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Structure of Molluscan Bivalve Hinge Ligament,
A Unique Calcified Elastic Tissue

by

Mary Marsh

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
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Thesis Director's Signature:

A handwritten signature in cursive script, reading "Ronald L. Sasser", is written over a horizontal line.

Houston, Texas

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INTRODUCTION

The molluscan bivalve exoskeleton consists of two valves connected dorsally by an elastic ligament. The ligament serves the function of a hinge and causes the valves to gape on relaxation of the adductor muscle(s). Although the form of the hinge ligament varies among bivalve families, the ligament is always under strain when the valves are closed (Russell-Hunter and Grant, 1962). The ligament is non-living and typically composed of protein and calcium carbonate. The ligament in vivo is hydrated; when dried, it becomes quite hard and loses all elasticity.

In general, the hinge ligament is divided into functionally different (and sometimes physically separated) parts. The ligament of Mytilus edulis and Ostrea edulis (Trueman, 1950a, 1951) is internal, i.e., situated between the valves and not projecting above them. See Figure 1a. These ligaments are divided structurally into an inner calcified layer and two adjacent uncalcified layers extending anteriorly and posteriorly along the dorsal edge of the valve. When the valves close, the uncalcified layers are subject to tensile strain and the calcified layer is compressed. The ligament of Tellina tenuis (Trueman, 1942, 1949) is external to the valves. See Figure 1b. The inner layer of the ligament is calcified; the outer covering is not. Trueman (1949) reports the inner calcified layer appears to be forced upwards, as if undergoing compression against the outer layer when the valves close. At the same time the outer layer is subjected to tensile strain. The calcified layer is elastic only to compression and tears readily when subject to tension. Both Spisula solidissima (Kahler,

Fig. 1. Diagrammatic sections through the ligamental area of bivalves. After Trueman (1951).

(a) Longitudinal section through *Ostrea edulis*.

(b) Transverse section through *Tellina tenuis*.

Abbreviations: OL - outer ligament layer; IL - inner ligament layer; GL - growth lamellae; V - valve.

1973) and Pecten maximum (Trueman, 1953) have two physically separate ligaments. See Figure 2. Both bivalves have a small uncalcified ligament subject to tensile strain when the valves are closed. Both have a large internal ligament which is calcified in Spisula and uncalcified in Pecten. The internal ligament is mounted on shelf-like extensions of the shell known as chondrophores and is compressed when the valves close.

The foregoing discussion covers the principal forms of ligaments in bivalves. It can be concluded that the calcified ligament (or ligament layer) functions as a compression spring. Dall (1895) described the calcified ligament as having "the essential nature of a c-spring". Only in the family Pectinidae has an uncalcified compression ligament been observed.

Hinge ligaments have growth lamellae running parallel to the growing margin, i.e., lines demarking intervals of rapid growth. The ligament growth lamellae superimpose upon the valve growth lamellae at the ligament-valve junction. This observation led Owens, et al. (1953) to consider the ligament as a local modification of the valve; the valve and ligament together constituting the shell. Trueman (1950a, 1951) and Stenzel (1962) observed fine fibers oriented with their long axis normal to the growing margin in sections from calcified ligaments.

Wada (1961) identified aragonite as the mineral component of the ligament of several bivalve species. Stenzel (1962) found the ligament of the oyster Crassostrea virginica contained only aragonite although the valves were principally calcite. Kahler (1973) determined the crystalline form of calcium carbonate in the shell and hinge

Fig. 2. Diagrammatic section through the ligamental area of bivalves. After Kahler (1973).

(a) Transverse section through *Pecten maximus*.

(b) and (c) Transverse and longitudinal sections through *Spisula solidissima*.

Abbreviations: OL - outer ligament; IL - inner ligament; GL - growth lamellae; C - chondrophores; and V - valve.

ligament of 26 species of bivalves representing 17 families. The ligaments of two species of Pectinidae, which contained less than 1% calcium by weight, were devoid of any crystalline phase. The remaining 24 species contained aragonite as the sole form of calcium carbonate in the ligament, although some of these species had calcite or calcite-aragonite shells. Thus the aragonite in the ligament is not a simple extension of the crystalline structure of the shell.

The organic phase of the hinge ligament is not so well defined. Kahler (1973) and Kelly and Rice (1967) identified this phase as protein with trace amounts of neutral sugars but no lipid. They were unable to solubilize the protein with any of the common protein solvents which they tested. Rice and Kelly (loc. cit.) have called the ligament protein "adductin" and Dall (1895) has called it "resilium" in order to characterize its properties. The amino acid composition of the ligament protein has been determined for several species (Kahler, 1973; Kelly and Rice, 1967; Hare, 1963). The outstanding feature of these analyses is the high concentration of glycine and methionine residues. The glycine residues of the ligament of Spisula solidissima and three species of Pectinidae were greater than 600 residues per thousand. The methionine residues ranged from 60 to 230 per thousand. The structural proteins - collagen, resilin, and elastin, for example - are characteristically 33% glycine with only a trace of methionine.

Like other structural proteins, the hinge ligament protein appears to be cross-linked. Trueman (1950b) and Brown (1952) described the ligament protein as "quinone-tanned" based on qualitative tests

designed to detect polyphenols. Anderson (1967) has isolated a cross-linking agent, 3,3'-methylene-bistyrosine, from hydrochloric acid hydrolysates of the ligament of Mytilus edulis, Spisula solidissima, and Pecten maximus. This cross-linking agent is different from any found in other structural proteins.

Kelly and Rice (1967) investigated the protein structure of Pectinidae ligaments with electron microscopy and X-ray diffraction. Electron micrographs showed this uncalcified ligament to be a "homogeneous amorphous substance of low electron density". Both high angle and low angle X-ray diffraction gave no distinct patterns for the ligament whether stretched or unstretched, wet or dry. Kahler (1973) recorded wide angle X-ray diffraction patterns of the protein of several uncalcified ligaments and of calcified ligaments after extraction of the mineral with EDTA or acid. The only clear reflexion from the protein of the internal and external ligaments of Spisula solidissima and the internal ligament of two species of Pectinidae was a diffuse ring at $4.6\text{\AA}$. They assigned the random β -conformation to these proteins on the basis of the $4.4\text{\AA}$ reflexion given by polyglycine films known to exist in the β -conformation (Harrington and Von Hippel, 1961). The pattern recorded from the outer ligaments of Crassostrea virginica and Mytilus edulis shows a diffuse ring at $5.19\text{\AA}$ for Crassostrea and $5.07\text{\AA}$ for Mytilus. They assigned the α -helix conformation to these ligament proteins based on the $5.1\text{\AA}$ spacing found in α -keratin.

Bevelander and Nakahara (1969) presented the first electron micrographs of a calcified hinge ligament. Their micrographs of the ligaments of Mytilus edulis and Pinctada radiata, which contain 40%

calcium carbonate by weight, illustrate single needle shaped aragonite crystals enclosed in envelopes and imbedded in an organic (electron-transluscent) matrix. The crystals, which are $1000\text{\AA}$ in width, are oriented with their long axis perpendicular to the growing margin of the ligament. The crystals are widely spaced in comparison to crystals in bivalve shells and dental enamel. They reported no fiber structure in the organic matrix. Kahler (1973) reported a similar aragonite orientation and distribution in the hinge ligament of Spisula solidissima based on electron micrographs, wide angle X-ray diffraction of oriented ligament sections and the extinction of polarized light by specific orientations of longitudinal sections with respect to the polarizer. By wide angle X-ray diffraction of oriented ligament sections they also determined the aragonite orientation in the calcified ligaments of five other species of bivalves. In all cases the crystallographic c (morphologically long) axis of the crystals was oriented normal to the growing margin. The a and b axes were randomly oriented.

Several investigators have studied the mechanical properties of the ligament. Trueman (1953) measured Young's modulus of elasticity for compression on isolated ligaments and found the uncalcified ligament of the Pectinidae much less resistant to compression forces than the calcified ligament of other bivalve families. He also studied the opening and closing moments as a function of the angle of gape of the valves after cutting the adductor muscles and removing the animal tissues. He observed very little work loss during an opening-closing cycle for the Pectinidae ligament but considerable work loss for the calcified ligaments. The small work loss and low resistance of the ligament of the Pectinidae is consistent with its light weight shell and swimming

mode of life. When swimming, the Pectinidae open and close their valves several times a minute. Alexander (1966) studied the force applied versus displacement of the isolated Pectinidae ligament as a function of temperature and concluded that this ligament responds to compression with rubber-like elasticity, i.e., that compression corresponds to an entropy decrease with little or no increase in internal energy.

Russell-Hunter and Grant (1962) measured the opening moments for the calcified compression ligament of Spisula solidissima, a surf clam, which burrows in the sand. They concluded that its ligament is powerful enough to open its valves against the forces of its environment. True-man (1954) measured the opening moments of the calcified ligament of Mya arenaria, a sedentary deep burrower which is almost immobile in adult life. He concluded that the opening moment was six times too small to open the valves against its environment, and the opening of the valves was found to be associated with a sudden increase of water pressure within the mantle cavity.

Kahler (1973) measured the resilience of several species of bivalve ligaments. Resilience was defined as:

$$\frac{\text{Work done by ligament in opening valves}}{\text{Work done on ligament in closing valves}} \times 100$$

They found the resilience was inversely related to the concentration of calcium carbonate and cystine/2 in the ligament and directly related to the concentration of glycine.

The purpose of this investigation is to extend the description of the bivalve hinge ligament to its prismatic or pseudo-prismatic structure. A correlation between this structure and the elastic properties of the ligament is proposed.

METHODS AND MATERIALS

The bivalves Spisula solidissima, Mercenaria mercenaria, and Placopecten magellanicus were obtained live from the Marine Biological Laboratory, Woods Hole, Massachusetts. Rangia cuneata was collected from the Texas gulf coast. The hinge ligaments were either removed and used immediately or else were removed and stored at 5 C in deionized water or in artificial sea water.

Twenty-five micron thick sections of the ligament were used for low angle X-ray diffraction and light microscopy. These sections were prepared by imbedding a portion of the ligament in 5% agar and sectioning with a Sorvall tissue chopper. After sectioning, the agar was easily removed from the ligament sections by washing.

For low angle X-ray diffraction an American Metals Research low angle diffractometer was used in conjunction with a Philips scintillation counter and pulse height discriminator. This diffractometer detects only reflexions in the equatorial plane. Therefore, to measure reflexions in other planes normal to the ligament section, the specimen was rotated between each measurement in a plane perpendicular to the beam. Copper K α radiation was used.

A Zeiss Wh microscope was used for normal light microscopy and an American Optical microscope equipped with Nicol prisms was used for polarized light microscopy. The ligament sections were examined wet.

For electron microscopy of calcified ligament, 200 micron thick sections were fixed for three hours in Karnovsky's fixative (Karnovsky, 1965) in 0.2 M cacodylate buffer, pH 7.4. The tissue was then washed 2 x 15 minutes in deionized water. The sections were dehydrated in a

series of solutions of increasing ethanol concentration according to the following program:

25% ethanol	15 minutes
50% ethanol	15 minutes
75% ethanol	15 minutes
95% ethanol	15 minutes
100% ethanol	4 x 15 minutes
propylene oxide	2 x 15 minutes

The sections were transferred to Epon*-propylene oxide (1:1) and were allowed to stand uncovered on top of a 60 C oven overnight. The next day the tissue was transferred to pure Epon and heated in a vacuum oven at 60 C for 2 x 10 minutes. The tissue was treated with fresh Epon twice again in the same manner. Finally, the sections were imbedded with Epon in a plastic mold, and the Epon was allowed to harden 48 hours at 60 C.

For some experiments the tissue was first decalcified with either 2M formic acid or 0.1 M hydrochloric acid. When either decalcified or uncalcified ligament was prepared for electron microscopy, the tissue was fixed for two hours in 1% OsO_4 in 0.2 M cacodylate buffer, pH 7.4, instead of or subsequent to fixation in Karnovsky's fixative. After removing the excess OsO_4 with a 2 x 5 minute treatment with deionized water, the tissue was dehydrated and imbedded as described for calcified ligament.

The imbedded ligament blocks were sectioned with a Sorvall MT2 ultramicrotome. For the calcified tissue a diamond knife was used; for uncalcified or decalcified tissue a glass knife was used. The calcified tissue was sectioned dry, because sectioning on to water caused the block

* Epon is 47% Epon 812, 15% dodecyl succinic anhydride, and 38% nadic methyl anhydride by volume with 1.6 ml of benzyldimethylamine catalyst per 100 ml. All chemicals were supplied by Ernest F. Fullam Inc., P. O. Box 444, Schenectady, N. Y.

face to absorb water and swell, making the preparation of thin sections impossible. The dry sections were then transferred to water for collecting on copper grids. The uncalcified and decalcified tissues were sectioned onto water; the fixation with OsO_4 greatly reduced the swelling of the tissue when wet. Calcified sections were cut 1000 to 1500Å thick; decalcified and uncalcified sections approximately 600Å. The sections were examined with an RCA EMU-3 electron microscope.

RESULTS

The internal calcified hinge ligament of Spisula solidissima (Figs. 2 and 3) weighs about one gram (dry wt.) in the adult animal and consists of 50 to 60% aragonite by weight in a protein matrix (Kahler, 1973). Visual observation of the ligament reveals that it is covered by a thin brown coat except on the lateral surfaces which are normally attached to the shell. The coat is only one to five microns thick on the ventral surface but is about 200 microns thick at the extreme dorsal end. X-ray and electron diffraction show the absence of any crystalline material in the coat.

I. CRYSTAL MORPHOLOGY

The Spisula hinge ligament (throughout this report "the ligament" or "the hinge ligament" will refer to the internal compression ligament) is physically one unit, but optical microscopy reveals two structurally distinct forms within the ligament. The lateral surfaces adjacent to the shell have one structure and the central region has another. The crystal shape in the lateral areas is quite different from the crystal shape in the central area (Fig. 4 and 5). In longitudinal section the crystals of the lateral area are very irregular. The cross bands of varying intensity observed in these crystals are probably artifacts. These bands are seen to move up and down the crystals as the sample is translated in the electron beam, or when the intensity of the beam is altered. This same effect was noted by Bevelander and Nakahara (1969). The crystals are about $1000\text{\AA}$ in diameter and each crystal is surrounded by an envelope approximately $100\text{\AA}$ thick, which is less electron dense than the crystals. The sheaths are not solubilized when thin sections are decalcified with

(a)

(b)

Fig. 3. Shell and ligament of Spisula solidissima.

- (a) Two valves with the ligament (lower center) removed from the shell. The ligament breaks in half when the valves are separated. Note the growth lamellae in the shelves, chronodrophores, which support the ligament and the growth lamellae in the ligament, X .25.
- (b) Two halves of broken ligament. Right half shows the lateral surface which is normally attached to the shell. The left half shows the broken surface in the central area, X 1.

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 4. Longitudinal section from the lateral area of the Spisula ligament, showing the aragonite crystals surrounded by envelopes. The dark bands and small holes in the crystals are artifacts. Unstained. Electron micrograph, X49,000.

Fig. 5. Same as Fig. 4 but taken from the central area of ligament, X49,000.

Fig. 6. Same as Fig. 5 after decalcifying thin section with 0.1N HCl. Notice the empty crystal envelopes, X49,000.

Fig. 7. Cross section from central area of ligament, showing hexagonal cross section of aragonite. Unstained. Electron micrograph, X49,000.

either EDTA or dilute acid on the electron microscope grid. Bevelander and Nakahara (loc. cit.) also reported crystal envelopes in the ligaments of Mytilus edulis and Pinctada radiata. The appearance of small pits in the crystals of Figures 4 and 5 is probably artifactual. Glimcher, et al. (1965) also found small holes in the apatite crystals of bovine dental enamel under high magnification which they attributed to sublimation in the electron beam. Figure 6 is an electron micrograph of a ligament section which had been treated with dilute acid on a copper grid after thin sectioning. The empty crystal sheaths are apparent. Figure 7 is an electron micrograph of a cross section of the crystals. The electron translucent holes may correspond to regions which once contained a crystal but which was dissolved out during the course of the animal's life. The crystals are hexagonal in cross section as was observed by Kahler (1973).

II. PRISMATIC STRUCTURE

The distribution of crystals in the lateral and central regions of the Spisula ligament are quite different. The structure of the lateral area is simpler and will be described first.

The lateral areas of the ligament as observed in the optical microscope are composed of long, roughly cylindrical, rods oriented with the long axis normal to the growing margin (the ventral surface) (Fig. 8). This structure is almost undiscernable when observed in the microscope with normal light, but is very striking when viewed between crossed Nicols. The rods range from 10 to 30 microns in diameter and may extend the entire length of the ligament. This structure may correspond to the fine fibers described by Trueman (1950a, 1951) and

Fig. 8

Fig. 9

Fig. 8. Longitudinal section from lateral area of Spisula ligament, showing columnar rods. Polarized light, crossed Nicols, X600.

Fig. 9. Longitudinal section from the lateral area of the ligament, showing the pseudo-prisms. Electron dense areas are the crystals; electron translucent area is protein matrix. Electron micrograph, X7000.

Stenzel (1962) in the calcified ligaments of Mytilus edulis, Ostrea edulis, and Crassostrea virginica.

Electron micrographs reveal the rods to be composed of aragonite crystals widely spaced in an organic (electron translucent) matrix (Fig. 9 and 10). The crystals are aligned with their morphologically long axis parallel to the long axis of the rods. This organization of crystal bundles into discrete units is referred to as "prismatic structure" in bivalve shells and vertebrate dental enamel, and the bundles are called "prisms". The hinge ligament rods will be called pseudo-prisms, because unlike the prisms of shell and enamel, the ligament pseudo-prisms are not delineated from each other by a noncalcified sheath. In electron micrographs individual pseudo-prisms are discernable only by a slightly different orientation of one prism with respect to another. When viewed in cross section (Fig. 11) very little space is seen between the pseudo-prisms; the interprismatic area which exists is uncalcified. This is unlike dental enamel which has relatively widely spaced prisms and a calcified interprismatic area. For fine electron micrographs of bovine dental enamel and bivalve shell see Glimcher, et al. (1965) and Travis (1968), respectively.

The central region of the Spisula ligament has an unusual structure. Figure 12 is an optical micrograph of a longitudinal section from this area. The horizontal lines are the growth lamellae, but notice the unusual cross hatching which gives the ligament a woven appearance. In order to observe this structure with transmission optical microscopy, the light entering the specimen must be of relatively low intensity. The cross hatched lines bisect each other at 60 degrees, and are not continuous but are actually composed of discrete units. Since this

Fig. 10

Fig. 11

Fig. 10. Longitudinal section from the lateral area of the Spisula hinge ligament, showing the pseudo-prisms. Electron dense areas are the crystals; electron translucent area is protein matrix. Electron micrograph, X7000.

Fig. 11. Cross section from lateral area of the ligament. Pseudo-prism cross sections are visible. Only a small amount of uncalcified interprismatic material is present. Electron micrograph, X3300.

Fig. 12. Longitudinal section from the central area of the Spisula ligament. The horizontal lines are the growth lamellae. Notice the unusual cross hatched structure, giving rise to lines which bisect each other at 60 degrees. Transmitted light, X580.

pattern is observed in all planes sectioned normal to the cross sectional plane, the basic structural unit of the pattern must be cylindrically symmetric.

Figure 13 is an electron micrograph of a prism from the central area of the ligament. Since this structure displays cylindrical symmetry, it can be interpreted as a cylinder constricted at regular intervals, i.e., as a three-dimensional standing wave. This structure will be referred to as noded prisms. The crystals are very closely packed at the nodes and widely separated at the lobes. Two dimensionally the noded prisms are arranged so that a node of one prism lies between lobes on the adjacent prisms. Figure 14 shows how this structure gives rise to rows of lobes (or nodes) which bisect each other at 60 degrees. Figure 15 is a similar micrograph of a very thick section which makes this structure even more apparent. The noded prisms have a diameter of about 4 microns at the lobes and are devoid of an obvious prismatic sheath. These prisms run perpendicular to the growing margin similar to the pseudo-prisms in the lateral areas of the ligament.

Consider the noded prisms as a three-dimensional array of lobes, nearly hexagonally closest packed. Figure 16 is an optical micrograph of a cross section from the ligament. The lobes are roughly ellipsoids of revolution and a cross section normal to the major axis is circular; a node is not circular in cross section, but rather fills the space between six lobes (Fig. 17). Four lobes are in the same cross sectional plane as the node, a fifth and sixth lobe are directly above and directly below the node and are part of the same prism. This means that for a particular lobe four of the six nearest neighbors in cross section are slightly closer than the other two (see drawing in Fig. 18). Since the

Fig. 13

Fig. 14

Fig. 13. Noded prism from the central area of the Spisula ligament. Since the structure is cylindrically symmetric, it resembles a cylinder constricted at regular intervals. Unstained longitudinal section, X8000.

Fig. 14. Same as Fig. 13 which shows a collection of the prisms. Notice how the prisms are arranged so that rows of lobes (nodes) bisect each other at 60 degrees, X10,000.

Fig. 15. Relatively thick longitudinal section from the noded prism area of the Spisula hinge ligament. Nodes and lobes are very apparent. Electron micrograph, X1100.

Fig. 16

Fig. 17

Fig. 16. Cross section from the noded prism region of the Spisula hinge ligament. The lobes form a nearly hexagonal array. Transmitted light, X2200.

Fig. 17. Electron micrograph of the same region as Fig. 16. The nodes are not circular but fill space between the nodes. Areas of high crystal density indicate the nodes, X6800.

Fig. 18. (a) Drawing of a cross section of noded prisms as if the nodes and lobes were exactly hexagonally packed, X17,000.
 (b) But the nodes are not circular in cross section; they fill space between the lobes. This causes four of the lobes' nearest neighbors to be slightly closer than the other two, X17,000.

lobes are ellipsoidal rather than spherical, nearest neighbors above and below a cross sectional plane are at a larger distance than the in plane neighbors. According to the packing structure proposed, only two planes mutually perpendicular to each other and to the cross section plane should display the cross hatched pattern when viewed with the optical microscope. The experimental observation is that all planes normal to the cross section plane have the cross hatched structure. The structure is not apparent throughout the entire length of the ligament but occurs in periodic bands running perpendicular to the long axis of the noded prisms, as if the entire three-dimensional array were periodically rotated about an axis in the longitudinal plane.

The individual noded prisms are internally structured. Since no single experiment directly elucidates this structure, the proposed structure will be presented first and then the experimental results consistent with this structure will be discussed. A noded prism is considered as not being simply a homogeneous mass of protein impregnated with mineral rods, rather the prism is a set of concentric protein cylinders appropriately constricted at regular intervals (Fig. 19). Whether the layers are actually discrete or consist of one sheet wound upon itself cannot be determined from the data available at this time. The former structure will be assumed because it is simpler. The crystals are imbedded longitudinally between lamellae (Fig. 19). The lamellae are about $1600\text{\AA}$ thick (about 1.5 times the crystal diameter) at the lobes and very thin at the nodes where the crystals are quite tightly packed. The prisms appear to consist of six to eight layers but only four are represented in the drawings. The longitudinal sites on the surface of the layers to which the crystals are restricted may extend the entire length

Fig. 19. Drawing of proposed structure of noded prisms. The prisms may be (a) a set of concentric cylinders constricted at regular intervals; or (b) a single sheet wound upon itself and constricted at regular intervals. (a) shows the location of the crystals as longitudinal lines on the surface of the layers, X10,000.

of the prisms. In some cases the crystals appear actually to curve to conform to the lamellar surface and at other instances two separate crystals are seen to meet end on end. In any event the crystal envelope surrounds all crystals constrained to a particular line of longitude. The following paragraphs describe the experimental observations upon which the proposed structure is based.

Figure 20a is a graphical representation of the low angle X-ray diffraction data from a longitudinal section of the noded prism region of the ligament. In polar coordinates r is proportional to the sine of the diffraction angle and θ is the angle between the equatorial plane and the plane in which diffraction is measured. The structural arrangement of the aragonite crystals is responsible for the X-ray scattering. Ligament sections which have been demineralized with either EDTA or acid give no low angle scattering. The square of the Fourier transform of two circles of the same radius and tangent to each other is $J_0^2(2\pi\alpha R/D) \cos^2(2\pi\alpha X/D)$ where α is the radius of the circles in the diffraction plane. $R = (X^2 + Y^2)^{\frac{1}{2}}$ Where X and Y are the coordinates in the image plane. D is the distance between the image and diffraction planes. (Refer to Fig. 20b.) The derivation of this transform is quite simple and can be found in many text books which include a discussion of Fraunhofer diffraction. See, for example, Born and Wolf (1970). Figure 20c is the optical transform of the two circle diffraction pattern. This secondary transform cannot be expected to yield an image of the ligament because the original transform is devoid of any phase or relative intensity information. However, certain features of the secondary transform are worth comment. First, the transform is radially symmetric, but superimposed on the radial symmetry is a set of

Fig. 20. (a) Drawing of low angle X-ray diffraction pattern of a longitudinal section from the noded prism region of the Spisula hinge ligament. r is proportional to the sine of the diffraction angle; θ equals the angle between the equatorial plane and the plane in which the diffraction is measured.

(b) Drawing of the diffraction plane and the image plane. See text.

(c) Optical transform of (a) bright spots correspond to maxima in the function $J_0^2(2\pi\alpha R/D) \cos^2(2\pi\alpha X/D)$ See text.

horizontal lines. This structure resembles a plane cut from a set of concentric spheres of low electron density each sphere containing on its surface a set of electron dense, regularly spaced, longitudinal lines. This is clearly not the structure of a noded prism, but is an approximate description of the prism lobes according to the proposed structure. The secondary transform predicts a distance of about 1600Å between adjacent crystals and this corresponds well to the observed crystal spacing in the prism lobes seen in the electron microscope.

A diagram of a plane intersecting a noded prism parallel to the prism long axis but not containing it is given in Figure 21a. Figure 21a is also a diagram of the proposed prism lamellae which would be visible in this plane. However, since the lamellae are electron translucent, Figure 21b is a diagram of the electron opaque crystals on the lamellae surfaces. Figure 21c is an electron micrograph of a longitudinal section through the noded prism area of the ligament. Note the correspondence between the micrograph, the diagram (Fig. 21a) and the optical transform (Fig. 20c). In the electron micrograph (Fig. 21c) the electron opaque areas are not the aragonite crystals. This tissue was demineralized and treated with OsO_4 before imbedding and thin sectioning. The electron opaque areas are the osmiophylic envelopes which enclosed the crystals. Comparison of electron micrographs of calcified tissue and decalcified OsO_4 treated tissue reveals exactly the same distribution of electron dense material. So several of the micrographs presented will be of decalcified, OsO_4 treated ligament, because this material is much easier to thin section than calcified ligament. Decalcified, OsO_4 fixed tissue is so indicated in the figure legends.

A plane slice taken from an array of noded prisms at 20 degrees

a

b

c

Fig. 21. (a) Drawing of a section from a noded prism parallel to the prism axis but not containing it, X10,000.
 (b) Representation of the crystals in this plane which are confined to lie at the lamellae interfaces, X10,000.
 (c) Electron micrograph of a longitudinal section from the noded prism area of the Spisula ligament. Tissue is decalcified, OsO_4 treated. Electron dense material is the crystal envelopes, X13,000.

to the prism long axis will display several different forms. A set of off-centered concentric ellipses (Fig. 22) is the easiest to recognize. Figure 22c is an electron micrograph of a ligament sectioned at about 20 degrees to the longitudinal plane. Several structure units are seen which correspond to crystals constrained to invisible, off-centered, concentric ellipses.

Finally, consider a plane cut perpendicular to the noded prism long axis. In this plane the cross sections of the prisms will be visible (Fig. 23) and should look like a set of concentric circles. Figure 23c is an electron micrograph of a cross sectional plane from the noded prisms area of the ligament. Notice how the crystal arrangements describe circular arcs. Since the crystal density is fairly uniform in this micrograph, this plane is not a nodal plane.

The lamellar structure of the noded prisms is clearly indicated by the electron micrographs of various planes through the central area of the ligament and from low angle X-ray diffraction of a longitudinal section. The observation that the crystals are constrained to lie between the lamellae rather than within them was accidental. A copper grid supporting thin sections of the calcified ligament was treated with hydrochloric acid to remove the mineral. During this process a portion of one layer was torn away from the adjacent layer (Fig. 24). Since portions of the same crystal envelopes could be seen in both layers, it was concluded that the crystals lie between the lamellae, attached to the outer surface of one layer and the inner surface of the adjacent layer. This micrograph also represents the only direct visualization of the lamellar structure of the ligament protein. Repeated attempts to again dissociate the lamellae in thin section were unsuccessful.

Fig. 22. (a) Drawing of a section cut from a noded prism at 20 degrees to the prism axis, X10,000.
 (b) Representation of the crystals in this plane which are confined to lie at the lamellae interfaces, X10,000.
 (c) Electron micrograph of a section from the noded prism region of the ligament cut 20 degrees to the prism axis. Decalcified OsO_4 treated tissue, X7,000.

a

b

c

Fig. 23. (a) Drawing of a cross sectional plane and a longitudinal plane cut from a noded prism, X10,000.
 (b) Representation of the crystals confined to lie between the lamellae interfaces in the cross sectional plane, X10,000.
 (c) Electron micrograph of a cross section plane from the noded prism region of the Spisula ligament. Decalcified, OsO_4 treated tissue, X19,000.

Fig. 24. Longitudinal section from the noded prism region of the Spisula ligament. During decalcification of the thin section with 0.1N HCl, one layer of the prism was torn away from the adjacent layer. The envelopes of the same crystals can be seen in each layer. Electron micrograph, X26,000.

Evidence for the crystals forming continuous longitudinal lines, extending virtually the length of the prisms, comes from electron micrographs of sections containing the noded prism long axis. According to the proposed structure, only in this plane would the entire length of the crystals be visible. See Figure 25a. Figure 25b is an electron micrograph of such a plane. Several crystal envelopes can be seen which extend the entire length of the micrograph.

The calcified compression ligaments of Rangia cuneata and Mercenaria mercenaria have been examined by optical microscopy and at this level the ligaments are divided into the same two structural units seen in Spisula solidissima. The uncalcified compression ligament of the Pectinidae, Placopecten magellanicus had no structure corresponding to the noded prisms as seen by optical microscopy. The ligament does have a fiber like structure which is similar to the structure of the pseudo-prisms but since this material displays no birefringence, the structure is difficult to discern.

III. LIGAMENT DEVELOPMENT

Some observations on the ligament structure give clues to its development. Since the crystals in the noded prisms are constrained to lie at such specific sites, it appears obvious that the ligament protein is formed first and is subsequently calcified. Electron micrographs of the uncalcified ligament coat taken from the dorsal end of the ligament display osmiophilic fiber like structures with the same orientations expected of crystals in noded prisms (Fig. 26). Whether these are sites of potential calcification or the remnants of once occupied crystal envelopes is not known. The uncalcified compression ligament of Placopecten magellanicus was prepared for electron microscopy to see

a

b

Fig. 25. (a) Drawing of a longitudinal plane through a noded prism containing the prism long axis. In this plane the location of the crystals corresponds to the junction between adjacent lamellae. Therefore, the longitudinal plane shown describes both the crystal sites and the lamellae junction, X10,000.

(b) Electron micrograph of a longitudinal plane from the noded prism region of Spisula ligament. Decalcified, OsO_4 treated tissue, X7,500.

Fig. 26

Fig. 27

Fig. 26. Approximately longitudinal section from the Spisula hinge ligament uncalcified coat. The arrangement of these fiber-like osmiophilic structure is the same as would be expected for the crystals in the bulk of the ligament. OsO_4 treated. Electron micrograph, X83,000.

Fig. 27. Longitudinal section from the uncalcified hinge ligament of Placopecten magellanicus. The tangled osmiophilic fibers may be artifacts (see text). OsO_4 treated. Electron micrograph, X30,000.

if it contained any osmiophylic fibers. However, this ligament behaves very differently to osmium fixation than does the decalcified Spisula ligament. The Spisula ligament becomes very brittle when treated with osmium, but when the Placopecten ligament is treated, it begins to disintegrate. Nevertheless, the tissue was imbedded and thin sectioned. Figure 27 is an electron micrograph of a section from this ligament. There are osmiophylic fibers, but they are in a tangled array. Whether or not this is an artifact of the osmium treatment is not known.

The first mineral to appear in the ligament after a new layer of protein is formed may not be aragonite. Cross sections taken from the growing margin of the ligament sometimes display amorphous looking electron dense material surrounded by envelopes (Fig. 28). This electron dense material is amorphous to electron diffraction. The existence of amorphous calcium carbonate is unknown to this investigator. The initial material could be a calcium phosphate, the phosphate later being replaced by carbonate.

Fig. 28. Cross section from the growing margin of the Spisula hinge ligament. The electron dense bodies are amorphous to electron diffraction. Unstained. Electron micrograph, X90,000.

DISCUSSION

The internal compression ligament of Spisula solidissima is composed of two types of prisms. The pseudo-prisms of the lateral surfaces which are normally attached to the shell are long and columnar. They are structurally similar to prisms of dental enamel except for the absence of a prismatic sheath and any appreciable amount of interprismatic material. The noded prisms constitute the central area of the ligament. Structurally a noded prism is a set of concentric cylinders constricted at regular intervals. The aragonite crystals lie longitudinally between the concentric organic layers.

The pseudo-prisms are probably rigid and contribute little to the elastic properties of the ligament. When attempting to thin section calcified ligament onto water, it was observed that the ligament area composed of pseudo-prisms swelled to a much lesser extent than the noded prism area of the ligament. However, the degree of swelling and the elasticity may not be related. The pseudo-prisms may reflect the prismatic structure of the shell in the ligament region, but this is pure speculation since the prismatic structure of the Spisula shell has not been studied.

When the valves close, the wedge shaped compression ligament is extended ventrally. The noded prisms probably account for the elastic properties of the ligament. When the adductor muscles close the valves, the ligament could readily be compressed at the ellipsoidal lobes of the noded prisms. Besides a ventral extension during compression, the ligament also bulges anteriorly and posteriorly. During compression the noded prisms would then tend to pass from a structural state of "circular cylinders with periodic constrictions" to a state of

"ellipsoidal cylinders with periodic constriction". The function of the rod shaped crystals is to give strength to the weak but elastic protein matrix, enabling the valves to open against the forces of the animal's environment. Structurally the noded prisms resemble an elliptical spring which is a compression spring characterized by its great strength. Dall (1895) had considerable insight when he described the calcified compression ligament as "having the essential nature of a c-spring". A c-spring is essentially an elliptical spring open on one end, and Dall formed his conclusion fifty years before the advent of electron microscopy to the study of biological systems.

The central area of the compression ligament of Rangia cuneata and Mercenaria mercenaria are also composed of noded prisms. This observation is not surprising for Rangia because it belongs to the same family Mactridae as does Spisula. Both of these species have an internal calcified compression ligament and a small external uncalcified ligament. The ligament of Mercenaria, however, is external, and the calcified and uncalcified layers are physically connected. This observation indicates that the noded prisms may be a structural feature common to all bivalves which have a calcified compression ligament. However, more species must be examined before this statement can be made with certainty. It is not surprising that other investigators have not noticed the woven appearance of the ligament caused by the noded prisms because it is nearly invisible in bright field microscopy, becoming visible only when the illumination is greatly reduced.

SUMMARY

- (1) The hinge ligament of Spisula solidissima is composed of two different forms of crystal prisms; the pseudo-prisms and noded prisms.
- (2) The noded prisms in the central area of the ligament consist of concentric protein cylinders constricted at regular intervals. Aragonite crystals are imbedded longitudinally between the protein lamellae. The crystal(s) restricted to a particular line of longitude is (are) surrounded by a single continuous osmiophilic envelope.
- (3) The pseudo-prisms in the lateral areas of the ligament are columnar and without constrictions. They closely resemble the apatite prisms of dental enamel. It has not been determined if the pseudo-prisms are lamellar.
- (4) There is very little interprismatic space in the pseudo-prism region of the ligament, and the space which does exist is uncalcified. In the central area of the ligament the noded prisms fill all space. The elliptical lobes are nearly hexagonally closest packed and the nodes fill the space between lobes.
- (5) The calcified compression ligaments of Rangia cuneata and Mercenaria mercenaria are composed of the same two types of prisms as Spisula.
- (6) It is proposed that the pseudo-prisms are rigid, and the noded prisms, which are structurally similar to an elliptical spring, are responsible for the elastic properties of ligament.
- (7) Observation of the mineral at the growing margin indicates that the initial mineral formed is not aragonite and is amorphous.

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