oral spines (cidaroid echinoids)	ambital primary spines	Cutress 1965

Table 6 (cont.).

Processes	Affected ossicles	References
shedding of damaged spine shaft (cidaroid echinoids)	primary spines	Prouhbo 1887, Märkel & Röser 1983b
shedding of the infected area of the test in the bald-sea-urchin disease (regular echinoids)	coronal plates	Höbaus *et al.* 1981, Maes & Jangoux 1984

the front face of the ridge and at the distal wall of the gill notches (Märkel 1975, 1976a, 1981).

Resorption processes of the corona of growing echinoids affect both the walls of the ambulacral pores and the margin of the tubercles (Jensen 1972, Smith 1980a, Märkel 1981). Both primary and secondary tubercles show resorption processes. Secondary tubercles grow at their leading margin and resorb at their trailing margin, shifting outwards from the growing primary tubercles (Smith 1980a). This leaves superficial trails of stereom different from the general stereom of the plate. Resorption and redeposition processes occurring during either the growth of primary tubercles or the shift of ambulacral pores will be described below (see part 5.2).

In the growing Aristotle's lantern, resorption maintains the isometry of both the rotulae and epiphyses (Märkel 1976b, 1979). Calcite is resorbed at the back edge of the articular surfaces whereas new calcite is deposited at the front edge. It is noteworthy that these articular surfaces are made of compact calcite which behaves like a polycrystalline aggregate between crossed nicol prisms, an observation demonstrating that microcrystals of articular surfaces of both rotulae and epiphyses are not aligned as in the stereom (such calcite was called 'polycrystalline calcite' as the authors believed – erroneously – that the stereom was as a rule monocrystalline; see part 3.4).

Spicule change. In the holothuroid *Neopentadactyla mixta* conspicuous differences occur in the shape of the spicules according to the age of the individuals, viz. juveniles have only table-like spicules with two columns while adults have only table-like spicules with four columns (Féral 1980). According to Oestergren (1938, in Féral 1980), tables with four columns are formed *de novo* without passing through a two-column stage. This implies that tables with two columns should have been resorbed during individual growth. Resorption could also be inferred in the molpadiid *Caudina chilensis*. Juveniles of this species have anal projections crowded with well-developed spicules whereas the anal projections of mature individuals are almost destitute of them (Hozawa 1928). One should note that very few

Table 7. Formulae of whole phosphatic granules of molpadiid holothuroids.

Species	Formulae	References
Molpadia oolitica	$Fe_{1.67}\,Ca_{0.59}\,(OH)_{3.19}\,PO_4\,(H_2O)_{2.78}$	Donnay 1975
Molpadia intermedia	$Fe_{1.25}\,Ca_{0.23}\,Mg_{0.20}\,(OH)_{1.58}\,PO_4\,(H_2O)_{1.88}\,(CH_2)_{0.23}$[1]	Lowenstam & Rossman 1975

1. (CH_2) represents the left over C and H.

studies deal with the spicular change during holothuroid growth (Féral 1980), and that spicular change and resorption in this group could be far more usual than believed.

Radical change in spiculation also occurs in molpadiid holothuroids. The juvenile skeleton of these holothuroids is composed of different types of calcite spicules. During the growth of individuals, the spicules are resorbed while deposition of phosphatic granules rich in iron occurs in the extracellular space of the dermis (Clark 1907, Donnay 1975, Lowenstam & Rossman 1975). The types, proportions, and numbers of resorbed spicules is species specific (Donnay 1975). However, in many species, the formation of calcite spicules continues although being confined to the anterior and posterior body areas (Clark 1907, Donnay 1975, Lowenstam & Rossman 1975). Partly resorbed spicules were observed inside phosphatic granules indicating that at least some of the early phosphatic granules develop around dissolving spicules (Donnay 1975, Lowenstam & Rossman 1975). However, most of the phosphatic granules of adult molpadiids are deposited in body areas where no spicules occur. Nucleation and accretion of phosphatic granules in adult individuals thus do not depend on the occurrence of calcite material (Lowenstam & Rossman 1975, Stricker 1986). Stricker (1986) suggested that the clusters of finely filamentous extracellular material observed on TEM sections in the vicinity of developing granules may serve as nucleation sites. According to him sclerocytes are not involved in the formation of phosphatic granules. The granules consist of concentric layers made of two types of spheroidal subunits (0.13-0.24 μm in diameter) made of amorphous minerals. The first and most abundant type is rich in water, ferric ions and phosphate while the second type consists of opaline hydrous silica (Lowenstam & Rossman 1975). The chemical formulae reported for whole phosphatic granules are given in Table 7. Extensive morphological and physico-chemical data on granules as well as a structural model for the iron-rich subunits are given by Lowenstam & Rossman (1975). The organic content of the granules (about 0.13% w/w) has been analysed by Weiner *et al.*

(1983) and its significance discussed by Lowenstam & Weiner (1983). The resorption mechanism of the calcareous spicules in molpadiids is still unknown.

Autotomy-related resorption. Autotomy-related resorption is either directly involved in the autotomy process (shedding of the spine shaft in cidaroids, and shedding of the test area in diseased echinoids) or is an epiphenomenon of the autotomy process (trabecular etching in crinoids, and stalk resorption of globiferous pedicellariae of echinoids).

Stalk resorption in globiferous (poisonous) pedicellariae has been described incidentally. After injecting their venom – e.g. into a potential predator – globiferous pedicellariae autotomize. In some species only the pedicellarial head autotomizes whereas the stalk remains fixed on the test and totally regresses before a new pedicellaria regenerates (Holland & Holland 1975). During the regression phase, the supporting rod of the stalk must obviously be resorbed too.

Arm autotomy in crinoids occurs at the level of particular brachial articulations, such as, e.g. the so-called syzygies (for review, see Emson & Wilkie 1980). Holland & Grimmer (1981) showed that some trabeculae of the brachial ossicles involved in syzygies were partly dissolved during the autotomy process. Etching affected the two outermost ranks of trabeculae of the articulation surface. Holland & Grimmer (1981) suggested that the neurosecretory granules involved in autotomy would include strong acid and/or chelating agents that would cause both the destabilization of the articular collagenic fibers and the etching of the trabeculae. The authors supposed that trabecular etching is of little significance in the autotomy process.

Cidaroid echinoids have two kinds of primary spines, namely, the ambital primary spines (which occur on most of the test plates) and the oral primary spines (which only occur on the interambulacral plates near the oral edge of the corona) (Cutress 1965). Cutress observed that the ambital primary spines of *Eucidaris tribuloides* located closest to the peristome fractured in a definite sequence through the proximal part of their collar. She reported that oral primary spines developed by regeneration from the fractured ambital spines. As the ambital spines break in a definite sequence, fracture cannot be attributed to accidental factors. This suggests that an autotomy-like process involving skeletal resorption occurs (Cutress 1965). Such endogenously caused breakage was reminiscent of Prouho's (1887) observations that a damaged spine of *Cidaris cidaris* has to cast off its distal part before regenerating. The breakage was due to a skeleton-resorbing 'membrane' – i.e. the so-called Prouho's membrane – that runs transversally through the collar of the spine. Ultrastructural investigations by Märkel & Röser (1983b) on experimentally damaged spines of *E. tribuloides* showed that the Prouho's

membrane is made of syncytial phagocytes which actively resorb the trabeculae. Märkel & Röser reported that soon after the spine was damaged, the phagocytes located in the spine shaft close to the collar migrate towards the distal part of the collar (i.e. at the level of the future membrane). During their migration, they absorb all other stromatic cells by phagocytosis. When reaching the distal part of the collar, the phagocytes extend in an horizontal network and merge into a syncytium forming the Prouho's membrane. The syncytium attaches distally to the trabeculae by means of junction-like structures characterized by numerous microtubules. Typical organelles of the syncytium are, namely, numerous SER-cisternae and phagosomes, coated vesicles associated with Golgi complexes, and numerous mitochondria. Resorption of the trabeculae starts at the level of the junction-like structures and results in the bulging of the latter into the trabeculae. Clear pinocytic vesicles (250 nm in diameter) appear within the bulges shortly after etching begins. A thin lamella (150 nm thick) arises from each bulge and carries on etching through the trabeculae, causing a 200 nm wide cleft. Thereafter, the lamellae thicken and clear vesicles as well as mitochondria enter them. Finally, the syncytium forms a continuous layer through the collar causing the shedding of the distal part of the spine. The stump is quickly covered by the epidermis and regeneration begins. Although the dissolving agent is unknown, Märkel & Röser (1983b) suggested that microtubules of the junction-like structures could play a role in its secretion and that the associated clear vesicles could take up the dissolved material. The shedding of the distal part of damaged cidaroid spines is the only resorption mechanism that has been described at the cellular level in postmetamorphic echinoderms.

The bald-sea-urchin disease often causes the shedding of the upper skeletal portion of the test plates which supports the lesion (see Maes & Jangoux 1984 for review). The process of skeleton elimination in the bald-sea-urchin disease could be similar to that involved in the shedding of damaged spines in cidaroids as a portion of the skeleton is isolated by a cellular layer made of phagocytes and red spherulous cells before being eliminated. The trabeculae are then dissolved along this layer and over a narrow slit causing the shedding of the upper skeletal layer (Höbaus *et al.* 1981, Maes pers. comm.).

5.2 *Skeletal dynamics*

Resorption in echinoderms is not only designed for clearing away stereom which has become unsuitable, but may also be followed by redeposition of new material making the stereom a true dynamic system. Occurrence of resorption/redeposition processes have been shown in at least three systems, viz. (1) the shift of the ambulacral pores in echinoid plates, (2) the growth of the echinoid primary tubercles, and (3) the growth of the Aristotle's lantern.

During growth of the ambulacral plates, the pores shift outwards and

follow the expanding plate margin. The trabeculae are resorbed at the front wall of the pores whereas new stereom – which is different from the surrounding one – is deposited at the back wall (Smith 1980a, Märkel 1981).

The enlargement of the mamelon and platform of a primary tubercle is preceded by resorption of the surrounding stereom of the boss whereas the development of the areole requires the resorption of the surrounding thorns together with the migration of secondary tubercles (Jensen 1972, Smith 1980a) (for tubercle terminology, see Smith 1980b).

Articular surfaces in Aristotle's lantern are resorbed in the course of growth (see part 5.1.2). In the epiphyses, the so-called articular 'polycrystalline' calcite is partly resorbed at its proximal edge before being covered by newly grown stereom. Evidence of reworking is also available from the hemipyramids. Ebert (1980) injected echinoids with tetracycline (a fluorescent dye which is incorporated into calcite during growth of the ossicles). After one year, the hemipyramids had increased in size regardless of whether the individuals were well-fed or not. However, tetracycline marks were only seen in the hemipyramids of well-fed individuals. Ebert (1980) pointed out that tetracycline has a very short life in sea water under tropical conditions. He consequently presumed that tetracycline was incorporated only during the first two days of the experiment in both groups of individuals as starvation was not yet effective. He suggested that the calcite of the hemipyramids of the starved echinoids were reworked during starvation causing the loss of the tetracycline marks.

The stereom may thicken after its formation. For instance, during regeneration of broken echinoid spines, a fine meshwork is first deposited and is afterwards thickened whereas the regeneration front is more advanced (Heatfield 1971a, Mischor 1975). Similarly, Smith (1980a) reported that when coronal plates grow rapidly an open stereom is deposited at both their inner and outer surfaces. When plates grow slowly or cease to grow, the trabeculae of the plate surfaces thicken, producing a dense, compact stereom layer. Such delayed thickening processes are all the more surprising since there are few sclerocytes either in the proximal part of the spine regenerates or in fully developed ossicles (see part 2.2). This means either that maintenance sclerocytes actually resume their activity and are able to ensure extensive deposition of calcite, or that they multiply and form typical active sclerocytes (see part 2.3).

The question now arises as to whether or not there is a permanent reworking of the calcite in the echinoderm stereom as it occurs in the Haversian bone of vertebrates. Nichols & Currey (1968) suggested that maintenance sclerocytes repair the flaws which arise in the trabeculae by erosion and redeposition of calcite (see part 2.2.3). This is, however, only hypothetical as most TEM studies have shown no evidence of active skeletoclastic cells in fully developed ossicles (see Table 1 for references). Stricker

(1985) found a permanent turn-over of holothuroid skeletal calcite unlikely as he did not find tetracycline marks in the fully developed ossicles of injected individuals. However, tetracycline labelling is not as sensitive as radio-tracers. As previously discussed, resorption/redeposition processes occur, namely (1) at places where the skeleton becomes unsuitable and/or needs remodelling (larval spicules, echinoid corona and Aristotle's lantern), (2) when skeletal elements are modified (holothuroid spicules), and (3) when part of a skeletal element is autotomized (cidaroid spines, test plates in bald-sea-urchin disease). Echinoderms, apparently, do not have a continuous turnover of their skeletal material although mechanisms of resorption and redeposition exist and function in places. Confirmation of this will need (1) morphological evidence on the occurrence and origin of skeletoclastic cells and (2) the demonstration of the static nature of echinoderm skeleton by radio-tracer experiments.

6 CONCLUSION

The skeletal system is one of the distinguishing features of the phylum Echinodermata. However, the process of calcification is one of the least understood of any of the animal phyla. The actual composition and the role of the organic matrix has only recently been investigated, ion transport has been studied almost only in echinoid larvae, and demonstration of skeletal dynamics is principally based on indirect clues. Regulatory processes are totally unknown. Only the morphology of the skeleton-forming cell is well characterized. In view of the quantitative and qualitative importance of the skeleton in the phylum and the special place of echinoderms among the invertebrates, the study of the calcifying mechanisms will be a major contribution to the understanding of the phylum biology and to comparative biology.

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REFERENCES

Akasaka, K., S. Amemiya & H. Terayama 1980. Scanning electron microscopical study of the inside of sea urchin embryos (*Pseudocentrotus depressus*). Effects of aryl β-xyloside, tunicamycin and deprivation of sulfate ions. *Exp. Cell. Res.* 129: 1-13.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts & J.D. Watson 1983. *Molecular Biology of the Cell.* Garland Publishing Inc., New York & London.

Anderson, H.A., J.W. Mathieson & R.H.Thomson 1969. Distribution of spinochrome pigments in echinoids. *Comp. Biochem. Physiol.* 28: 333-345.

Anstrom, J.A., J.E. Chin, D.S. Leaf, A.L. Parks & R.A. Raff 1987. Localization and expression of msp130, a primary mesenchyme lineage-specific cell surface protein of the sea urchin embryo. *Development* 101: 255-266.

Benson, S., N. Benson & F. Wilt 1986. The organic matrix of the skeletal spicule of sea-urchin *Strongylocentrotus purpuratus* embryos. *J. Cell Biol.* 102: 1878-1886.

Benson, S., E.M.E. Jones, N. Crise-Benson & F. Wilt 1983. Morphology of the organic matrix of the spicule of the sea urchin larva. *Exp. Cell Res.* 148: 249-253.

Benson, S., H. Sucov, L. Stephens, E. Davidson & F. Wilt 1987. A lineage-specific gene encoding a major matrix protein of the sea urchin embryo spicule. I. Authentication of the cloned gene and its developmental expression. *Dev. Biol.* 120: 499-506.

Berman, A., L. Addadi & S. Weiner 1988. Interactions of sea-urchin skeleton macromolecules with growing calcite crystals. A study of intracrystalline proteins. *Nature, Lond.* 331: 546-548.

Bevelander, G. & H. Nakahara 1960. Development of the skeleton of the sand dollar *Echinarachnius parma*. In R.F. Sognnaes (ed.), *Calcification of biological Systems*, pp. 41-56. Am. Assoc. Adv. Sci. Publ., Washington.

Bignot, C. 1976. L'observation des tissus organiques minéralisés au microscope électronique à balayage (MEB). Le squelette des échinodermes vivants et fossiles. *Travaux Lab. Micropaléont.* 5: 207-245.

Binyon, J. 1978. Some observations upon the chemical composition of the starfish *Asterias rubens* L. with particular reference to strontium uptake. *J. mar. biol. Ass. UK* 58: 441-449.

Blake, D.F. & D.R. Peacor 1981. Biomineralization in crinoid echinoderms: characterization of crinoid skeletal elements using TEM and STEM microanalysis. *Scanning Electron Microsc.* 3: 321-328, 224.

Blake, D.F., D.R. Peacor & L.F. Allard 1984. Ultrastructural and microanalytical results from echinoderm calcite: implications for biomineralization and diagenesis of skeletal material. *Micron Microsc. Acta* 15: 85-90.

Blankenship, J. & S. Benson 1984. Collagen metabolism and spicule formation in sea urchin micromeres. *Exp. Cell Res.* 152: 98-104.

Bonar, D.B. 1978. Regeneration in sea urchin larvae. *Amer. Zool.* 18: 43.

Bouxin, H. 1926. Action des acides sur les larves de l'oursin *Paracentrotus lividus*. Etude morphologique. *C.R. Séanc. Soc. Biol. (Paris)* 94: 451-453.

Brookbank, J.W. & A.H. Whiteley 1954. Studies on the urease of the eggs and embryos of the sea urchin, *Strongylocentrotus purpuratus*. *Biol. Bull.* 107: 57-63.

Cameron, R.A. & R.T. Hinegardner 1978. Early events in sea urchin metamorphosis, description and analysis. *J. Morph.* 157: 21-32.

Carson, D.D., M.C. Farach, D.S. Earles, G.L. Decker & W.J. Lennarz 1985. A monoclonal antibody inhibits calcium accumulation and skeleton formation in cultured embryonic cells of the sea urchin. *Cell* 41: 639-648.

Chave, K.E. 1952. A solid solution between calcite and dolomite. *J. Geol.* 60: 190-192.
Chave, K.E. 1954. Aspects of the biogeochemistry of magnesium. I. Calcareous marine organisms. *J. Geol.* 62: 266-283.
Chen, C.P. 1985. The role of carbonic anhydrase in the calcification of the tooth of the sea urchin *Lytechinus variegatus*. Ph.D. Thesis, Univ. of South Florida, Tampa, USA.
Chen, C.P. & J.M. Lawrence 1986a. The ultrastructure of the plumula of the tooth of *Lytechinus variegatus* (Echinodermata: Echinoidea). *Acta Zool.* 67: 33-41.
Chen, C.P. & J.M. Lawrence 1986b. Localization of carbonic anhydrase in the plumula of the tooth of *Lytechinus variegatus* (Echinodermata: Echinoidea). *Acta Zool.* 67: 27-32.
Chen, C.-P. & J.M. Lawrence 1987. The role of carbonic anhydrase in facilitating the transport of CO_2 in the tooth of *Lytechinus variegatus* (Echinodermata: Echinoidea). *Comp. Biochem. Physiol.* (A)87: 327-331.
Chia, F.-S. & R.D. Burke 1978. Echinoderm metamorphosis: fate of larval structures. In F.-S. Chia & M.E. Rice (eds), *Settlement and Metamorphosis of marine invertebrate Larvae*, pp. 219-234. Elsevier, New York.
Chow, G. & S.C. Benson 1979. Carbonic anhydrase activity in developing sea urchin embryos. *Exp. Cell Res.* 124: 451-453.
Clark, H.L. 1907. The apodous holothurians. *Smithson. Contrib. Knowl.* 35: 1-231.
Clarke, F.W. & W.C. Wheeler 1922. The inorganic constituents of marine invertebrates. *US Geol. Survey Prof. Paper* 124: 1-62.
Crenshaw, M.A. 1982. Mechanisms of normal biological mineralization of calcium carbonates. In G.H. Nancollas (ed.), *Biological Mineralization and Demineralization*, pp. 243-257. Springer-Verlag, Berlin.
Currey, J.D. & D. Nichols 1967. Absence of organic phase in echinoderm calcite. *Nature, Lond.* 214: 81-83.
Currey, J.D. & D. Nichols 1969. The question of the organic matrix of echinoderm skeletons. *Proc. malac. Soc. Lond.* 38: 546-547.
Cutress, B.M. 1965. Observations on growth in *Eucidaris tribuloides* (Lamarck), with special reference to the origin of the oral primary spines. *Bull. mar. Sci.* 15: 797-834.
Dafni, J. 1985. Effect of mechanical stress on the calcification pattern in regular echinoid skeletal plates. In B.F. Keegan & B.D.S. O'Connor (eds), *Proceedings of the fifth international echinoderm Conference, Galway*, pp. 233-236. A.A. Balkema, Rotterdam.
Dafni, J. & J. Erez 1982. Differential growth in *Tripneustes gratilla* (Echinoidea). In J.M. Lawrence (ed.), *Echinoderms: Proceedings of the international Conference, Tampa Bay*, pp. 71-75. A.A. Balkema, Rotterdam.
Dan-Sohkawa, M., G. Tamura & H. Mitsui 1980. Mesenchyme cells in starfish development: effect of tunicamycin on their differentiation, migration and function. *Develop. Growth Differ.* 22: 495-502.
Davies, T.T., M.A. Crenshaw & B.M. Heatfield 1972. The effect of temperature on the chemistry and structure of echinoid spine regeneration. *J. Paleontol.* 46: 874-883.
Decker, G.L. & W.J. Lennarz 1988. Growth of linear spicules in cultured primary mesenchyme cells of sea urchin embryos is bidirectional. *Dev. Biol.* 126: 433-436.
Decker, G.L., J.B. Morrill & W.J. Lennarz 1987. Characterization of sea urchin primary mesenchyme cells and spicules during biomineralization *in vitro*. *Development* 101: 297-312.
Decker, G.L. & M.C. Valdizan 1987. Immunogold and immunoblot studies on a cell surface protein found in primary mesenchyme cells and in the cortical secretory

vesicles of the sea urchin egg. In *Proceedings of the 45th annual Meeting of the electron microscopy Society of America*, pp. 832-833. San Francisco Press: San Francisco.

Degens, E.T. 1976. Molecular mechanisms of carbonate, phosphate and silica deposition in the living cell. *Top. Curr. Chem.* 64: 1-112.

De Greef, Y. & M. Jangoux 1985. Skeletal growth systems in echinoid larvae (*Paracentrotus lividus*). In B.F. Keegan & B.D.S. O'Connor (eds), *Proceedings of the fifth international echinoderm Conference, Galway*, p. 380. A.A. Balkema, Rotterdam.

Dillaman, R.M. & H.V. Hart 1981. X-ray evaluation of a SEM technique for determining the cristallography of echinoid skeletons. *Scanning electron Microscopy* 3: 313-320.

Dimelow, E.J. 1958. Pigments present in arms and pinnules of the crinoid *Antedon bifida* (Pennant). *Nature, Lond.* 182: 812.

Donachy, J.E. & N. Watabe 1986. Effects of salinity and calcium concentration on arm regeneration by *Ophiothrix angulata* (Echinodermata, Ophiuroidea). *Mar. Biol.* 91: 253-258.

Donnay, G. 1975. Biocrystallographic studies. *Biomineralization* 8: 14-20.

Donnay, G. & D.L. Pawson 1969. X-ray diffraction studies of echinoderm plates. *Science* 166: 1147-1150.

Dubois, Ph. 1988. Tissu squelettique et calcification chez *Asterias rubens* L. (Echinodermata: Asteroidea). Thèse de Doctorat, Université Libre de Bruxelles, Bruxelles, Belgique, 163 pp.

Dubois, Ph. & M. Jangoux 1985. The microstructure of the asteroid skeleton (*Asterias rubens*). In B.F. Keegan & B.D.S. O'Connor (eds), *Proceedings of the fifth international echinoderm Conference, Galway*, pp. 507-512. A.A. Balkema, Rotterdam.

Eastoe, J.E. 1968. Chemical aspects of the matrix concept in calcified tissue organization. *Calcif. Tiss. Res.* 2: 1-19.

Ebert, T.A. 1967. Negative growth and longevity in the purple sea urchin *Strongylocentrotus purpuratus* (Stimpson). *Science* 157: 557-558.

Ebert, T.A. 1968. Growth rates of the sea urchin *Strongylocentrotus purpuratus* related to food availability and spine abrasion. *Ecology* 49: 1075-1091.

Ebert, T.A. 1980. Relative growth of sea urchin jaws: an example of plastic resource allocation. *Bull. mar. Sci.* 30: 467-474.

Ellis, C.H. & R.J. Winter 1967. Protein synthesis and skeletal spicule formation in the sea urchin larvae. *Amer. Zool.* 7: 750.

Emlet, R.B. 1982. Echinoderm calcite: a mechanical analysis from larval spicules. *Biol. Bull.* 163: 264-275.

Emlet, R.B. 1985. Crystal axes in recent and fossil adult echinoids indicate trophic mode in larval development. *Science* 230: 937-940.

Emson, R.H. & I.C. Wilkie 1980. Fission and autotomy in echinoderms. *Oceanogr. mar. Biol. ann. Rev.* 18: 155-250.

Evola-Maltese, C. 1957a. Histochemical localization of alkaline phosphatase in the sea urchin embryo. *Acta Embryol. Morphol. exp.* 1: 99-104.

Evola-Maltese, C. 1957b. Effects of beryllium on the development and alkaline phosphatase activity of *Paracentrotus lividus* embryos. *Acta Embryol. Morphol. exp.* 1: 143-149.

Farah, M.C., M. Valdizan, H.R. Park, G.L. Decker & W.J. Lennarz 1987. Development expression of a cell-surface protein involved in calcium uptake and skeleton formation in sea urchin embryos. *Dev. Biol.* 122: 320-331.

Féral, J.P. 1980. *Neopentadactyla mixta* (Holothuroidea: Phyllophoridae). Spiculation et

croissance. In M. Jangoux (ed.), *Echinoderms: Present and Past*, pp. 111-113. A.A. Balkema, Rotterdam.

Fox, D.L. & T.S. Hopkins 1966. The comparative biochemistry of pigments. In R.A. Boolootian (ed.), *Physiology of Echinodermata*, pp. 277-300. Interscience Publishers, New York.

Fujino, Y., K. Mitsunaga, A. Fujiwara & I. Yasumasu 1985a. Inhibition of $^{45}Ca^{2+}$ uptake in the eggs and embryos of the sea urchin, *Anthocidaris crassispina*, by several calcium antagonists, anion transport inhibitor, and chloride transport inhibitors. *J. exp. Zool.* 235: 281-288.

Fujino, Y., R. Makihara, K. Mitsunaga & I. Yasumasu 1985b. Roles of cytoskeletons on spicule formation in cultured micromeres isolated from sea-urchin eggs. *Zool. Sci., Tokyo* 2: 945.

Garrido, J. & J. Blanco 1947. Structure cristalline des piquants d'oursin. *C.R. Acad. Sci., Paris* 224: 485.

Gibbins, J.R., L.G. Tilney & K.R. Porter 1969. Microtubules in the formation and development of the primary mesenchyme in *Arbacia punctulata*. *J. Cell Biol.* 41: 201-226.

Giudice, G. 1973. *Developmental Biology of the Sea Urchin Embryo*. Academic Press, New York and London.

Goldsmith, J.R., D.L. Graf & O.I. Joensuu 1955. The occurrence of magnesium calcites in nature. *Geochim. Cosmochim. Acta* 7: 212-230.

Goodwin, T.W. 1969. Pigments in Echinodermata. In M. Florkin & B.T. Scheer (eds), *Chemical Zoology vol. III, Echinodermata, Nematoda, and Acantocephala*, pp. 135-147. Academic Press, New York and London.

Goodwin, T.W. & S. Srisukh 1950. A study of the pigments of the sea urchins, *Echinus esculentus* L. and *Paracentrotus lividus* Lamarck. *Biochem. J.* 47: 69-76.

Gould, D. & S.C. Benson 1978. Selective inhibition of collagen synthesis in sea urchin embryos by a low concentration of actinomycin D. *Exp. Cell. Res.* 112: 73-78.

Grant, S.R., M.C. Farach, G.L. Decker, H.D. Woodward, H.A. Farach & W.J. Lennarz 1985. Developmental expression of cell-surface (glyco)proteins involved in gastrulation and spicule formation in sea urchin embryos. *Cold Spring Harbor Symposia on quantitative Biology* 50: 91-98.

Grimmer, J.C., N.D. Holland & I. Hayami 1985. Fine structure of the stalk of an isocrinid sea lily (*Metacrinus rotundus*) (Echinodermata, Crinoidea). *Zoomorphology* 105: 39-50.

Grimmer, J.C., N.D. Holland & H. Kubota 1984a. Fine structure of the stalk of the pentacrinoid larva of a feather star *Comanthus japonica* (Echinodermata: Crinoidea). *Acta Zool.* 65: 41-58.

Grimmer, J.C., N.D. Holland & C.G. Messing 1984b. Fine structure of the stalk of the bourgueticrinid sea lily *Democrinus conifer* (Echinodermata: Crinoidea). *Mar. Biol.* 81: 163-176.

Gunatilaka, A. 1975. The chemical composition of some carbonate secreting marine organisms from Connemara. *Proc. R. Irish Acad.* B 75: 543-556.

Gustafson, T. & L. Wolpert 1961. Studies on the cellular basis of morphogenesis in the sea urchin embryo. Directed movements of primary mesenchyme cells in normal and vegetalized larvae. *Exp. Cell Res.* 24: 64-79.

Harkey, M.A. & A.H. Whiteley 1980. Isolation, culture and differentiation of echinoid primary mesenchyme cells. *Wilhem Roux's Arch.* 189: 111-122.

Harris, R.C. & O.H. Pilkey 1966. Temperature and salinity control of the concentration of

skeletal Na, Mn, and Fe in *Dendraster excentricus*. *Pacific Sci.* 20: 235-238.
Heatfield, B.M. 1970. Calcification in echinoderms: effects of temperature and diamox on incorporation of calcium-45 in vitro by regenerating spines of *Strongylocentrotus purpuratus*. *Biol. Bull.* 139: 151-163.
Heatfield, B.M. 1971a. Growth of the calcareous skeleton during regeneration of spines of the sea urchin, *Strongylocentrotus purpuratus*: a light and scanning electron microscope study. *J. Morph.* 134: 57-90.
Heatfield, B.M. 1971b. Origin of calcified tissue in regenerating spines of the sea urchin, *Stronglycentrotus purpuratus* (Stimpson): a quantitative radioautographic study with tritiated thymidine. *J. exp. Zool.* 178: 233-246.
Heatfield, B.M. & D.F. Travis 1975a. Ultrastructural studies of regenerating spines of the sea urchin *Strongylocentrotus purpuratus*. I. Cell types without spherules. *J. Morph.* 145: 13-50.
Heatfield, B.M. & D.F. Travis 1975b. Ultrastructural studies of regenerating spines of the sea urchin *Strongylocentrotus purpuratus*. II. Cell types with spherules. *J. Morph.* 145: 51-72.
Heatfield, B.M. & K.M. Wilbur 1972. Effects of a carbonic anhydrase inhibitor on skeleton growth and microstructure in regenerating sea urchin spines. *Am. Zool.* 12: 718.
Heifetz, A. & W.J. Lennarz 1979. Biosynthesis of N-glycosidically linked glycoproteins during gastrulation of sea urchin embryos. *J. biol. Chem.* 254: 6119-6127.
Hendler, G. 1975. Adaptational significance of the patterns of ophiuroid development. *Am. Zool.* 15: 691-715.
Höbaus, E., L. Fenaux & M. Hignette 1981. Premières observations sur les lésions provoquées par une maladie affectant le test des oursins en Méditerranée occidentale. *Rapp. Comm. int. Expl. Mer Médit.* 27: 221-222.
Holland, L.Z. & N.D. Holland 1975. The fine structure of epidermal glands of regenerating and mature globiferous pedicellariae of a sea urchin (*Lytechinus pictus*). *Tissue Cell* 7: 723-737.
Holland, N.D. 1965. An autoradiographic investigation of tooth renewal in the purple sea urchin (*Strongylocentrotus purpuratus*). *J. exp. Zool.* 158: 275-282.
Holland, N.D. & J.C. Grimmer 1981. Fine structure of syzygial articulations before and after arm autotomy in *Florometra serratissima* (Echinodermata: Crinoidea). *Zoomorphology* 98: 169-183.
Hozawa, S. 1928. On the change occurring with advancing age in the calcareous deposits of *Caudina chilensis* (J. Müller). *Sci. Rep. Tôhoku Imperial Univ.* (4) 3: 361-378, pls 14-17.
Hsiao, S.C. & W.K. Fujii 1963. Early ontogenic changes in the concentration of alkaline phosphatase in Hawaiian sea urchins. *Exp. Cell Res.* 32: 217-231.
Hyman, L.H. 1955. *The Invertebrates. Vol. 4. Echinodermata*. MacGraw-Hill: New York.
Iwata, M. & E. Nakano 1985a. Fibronectin-binding acid polysaccharide in the sea urchin embryos. *Wilhelm Roux Arch.* 194: 377-384.
Iwata, M. & E. Nakano 1985b. Enhancement of spicule formation and calcium uptake by monoclonal autibodies to fibronectin-binding acid polysaccharide in cultured sea urchin *Hemicentrotus pulcherrimus* embryonic cells. *Cell Differ.* 17: 57-62.
Iwata, M. & E. Nakano 1986. A large calcium-binding protein associated with the larvel spicules of the sea urchin embryo. *Cell Differ.* 19: 229-236.
Jensen, M. 1969. Age determination of echinoids. *Sarsia* 37: 41-44.

Jensen, M. 1972. The ultrastructure of the echinoid skeleton. *Sarsia* 48: 39-48.
Kaneko, I., Y. Ikeda & H. Ozaki 1981a. Absorption of calcium through digestive tract in sea urchin. *Bull. Jap. Soc. scient. Fish.* 47: 1421-1424.
Kaneko, I., Y. Ikeda & H. Ozaki 1981b. Absorption of calcium from sea water and its excretion in sea urchin. *Bull. Jap. Soc. scient. Fish.* 47: 1425-1430.
Katow, H. 1986. Behavior of sea-urchin *Pseudocentrotus depressus* primary mesenchyme cells in artificial extracellular matrices. *Exp. Cell Res.* 162: 401-410.
Katow, H. & M. Hayashi 1985. Role of fibronectin in primary mesenchyme cell migration in the sea-urchin *Pseudocentrotus depressus*. J. Cell Biol. 101: 1487-1491.
Kennedy, G.Y. & H.G. Vevers 1972. Tetrapyrrol pigments in the test of the echinoid *Arbacia lixula*. *J. Zool., Lond.* 168: 527-533.
Kerkis, A.Y. & V.V. Isaeva 1984. Electron-microscopic study of spiculogenesis in a culture of embryonic cells of the sea urchin *Strongylocentrotus nudus*. *Ontogenez* 15: 24-29 (in Russian with English summary).
Kindred, J.E. 1924. The cellular elements in the perivisceral fluid of echinoderms. *Biol. Bull.* 46: 228-251.
Kitajima, T. & K. Okazaki 1980. Spicule formation *in vitro* by the descendants of precocious micromere formed at the 8-cell stage of sea urchin embryo. *Develop. Growth Differ.* 22: 265-279.
Klein, L. & J.D. Currey 1970. Echinoid skeleton: absence of a collagenous matrix. *Science* 169: 1209-1210.
Kniprath, E. 1974. Ultrastructure and growth of the sea urchin tooth. *Calcif. Tissue Res.* 14: 211-228.
Krampitz, G.P. 1982. Structure of the organic matrix in mollusc shells and avian eggshells. In G.H. Nancollas (ed.), *Biological Mineralization and Demineralization*, pp. 219-232. Springer-Verlag, Berlin.
Leaf, D.S., J.A. Anstrom, J.E. Chin, M.A. Harkey, R.M. Showman & R.A. Raff 1987. Antibodies to a fusion protein identify a cDNA clone encoding msp130, a primary mesenchyme-specific cell surface protein of the sea urchin embryo. *Dev. Biol.* 121: 29-40.
Lederer, E. 1952. Sur les pigments naphtoquinoniques des épines et du test des oursins *Paracentrotus lividus* et *Arbacia pustulosa*. *Biochim. Biophys. Acta* 9: 92-101.
Loest, R.A. 1979. Urease from a sea urchin *Lytechinus variegatus*: partial purification and kinetics. *Comp. Biochem. Physiol.* (B) 63: 103-107.
Lowenstam, H.A. 1981. Minerals formed by organisms. *Science* 211: 1126-1131.
Lowenstam, H.A. & G.R. Rossman 1975. Amorphous, hydrous, ferric phosphatic dermal granules in *Molpadia* (Holothuroidea): physical and chemical characterization and ecologic implications of the bioinorganic fraction. *Chem. Geol.* 15: 15-51.
Lowenstam, H.A. & S. Weiner 1983. Mineralization by organisms and the evolution of biomineralization. In P. Westbroek & E.W. de Jong (eds), *Biomineralization and biological metal Accumulation*, pp. 191-203. D. Reidel Publishing Company, Dordrecht, Holland.
Maes, P. & M. Jangoux 1984. The bald-sea-urchin disease: a biopathological approach. *Helgoländer Meeresunters.* 37: 217-224.
Makihara, R., K. Mitsunaga, Y. Fujino & I. Yasumasu 1985. Inhibitory effects of calcium antagonists and ruthenium red on spicule formation in sea-urchin *Hemicentrotus pulcherrimus embryos*. *Zool. Sci., Tokyo* 2: 945.
Mann, S. 1983. Mineralization in biological systems. *Struct. Bonding* 54: 125-174.
Mann, S., S.B. Parker, M.D. Ross, A.J. Skarnulis & R.J.P. Williams 1983. The ultrastruc-

ture of the calcium carbonate balance organs of the inner ear: an ultra-high resolution electron microscopy study. *Proc. R. Soc. Lond.* B 218: 415-424.
Märkel, K. 1970. The tooth skeleton of *Echinometra mathaei* (Blainville) (Echinodermata, Echinoidea). *Annot. Zool. Jap.* 43: 188-199.
Märkel, K. 1975. Wachstum des Coronarskeletes von *Paracentrotus lividus* Lmk. (Echinodermata, Echinoidea). *Zoomorphologie* 82: 259-280.
Märkel, K. 1976a. Struktur und Wachstum des Coronarskeletes von *Arbacia lixula* L. (Echinodermata, Echinoidea). *Zoomorphologie* 84: 279-299.
Märkel, K. 1976b. Das Wachstum der 'Laterne des Aristoteles' und seine Anpassung and die Funktion der Laterne (Echinodermata, Echinoidea). *Zoomorphologie* 86: 25-40.
Märkel, K. 1979. Structure and growth of the cidaroid socket-joint lantern of Aristotle compared to the hinge-joint lanterns of non-cidaroid regular echinoids (Echinodermata, Echinoidea). *Zoomorphologie* 94: 1-32.
Märkel, K. 1981. Experimental morphology of coronar growth in regular echinoids. *Zoomorphology* 97: 31-52.
Märkel, K. & U. Röser 1983a. The spine tissues in the echinoid *Eucidaris tribuloides*. *Zoomorphology* 103: 25-41.
Märkel, K. & U. Röser 1983b. Calcite resorption in the spine of the echinoid *Eucidaris tribuloides*. *Zoomorphology* 103: 43-58.
Märkel, K. & U. Röser 1985. Comparative morphology of echinoderm calcified tissues: histology and ultrastructure of ophiuroid scales (Echinodermata, Ophiuroida). *Zoomorphology* 105: 197-207.
Märkel, K., U. Röser, U. Mackenstedt & M. Klostermann 1986. Ultrastructural investigations of matrix-mediated biomineralization in echinoids (Echinodermata, Echinoidea). *Zoomorphology* 106: 232-243.
Mauchline, J. & W.L. Templeton 1966. Strontium, calcium and barium in marine organisms from the Irish sea. *J. Cons. perm. int. Explor. Mer* 30: 161-170.
Millonig, G. 1970. A study on the formation and structure of the sea urchin spicule. *J. submicr. Cytol.* 2: 157-165.
Minafra, S., R. Galante, S. D'Antoni, M. Fanara, L. Coppola & I. Pucci-Minafra 1980. Collagen associated protein-polysaccharide in the Aristotle's lanternae of *Paracentrotus lividus*. *J. submicrosc. Cytol.* 12: 255-265.
Mintz, G.R., S. de Francesco & W.J. Lennarz 1981. Spicule formation by cultured embryonic cells from the sea urchin. *J. biol. Chem.* 256: 13105-13111.
Mintz, G.R., D.S. Earles & W.J. lennarz 1982. Regulation of calcium incorporation into embryonic cells from sea-urchin embryos. *J. Cell Biol.* 95: 157 A.
Mintz, G.R. & W.J. Lennarz 1982. Spicule formation by cultured embryonic cells from the sea urchin. *Cell Differ.* 11: 331-333.
Mischor, B. 1975. Zur Morphologie und Regeneration der Hohlstacheln von *Diadema antillarum* Philippi und *Echinothrix diadema* (L.) (Echinoidea, Diadematidae). *Zoomorphologie* 82: 243-258.
Mitsunaga, K., Y. Fujino, A. Fujiwara & I. Yasumasu 1984. Anion transport participates in spicule calcification of sea-urchin embryos. *Dev. Growth Differ.* 26: 375.
Mitsunaga, K., R. Makihara, Y. Fujino & I. Yasumasu 1986a. Inhibitory effects of ethacrynic acid, furosemide, and nifedipine on the calcification of spicules in cultures of micromeres isolated from sea-urchin *Hemicentrotus pulcherrimus* eggs. *Differentiation* 30: 197-204.
Mitsunaga, K., K. Akasaka, H. Shimada, Y. Fujino, I. Yasumasu & H. Num Anoi 1986b. Carbonic anhydrase activity in developing sea-urchin embryos with special reference to calcification in spicules. *Cell Differ.* 18: 258-262.

Mitsunaga, K., Y. Fujino & I. Yasumasu 1986c. Change in the activity of Cl^-/HCO^-_3 ATPase in microsome fraction during early development of the sea urchin, *Hemicentrotus pulcherrimus*. *J. Biochem.* 100: 1607-1615.

Mitsunaga, K. Y. Fujino & I. Yasumasu 1987a. Distributions of H^+/K^+ ATPase and Cl^-/HCO^-_3 ATPase in micromere-derived cells of urchin embryos. *Differentiation* 35: 190-196.

Mitsunaga, K., Y. Fujino & I. Yasumasu 1987b. Probable role of allylisothiocyanate-sensitive H^+/K^+ ATPase in spicule calcification in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. *Develop. Growth Differ.* 29: 57-70.

Miyachi, Y., M. Iwata, H. Sata & E. Nakano 1984. Effect of fibronectin on cultured cells derived from isolated micromeres of the sea urchin, *Hemicentrotus pulcherrimus*. *Zool. Sci., Tokyo* 1: 265-271.

Mizoguchi, A., T. Mizuochi, Y. Kitazume, G. Tamura & A. Kobata 1981. Abnormal spicule formation induced by tunicamycin in the early development of the sea urchin embryo. *Cell Struct. Funct.* 6: 341-346.

Mladenov, Ph.V. 1985. Development and metamorphosis of the brittle star *Ophiocoma pumila*: evolutionary and ecological implications. *Biol. Bull.* 168: 285-295.

Moore, H.B. 1935. A comparison of the biology of *Echinus esculentus* in different habitats. Part II. *J. mar. biol. Ass. UK* 20: 109-128.

Moore, H.B. 1966. Ecology of echinoids. In R.A. Boolootian (ed.), *Physiology of Echinodermata*, pp. 73-85. Interscience Publishers, New York.

Moore, R.E., H. Singh & P.J. Scheuer 1966. Isolation of eleven new spinochromes from the sea urchin *Echinothrix diadema*. *J. org. Chem.* 31: 3645-3650.

Mortensen, Th. 1931. Contributions to the study of the development and larval forms of echinoderms I-II. *K. Danske Vidensk. Selsk. Skr. (Naturv. Math. Afd.)* (9) 4: 1-39, pls 1-7.

Mortensen, Th. 1937. Contributions to the study of the development and larval forms of echinoderms III. *K. Danske Vidensk. Selsk. Skr. (Naturv. Math. Afd.)* (9) 7: 1-65, pls 1-15.

Mukai, T. 1958. A new pigment from the sea cucumber, *Polycheira rufescens* (Brandt). *Mem. Fac. Sci. Kyusku Univ.* (C) 3: 29-33.

Mukai, T. 1960. Structure of namakochrome. I. *Bull. chem. Soc. Japan* 33: 453-456.

Nakano, E., K. Okazaki & T. Iwamatsu 1963. Accumulation of radioactive calcium in larvae of the sea urchin *Pseudocentrotus depressus*. *Biol. Bull.* 125: 125-132.

Nauen, C.E. & L. Böhm 1979. Skeletal growth in the echinoderm *Asterias rubens* L. (Asteroidea, Echinodermata) estimated by ^{45}Ca-labelling. *J. exp. mar. Biol. Ecol.* 38: 261-269.

Nichols, D. & J.D. Currey 1968. The secretion, structure and strength of echinoderm calcite. In S.M. McGee-Russell & K.F.A. Ross (eds), *Cell Structure and its Interpretation*, pp. 251-261. Edward Arnold, London.

Nichols, D., A.A.T. Sime & G.M. Bishop 1985. Growth in populations of the sea-urchin *Echinus esculentus* L. (Echinodermata: Echinoidea) from the English Channel and Firth of Clyde. *J. exp. mar. Biol. Ecol.* 86: 219-228.

Nissen, H.U. 1963. Röntgengefügeanalyze am Kalzit von Echinodermenskeletten. *Neues Jahrb. Geol. Paläontol.* 117: 230-234.

Nissen, H.U. 1969. Crystal orientation and plate structure in echinoid skeletal units. *Science* 166: 1150-1152.

Okazaki, K. 1956. Skeleton formation of sea-urchin larvae. I. Effect of Ca concentration of the medium. *Biol. Bull.* 110: 320-333.

Okazaki, K. 1960. Skeleton formation of sea urchin larvae. II. Organic matrix of the spicule. *Embryologia* 5: 283-320.
Okazaki, K. 1961. Skeleton formation of sea urchin larvae. III. Similarity of effect of low calcium and high magnesium on spicule formation. *Biol. Bull.* 120: 177-182.
Okazaki, K. 1962. Skeleton formation of sea urchin larvae. IV. Correlation in shape of spicule and matrix. *Embryologia* 7: 21-38.
Okazaki, K. 1965. Skeleton formation of sea urchin larvae. V. Continuous observation of the process of matrix formation. *Exp. Cell Res.* 40: 585-596.
Okazaki, K. 1975a. Normal development to metamorphosis. In G. Czihak (ed.), *The Sea Urchin Embryo*, pp. 177-232. Springer-Verlag, Berlin.
Okazaki, K. 1975b. Spicule formation by isolated micromeres of the sea-urchin embryo. *Am. Zool.* 15: 567-581.
Okazaki, K., R.M. Dillaman & K.M. Wilbur 1981. Crystalline axes of the spine and test of the sea urchin *Strongylocentrotus purpuratus*: determination by crystal etching and decoration. *Biol. Bull.* 161: 402-415.
Okazaki, K. & S. Inoué 1976. Crystal property of the larval sea urchin spicule. *Dev. Growth Differ.* 18: 413-434.
O'Neill, P.L. 1981. Polycrystalline echinoderm calcite and its fracture mechanics. *Science* 213: 646-648.
Pagett, R.M. 1985. Some observations upon the distribution of strontium in four species of ophiuroids: *Ophiocomina nigra* (Abildgaard), *Ophiura albida* (Forbes), *Ophiothrix suensoni* (Lütken), and *Ophioderma cinereum* (Müller and Troschel). *J. mar. biol. Ass. UK* 65: 293-303.
Pearse, J.S. & V.B. Pearse 1975. Growth zones in the echinoid skeleton. *Am. Zool.* 15: 731-753.
Pilkey, O.H. & J. Hower 1960. The effect of environment on the concentration of skeletal magnesium and strontium in *Dendraster*. *J. Geol.* 68: 203-216.
Pilkington, J.B. 1969. The organization of skeletal tissues in the spines of *Echinus esculentus*. *J. mar. biol. Assoc. UK* 49: 857-877.
Prenant, M. 1926. Notes histologiques sur la structure et la croissance des dents d'oursin. *Arch. Zool. exp. gén.* 65: 25-38.
Prouho, H. 1887. Recherches sur le *Dorocidaris papillata* et quelques autres échinides de la Méditerranée. *Arch. Zool. exp. gén.* 15: 213-380, pls 14-26.
Pucci-Minafra, I., C. Casano & C. la Rosa 1972. Collagen synthesis and spicule formation in sea urchin embryos. *Cell Differ.* 1: 157-165.
Pucci-Minafra, I., M. Fanara & S. Minafra 1980. Chemical and physical changes in the organic matrix of mineralized tissues from embryos to adult of *Paracentrotus lividus*. *J. submicrosc. Cytol.* 12: 267-273.
Pucci-Minafra, I., R. Galante & S. Minafra 1978. Identification of collagen in the Aristotle's lanternae of *Paracentrotus lividus*. *J. submicrosc. Cytol.* 10: 53-63.
Raup, D.M. 1959. Crystallography of echinoid calcite. *J. Geol.* 67: 661-674.
Raup, D.M. 1966. The endoskeleton. In R.A. Boolootian (ed.), *Physiology of Echinodermata*, pp. 379-395. Interscience Publishers, New York.
Régis, M.-B. 1979. Croissance négative de l'oursin *Paracentrotus lividus* (Lamarck) (Echinoidea, Echinidae). *C.R. Acad. Sci., Paris* D 288: 355-358.
Riley, J.P. & B.A. Segar 1970. The distribution of the major and some minor elements in marine animals. I. Echinoderms and coelenterates. *J. mar. biol. Ass. UK* 50: 721-730.
Ross, M.D. & K.G. Pote 1984. Some properties of otoconia. *Phil. Trans. R. Soc. Lond.* B 304: 445-452.

Runnström, J. 1912. L'effet de l'inanition sur la larve de l'oursin. *Bull. Inst. océanogr., Monaco* 245: 1-20.

Russell, R.G.G., A. Monod, J.P. Bonjour & H. Fleisch 1972. Relation between alkaline phosphatase and Ca-ATPase in calcium transport. *Nature (New Biol.)* 240: 126-127.

Schneider, E.G., H.T. Nguyen & W.J. Lennarz 1978. The effect of tunicamycin, an inhibitor of protein glycosylation, on embryonic development in the sea urchin. *J. biol. Chem.* 253: 2348-2355.

Schroeder, J.H., E.J. Dwornik & J.J. Papike 1969. Primary protodolomite in echinoid skeletons. *Geol. Soc. Am. Bull.* 80: 1613-1616.

Schroeder, T.E. 1981. Development of a 'primitive' sea urchin (*Eucidaris tribuloides*): irregularities in the hyaline layer, micromeres, and primary mesenchyme. *Biol. Bull.* 161: 141-151.

Shimizu, M. & J. Yamada 1976. Light and electron microscope observations of the regenerating test in the sea urchin, *Strongylocentrotus intermedius*. In N. Watabe & K.M. Wilbur (eds), *The Mechanisms of Mineralization in the Invertebrates and Plants*, pp. 261-281. Univ. South Carolina Press, Columbia s.c.

Shizimu, M. & J. Yamada 1980. Sclerocytes and crystal growth in the regeneration of sea urchin test and spines. In M. Omori & N. Watabe (eds), *The Mechanisms of Biomineralization in Animals and Plants*, pp. 169-178. Tokai Univ. Press, Tokyo.

Sikes, C.S. & A.P. Wheeler 1983. A systematic approach to some fundamental question of carbonate calcification. In P. Westbroek & E.W. de Jong (eds), *Biomineralization and biological metal Accumulation*, pp. 285-289. D. Reidel Publishing Company, Dordrecht, Holland.

Sikes, C.S., K. Okazaki & R.D. Fink 1981. Respiratory CO_2 and the supply of inorganic carbon for calcification of sea urchin embryos. *Comp. Biochem. Physiol.* 70A: 285-291.

Sime, A.A.T. 1982. Growth ring analyses in regular echinoids. *Progr. underwat. Sci.* 7: 7-14.

Simkiss, K. 1980. Detoxification, calcification and the intercellular storage of ions. In M. Omori & N. Watabe (eds), *The Mechanisms of Biomineralization in Animals and Plants*, pp. 13-18. Tokai Univ. Press, Tokyo.

Singh, H., R.E. Moore & P.J. Scheuer 1967. The distribution of quinone pigments in echinoderms. *Experientia* 23: 624-626.

Smith, A.B. 1980a. Stereom microstructure of the echinoid test. *Spec. Pap. Palaeont.* 25: 1-81.

Smith, A.B. 1980b. The structure and arrangement of echinoid tubercles. *Phil. Trans. R. Soc. Lond.* B 289: 1-54.

Stevenson, R.A. & S.L. Ufret 1966. Iron, manganese, and nickel in skeletons and food of the sea urchins *Tripneustes esculentus* and *Echinometra lucunter*. *Limnol. Oceanogr.* 11: 11-17.

Stolkowski, J. 1948. Sur l'inhibition du développement des spicules chez la larve d'oursin. *C.R. Acad. Sci., Paris* 227: 867-869.

Stricker, S.A. 1985. The ultrastructure and formation of the calcareous ossicles in the body wall of the sea cucumber *Leptosynapta clarki* (Echinodermata, Holothuroida). *Zoomorphology* 105: 209-222.

Stricker, S.A. 1986. The fine structure and development of calcified skeletal elements in the body wall of holothurian echinoderms. *J. Morph.* 188: 273-288.

Sucov, H.M., S. Benson, J.J. Robinson, R.J. Britten, F. Wilt & E.H. Davidson 1987. A lineage-specific gene encoding a major matrix protein of the sea urchin embryo

spicule. II. Structure of the gene and derived sequence of the protein. *Dev. Biol.* 120: 507-519.
Sumich, J.L. & J.E. McCauley 1972. Calcium magnesium ratios in the test plates of *Allocentrotus fragilis*. *Mar. Chem.* 1: 55-59.
Swift, D.M., C.S. Sikes & A.P. Wheeler 1986. Analysis and function of organic matrix from sea urchin tests. *J. exp. Zool.* 240: 65-73.
Tashian, R.E. & D. Hewett-Emmett (ed.) 1984. Biology and chemistry of the carbonic anhydrase. *Ann. NY Acad. Sci.* 429: 640 pp.
Théel, H. 1892. On the development of *Echinocyamus pusillus*. *Nova Acta Reg. Soc. Sci. Upsala* (3) 15: 1-57, pls 1-9.
Théel, H. 1894. Notes on the formation and absorption of the skeleton in the echinoderms. *Kongl. Vet. Akad. Förhandl.* 8: 345-354.
Thompson, T.G. & T.J. Chow 1955. The strontium-calcium atom ratio in carbonate-secreting marine organisms. *Deep-Sea Res.* 3 (suppl.): 20-39.
Towe, K.M. 1972. Invertebrate shell structure and the organic matrix concept. *Biomineralization* 4: 1-14.
Travis, D.F. 1970. The comparative ultrastructure and organization of five calcified tissues. In H. Schraer (ed.), *Biological Calcification: cellular and molecular Aspects*, pp. 203-312. Appleton, Century, and Crofts, New York.
Travis, D.F., C.F. François, L.C. Bonar & J. Glimcher 1967. Comparative studies of the organic matrices of invertebrate mineralized tissues. *J. ultrastruct. Res.* 18: 519-550.
Uto, N., M. Fujie & Y. Muranaka 1984. Ultrastructural observation concerning effects of penicillamine on spicule formation in sea-urchin embryos. *Zool. Sci., Tokyo* 1: 927.
Uto, N. & Y. Muranaka 1985. X-ray microanalysis on calcium in matrix cell or spicule in sea-urchin egg treated with collagen crosslinking inhibitors. *Zool. Sci., Tokyo* 2: 943.
Veis, D.J., T.M. Albinger, J. Clohisy, M. Rahima, B. Sabsay & A. Veis 1986. Matrix proteins of the teeth of the sea urchin *Lytechinus variegatus*. *J. exp. Zool.* 240: 35-46.
Venkatesan, M. & R.T. Simpson 1986. Isolation and characterization of spicule proteins from *Strongylocentrotus purpuratus*. *Exp. Cell Res.* 166: 259-264.
Vinogradov, A.P. 1953. The elementary chemical composition of marine organisms. *Sears Found. mar. Res. Mem.* 2: 1-647.
Vocisano, R.A. 1971. Calcification in echinoderms: regeneration of the test of the sea urchin *Strongylocentrotus droebachiensis*. Master thesis, McGill University, Montreal, Canada, 207 pp.
von Ubisch, L. 1937. Die normale Skelettbildung bei *Echinocyamus pusillus* und *Psammechinus miliaris* und die Bedeutung dieser Vorgänge für die Analyse der Skelette von Keimblatt-Chimären. *Z. wiss. Zool.* 149: 402-476.
von Ubisch, L. 1939. Keimblattchimärenforschung an Seeigellarven. *Biol. Rev.* 14: 88-103.
Weber, J.N. 1969. The incorporation of magnesium into the skeletal calcite of echinoderms. *Am. J. Sci.* 267: 537-566.
Weber, J.N. 1973. Temperature dependence of magnesium in echinoid and asteroid skeletal calcite: a reinterpretation of its significance. *J. Geol.* 81: 543-556.
Weiner, S. 1984. Organization of organic matrix components in mineralized tissues. *Am. Zool.* 24: 945-951.
Weiner, S. 1985. Organic matrix-like macromolecules associated with the mineral phase of sea-urchin skeletal plates and teeth. *J. exp. Zool.* 234: 7-15.
Weiner, S., W. Traub & H.A. Lowenstam 1983. Organic matrix in calcified exoskeletons. In P. Westbroek & E.W. de Jong (eds), *Biomineralization and biological metal*

Accumulation, pp. 205-224. D. Reidel Publishing Company, Dordrecht, Holland.
Wheeler, A.P. & C.S. Sikes 1984. Regulation of carbonate calcification by organic matrix. *Am. Zool.* 24: 933-944.
Wolpert, L. & T. Gustafson 1961. Studies on the cellular basis of morphogenesis of the sea urchin embryo. Development of the skeletal pattern. *Exp. Cell Res.* 2: 311-325.
Woodland, W. 1906. Studies in spicule formation. IV. The scleroblastic development of the spicules in Cucumariidae; with a note relating to the plate-and-anchor spicules of Synapta inhaerens. Quart. J. micr. Sci. 49: 533-559, pls 32-34.
Woodland, W. 1907a. Studies in spicule formation. V. The scleroblastic development of the spicules in Ophiuroidea and Echinoidea, and in the genera *Antedon* and *Synapta*. *Quart. J. micr. Sci.* 51: 31-43, pls 3-4.
Woodland, W. 1907b. Studies in spicule formation. VII. The scleroblastic development of the plate-and-anchor spicules of *Synapta*, and of the wheel spicules of the auricularia larva. *Quart. J. micr. Sci.* 51: 483-509, pls 29-30.
Wuthier, R.E., G.W. Cyboren, G.P. Warner, H.L. Hubbard & M.S. Vejins 1981. Properties of alkaline phosphatase and the effects of its inhibitors on ^{45}Ca and ^{32}P-inorganic P uptake by isolated matrix vesicles. In A. Veis (ed.), *The Chemistry and Biology of mineralized connective Tissues*, pp. 577-581. Elsevier, North Holland.
Yasumasu, I., K. Mitsunaga, A. Fujiwara & Y. Fujino 1985a. Effects of anion transport inhibitors on calcification of spicule in sea-urchin embryos. *Dev. Growth Differ.* 27: 184-185.
Yasumasu, I., K. Mitsunaga & Y. Fujino 1985b. Mechanism for electrosilent Ca^{2+} transport to cause calcification of spicules in sea urchin embryos. *Exp. Cell Res.* 159: 80-90.