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Evolution

Phosphatic shell plate of the barnacle *Ibla* (Cirripedia): A bone-like structure

(biomineralization/carbonate apatite/organic matrix/crystals/plywood structure)

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ABSTRACT **The carbonate apatite crystals and the segmented structure of the shell plates of the barnacles in the genus *Ibla* distinguish them from the shell plates of all other barnacles, which are coherent calcitic structures. A detailed study of the hierarchical organization of one of the two shell plate types, the tergum, reveals a remarkably complex structure. Cylinders composed of a chitin–protein complex and nodes of plate-shaped crystals constitute the basic building blocks. The crystals are organized into layered stacks in which the *c* crystallographic axes are all aligned perpendicular to a 25-nm banded structure. The cylinders are, in turn, ordered in arrays such that parts of each cylinder are aligned in a plane, and parts form arcuate out-of-plane structures. The overall result is a lamellar structure, with a plywood-like motif, that is present throughout an individual segment. A series of segments forms an interlocking mineralized core, which is enclosed within a thick organic envelope. The flexible and complex skeletal structure of the tergum shows some marked similarities to the structure of lamellar bone. Although this is undoubtably a product of convergent evolution, the iblid tergum provides a unique perspective on bone structure, which was heretofore unavailable.**

Barnacles are a group of marine crustaceans that are widely distributed, particularly in the intertidal zones of rocky shores. One characteristic feature of many barnacles is their exoskeleton, which is composed of an array of shell plates. These plates generally contain the calcium carbonate mineral calcite in an organic matrix of protein and chitin (1, 2). In a few cases the mineral component of the basal plates is aragonite, another polymorph of calcium carbonate (3). It was recently reported that a species of one group of stalked barnacles, the Iblidae, differs from all others, in that its shell plates contain an amorphous phosphatic mineral (4) and not carbonate. Lowenstam *et al.* (5) subsequently demonstrated that the mineral is not amorphous but is composed of crystalline carbonate apatite, also called dahllite. This is the same mineral that is found in most vertebrate mineralized skeletons. The only other invertebrates known to form a mineralized skeleton containing carbonate apatite are the inarticulate brachiopods (6). Several other nonskeletal deposits of dahllite are found among invertebrates, although most of the phosphatic minerals deposited by invertebrates are amorphous (7).

Lowenstam *et al.* (5) also reported that the shell plates of two species of the Iblidae have a unique segmented structure, quite different from all other barnacles. The major organic components are chitin, possibly in the β form, and proteins. Collagen was not detected, as shown by the absence of hydroxyproline using gas chromatography and ion exchange (5). Here we describe the structure of the tergum, one of the two types of shell plates, from the species *Ibla cumingi*. We place particular emphasis on the lamellar structure, which, in many respects, is remarkably similar to bone.

MATERIALS AND METHODS

Specimens of *I. cumingi* Darwin 1854 were collected in Eilat, Israel (Gulf of Aqaba), where they are located in the rocky intertidal zone. They are usually found beneath shell remnants. The specimens were kept frozen prior to examination.

Scanning Electron Microscopy (SEM). The tergum was cleaned of all adhering tissue, washed in distilled water, air dried, and mechanically fractured (not cut) in the desired orientation. Some specimens were treated after fracture with 2.5% sodium hypochlorite (NaOCl) for 4 hr or 24 hr. During the oxidation treatment they were kept in constant motion on a rocking table, then washed with distilled water, and air dried. The specimens were examined in a JEOL 6400 SEM after being coated with gold/palladium. Note that one ethanol-preserved specimen from Heron Island, Australia, was examined uncoated in a JEOL 6400F field emission SEM at 2 kV, after being treated with NaOCl for 24 hr.

Transmission Electron Microscopy (TEM). Adhering tissue, as well as the organic envelope, was removed as much as possible by scraping with a scalpel. The tergum was then treated for 4 hr or 24 hr in 2.5% NaOCl while placed on a rocking table, washed in distilled water, air dried, and crushed in an agate mortar and pestle under liquid nitrogen. The powder was then resuspended in 100% ethanol (about 0.25 ml) and sonicated in a cup horn water bath (Heat Systems Ultrasonicator, Farmingdale, NY) until a milky white suspension was obtained. A drop was placed on a 300-mesh grid with a support film of pioloform reinforced with a thin layer of carbon and dried by gently blowing on the sample. The samples were examined using a Philips CM12 TEM. Samples extensively treated with NaOCl were also examined at −174°C in the TEM using a Gatan (Pleasanton, CA) cryospecimen holder.

Amino Acid Composition Analyses. Tergum samples were decalcified in 1 M HCl at room temperature for about 1 hr (they do not decalcify in EDTA). The insoluble organic material was weighed, then hydrolyzed at 110°C for 24 hr in 6 M HCl vapor, and analyzed using a Dionex BIOLC amino acid analyzer.

Infrared Analyses. A portion of the 24-hr NaOCl-treated specimen prepared for TEM was analyzed by placing about 1 μl of the suspension in ethanol in a diamond pressure cell (High Pressure Diamond Optics, Tucson, AZ), drying, and then analyzing using a Midac Fourier-transform infrared spectrometer.

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Abbreviations: SEM, scanning electron microscope (microscopy); TEM, transmission electron microscope (microscopy).

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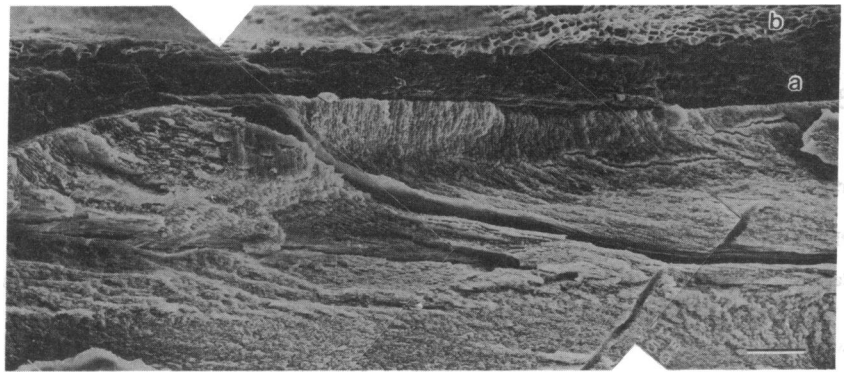

FIG. 1. Longitudinal cross section of a portion of the tergum, showing parts of two mineralized segments and the organic envelope (top). The latter is subdivided into two layers: an inner organic layer (a) and an outer cellular layer (b). (Bar = 20 μm.)

RESULTS

The tergum is composed of a segmented mineralized core, which is surrounded by a continuous envelope of organic material. The envelope comprises two layers: an inner organic layer that abuts the core and an outer cellular structure (Fig. 1). The envelope is much thicker on the outward facing surface, as compared to the inner surface. One of the characteristics that distinguishes the tergum of *I. cumingi* from shell plates of other barnacles is its flexibility. This is derived from the interlocking segmented structure of the mineralized core and the flexible organic envelope. The horse hoof-shaped mineralized segments are separated from each other by a thin organic layer (Fig. 1), which presumably facilitates relative motion between the segments. See ref. 5 for more details.

The fracture surface of the mineralized core examined in the SEM (Fig. 1) shows that the entire segment is composed of a regular lamellar structure. Individual lamellae are about a micrometer thick. The lamellae are generally inclined at a low angle to the segment surfaces, except at the thick end, where they curve to form an angle of about 90° to the surface (Fig. 1). The surface expression of the latter structure is a raised rim, which is part of the interlocking system of adjacent segments.

An examination of the fracture surfaces at higher magnification (Fig. 2) shows that the lamellae are composed of a complex and somewhat irregular structure. The basic building blocks are elongated and roughly symmetrical cylinders about 200 nm in diameter. They occasionally twist around each other to form a rope-like structure. The overall orientation of cylinder arrays in successive lamellae often differs, giving the structure a plywood-like character. An individual lamella is composed of a planar portion and an inclined portion, in which the cylinders curve sharply downward (Fig. 3). In fact, individual cylinders are continuous between the two parts. The major part of each cylinder is in the planar portion, where it is generally aligned with its neighbors.

Fig. 4 shows the fracture surface of a specimen not treated with NaOCl. It is still dominated by the cylindrical motif, but with much less space between the cylinders, when compared to the untreated specimen in Fig. 3. An amino acid composition analysis of an extensively (about 24 hr) oxidized specimen confirms that only chitin remains, as evidenced by the presence of two hydrolysis products identical with those

FIG. 2. Fractured surface of the tergum after 4-hr NaOCl treatment, showing the lamellar structure, composed of the in-plane and out-of-plane curved cylinder arrays. (Bar = 1 μm.)

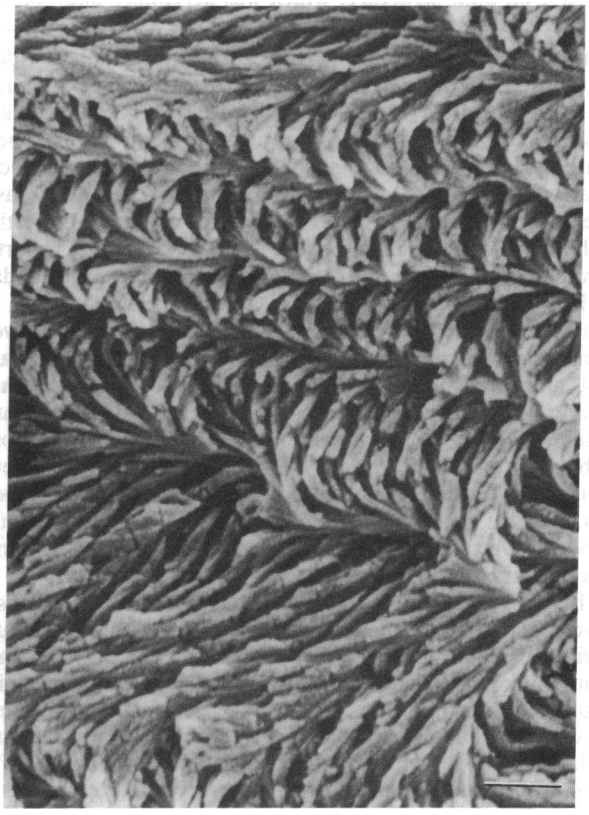

FIG. 3. Four-hour NaOCl-treated tergum surface, showing the individual cylinders partially aligned in the plane of the lamellar boundaries and in part curving out of the plane. (Bar = 1 μm.)

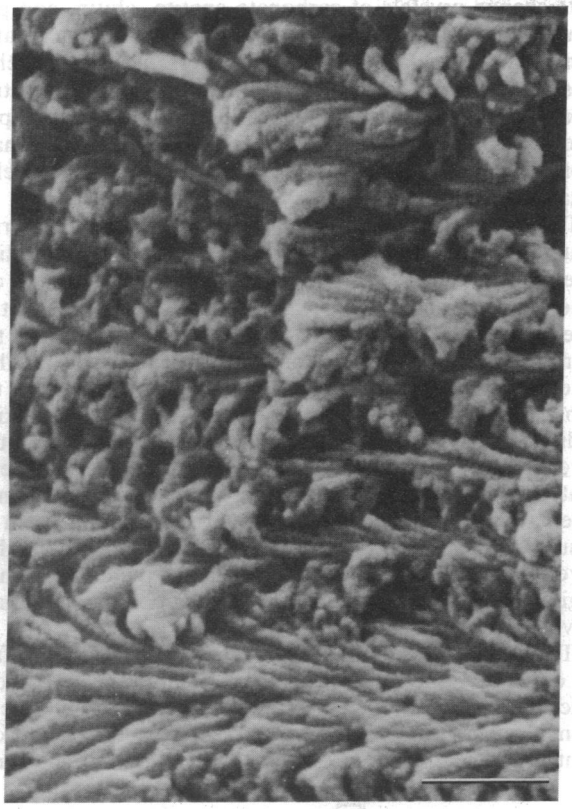

FIG. 4. Untreated fracture surface, showing much less space between cylinders as compared to a NaOCl-treated surface (Fig. 3). Note also the banding pattern on the cylinder surfaces. (Bar = 1 μm.)

formed when standard chitin is treated in the same way. The untreated sample contains as a second major component, in addition to chitin, an assemblage of proteins rich in glycine (24.4%), alanine (11.7%), proline (9.0%), aspartic acid (7.9%), glutamic acid (7.4%), valine (6.8%), serine (5.3%), and methionine (5.2%). (Values are in mol %. Protein comprises 19% of the dry weight of the insoluble matrix, which in turn is about 55% by weight of the mineralized segments.) The proteins are intimately associated with the chitin to form a complex that makes up the cylinders.

A most unusual feature of Fig. 4 is a banded structure on the cylinder surfaces. The bands are perpendicular to the long axis of the cylinders. These bands can be clearly seen at higher magnification when specimens extensively treated with NaOCl are viewed uncoated in the field emission SEM (Fig. 5). The structures are not continuous and their ends

FIG. 5. High-magnification view of a 24-hr NaOCl-treated specimen, showing discontinuous clusters of banded material, which in part is covered with an apparently organic coating. Note also the sharp ends of some of the clusters. The specimen was imaged uncoated in a field emission SEM. (Bar = 100 nm.)

splay out radially. Furthermore, the entire remaining material is well banded. We interpret these structures as nodes of mineralized crystal clusters and their intimately associated chitinous matrix. The splayed-out ends appear to be views of the elongated crystals themselves, and we assume the NaOCl has destroyed the nonmineralized parts of the cylinders between the mineralized nodes. To examine these postulations, it is necessary to use the TEM.

Fig. 6*A* is a stereo view of a tergum particle after extensive treatment with NaOCl, viewed unstained in the TEM. Thus most of the electron density is due to the mineral phase. The crystals all have the shape of thin plates and are organized in a stack of parallel layers. The banding is clearly visible except at the thin end of the particle (Fig. 6 top). It has a repeat distance of around 25 nm. This is due primarily to small holes arranged in rows that perforate through several crystal layers. In the thicker parts of the particle the successive crystal layers are apparently not perfectly aligned, and as a result the holes have the appearance of continuous lines. Fig. 6*B* is an electron diffraction pattern of the same particle, showing that it is composed mainly of carbonate apatite crystals oriented

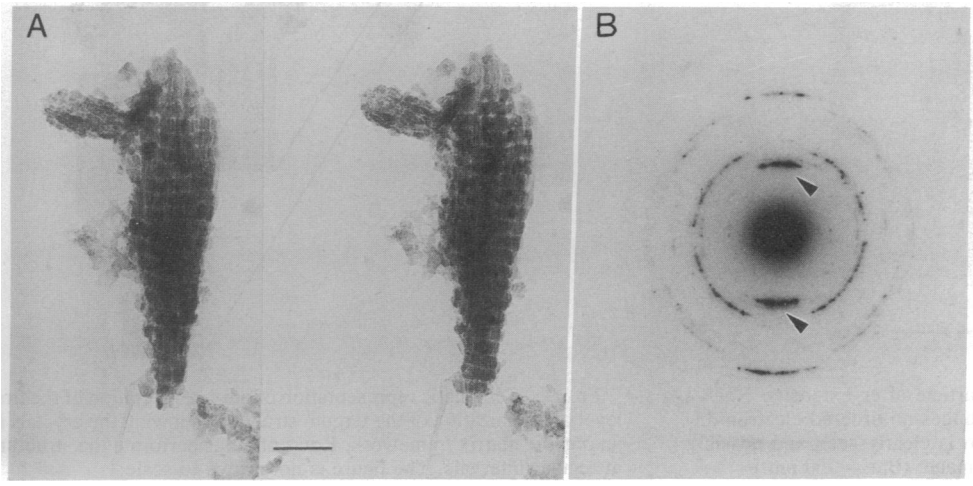

FIG. 6. (*A*) TEM stereo view of a particle in a 24-hr NaOCl-treated and dispersed sample. As the sample is unstained, most of the electron density must be due to the crystals. The plate-shaped crystals are organized in layers. The holes in the structure that give rise to the banded pattern can also be seen. Note the small irregular holes in the crystals are probably due to radiation damage. (Bar = 100 nm.) (*B*) Electron diffraction pattern of the same particle, showing that the *c* crystallographic axes of the crystals are oriented perpendicular to the banding. The arrowheads mark the 002 reflections and, hence, the *c* axis direction.

FIG. 7. TEM view of particles prepared as described in the legend to Fig. 6, showing the elongated crystals tapering slightly at their ends. The rows of holes that perforate the structure are clearly visible in the thicker parts of the cluster where they traverse several crystal layers. (Bar = 100 nm.)

with their *c* axes perpendicular to the banding and parallel to the elongation of the crystals. These phenomena can also be seen in Fig. 7. The upright particle in particular shows the rows of holes, and the crystals tend to taper at their ends.

Particles examined in the TEM after extensive sonication and NaOCl treatment showed a banding pattern (Fig. 8), but no ordered electron diffraction pattern. The same was true of such particles cooled to −174°C to provide radiation protection. An infrared spectrum of the same sample showed that it was composed almost entirely of chitin (not shown). We therefore infer that the chitin also has a periodic banding pattern. Careful examination of Fig. 8 also shows that the chitin is arranged in thin sheets, that are presumably located between the crystals.

DISCUSSION

The structure of the tergum, like many other biological tissues, is hierarchical. The major components are elongated plate-shaped crystals of carbonate apatite, chitin, and proteins. The crystals are located within an organic matrix framework. They are arranged in layers, each of which is presumably separated by a thin layer of chitinous matrix. Small holes, located in rows separated by about 25 nm, pass through the crystal–matrix layers. Fig. 9 is a schematic representation of the structure, at this most basic level of organization.

The chitin–protein complex is organized into cylindrical structures that constitute the second level of organization. The mineralized portions are not continuous along the cylinder but form nodes of crystal clusters in the organic matrix. The cylinders in turn are organized into complex arrays that form the lamellar structure. This can be regarded as the third level of organization. Each lamella is composed of two substructures: the planar portion and the curved portion. Individual cylinders pass from one to the other. The fourth level of organization comprises the regular array of lamellae that make up entire mineralized segments. The cylinder orientations can vary between successive lamellae with the result that a plywood-like structure is formed. The highest levels of organization can be regarded as the array of interdigitating segments and the surrounding continuous organic envelope.

Three properties of the tergum distinguish the Iblidae from all other barnacles: the segmented mineralized core and its thick organic envelope, the carbonate apatite mineral component, and the high proportion of organic to mineral content. The shell plates of other barnacles are not segmented

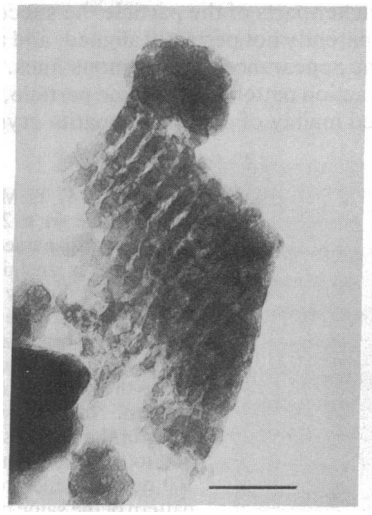

FIG. 8. TEM micrograph of a particle after extensive NaOCl treatment and sonication, which produces no ordered electron diffraction pattern. The banding pattern is clearly seen and possibly even enhanced by some radiation damage. (Bar = 100 nm.)

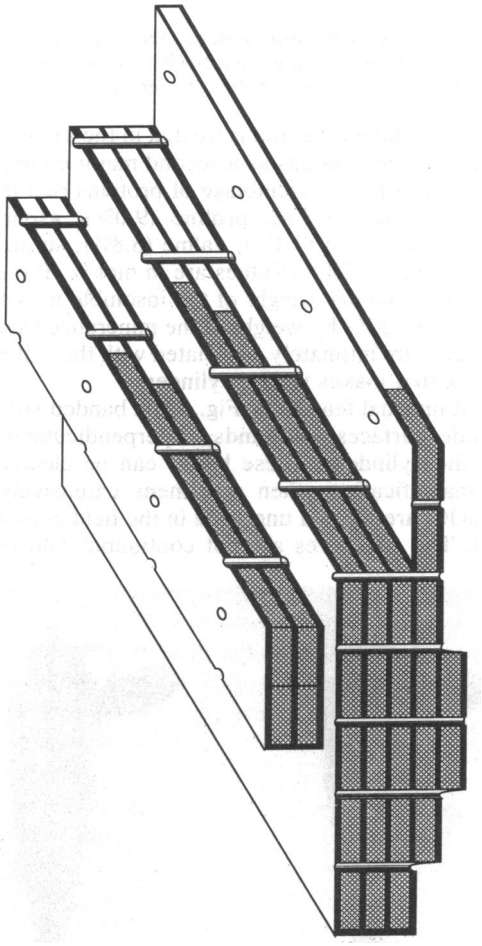

FIG. 9. Schematic representation of our interpretation of the first level of organization of the tergum structure, showing the crystals in an organic matrix framework. Rows of holes perforate the structure at regular intervals. The figure is not drawn to scale.

but are composed of a carbonate mineral, usually calcite, and contain only small amounts of chitinous matrix. Lowenstam *et al.* (5) noted that structures reminiscent of the plates of *Ibla* can be recognized among the phosphatic "problematica" found in Cambrian sediments (8), suggesting that the plates constitute a primitive trait. The iblids are known to possess various other features thought to represent primitive traits. In contrast, the structural complexity of the tergum, and in particular its flexibility, suggest that it has evolved into a well-adapted structure. To clarify this apparent paradox, much more needs to be learned about the functions of the iblid shell plates and, in fact, about the ecology of the animals themselves.

The presence of carbonate apatite crystals in a skeletal tissue of an invertebrate is most unusual. The only other invertebrates that form crystalline phosphatic skeletal structures are the inarticulate brachiopods (6). Chitin is also a major component of their shells. Their shell structures, however, appear to be quite different (9). Other nonskeletal carbonate apatite-containing invertebrate structures known are the inner cores of some chiton teeth, periostracal deposits of certain bivalves, and concretions in an annelid (7). The one mineralized tissue that the iblid tergum does resemble is vertebrate bone.

In comparing the tergum structure with that of the material bone, we can assume that the similarities are not the product of divergent evolution but of convergent evolution. Hence a comparison of this nature is somewhat superficial but does provide an invaluable perspective on both tissues, which was heretofore unavailable. For reviews of bone structure, see refs. 7 and 10.

At the first level of organization, the most striking similarity between bone and the tergum is the presence of thin plate-shaped crystals of carbonate apatite (dahllite) in both tissues. The tergum crystals are, however, much longer and more elongated than those in bone. The major matrix constituents are quite different—type I collagen in bone and chitin and various proteins in the tergum, excluding collagen.

Perhaps the most significant structural similarities between the two tissues are the organization of the plate-shaped crystals into arrays of parallel layers, each presumably separated by a layer of matrix, the regularly repeating banding pattern that in part is derived from the three-dimensional organization of the crystals, and the orientation of the *c* crystallographic axes of the crystals perpendicular to the banding. Despite the fact that the structural basis for the banding in the two tissues is quite different at the molecular level, these three striking similarities raise many challenging questions about common underlying formation processes as well as the functional significance of these structures.

At the second level of organization of the tergum, the cylinders can be compared to the collagen fibrils in bone. The organization of the cylinders into ordered arrays is, however, quite different in the two tissues. A common motif in lamellar bone (and we acknowledge that generalizations about bone structure at this level are difficult) is the organization of mineralized collagen fibrils into layered arrays in which they are all aligned in a preferred direction. In the tergum the cylinders do often form locally into aligned arrays, but the predominant theme is one of cylinder arrays curving to form arcuate structures in the plane of the lamellar boundaries and out of the plane. The curvature of the cylinders in the plane is not a result of viewing the structure at oblique angles. It is most interesting to note that the curvature of the cylinders out of the plane of the lamellar boundaries can be compared to the recently described rotated plywood structure of rat bone lamellae (11), in which the crystal planes are rotated such that they too are out of the plane of the lamellar boundaries. This feature is thought to contribute significantly to the mechanical properties of the bone (12).

At the next level of organization, the tergum cylinders do form well-developed and regular lamellar arrays. These can be compared with the lamellar structure found in many bones, particularly among the vertebrates. Another structural motif that the two tissues have in common at this level of organization is that the predominant orientations of the tergum cylinders and bone fibrils are different in successive lamellae. This results in a plywood-like organizational motif being present in both tissues.

We also note that in many instances the two mineralized tissues are not only similar in structure but also in scale as well. The crystal plates appear to have roughly the same thickness as those in bone (2–4 nm). The banding pattern has a repeat distance of about 25 nm in the tergum and 64 nm in type I bone collagen. The tergum cylinder diameters and the collagen fibril diameters are quite variable, but both generally fall in the range of 100–300 nm. The lamellae thicknesses in bone vary between around 0.5 and 3.0 μm and those in the tergum around 1.0 μm.

CONCLUSIONS

The iblid tergum has an extremely complex hierarchical structure, quite different from the shell plates of other barnacles. It is, in many respects, surprisingly similar to lamellar bone. Even though it is clear that the similarities are not due to any common evolutionary pathway, the convergence in structure of these two mineralized tissues raises tantalizing questions about the common underlying processes responsible for their formation. The structural similarities also open the way to addressing questions about the functional significance of structures that have evolved quite independently in both tissues. The tergum is thus not only a most fascinating mineralized tissue in its own right but also provides a unique perspective on bone, which was, to date, unavailable.

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