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NOTES ON THE MICROSTRUCTURE OF THE *NAUTILUS* SHELL

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Abstract

The shell of the *Nautilus* was examined using scanning electron microscopy, transmission electron microscopy and polarized light microscopy. The structure consisted of two major layers: a porcellaneous outer layer and a nacreous inner layer. Most of the porcellaneous layer was composed of granular crystals randomly distributed with a substructure suggestive of bundles of acicular crystallites. A separate prismatic sublayer of the porcellaneous material was composed of a more regular arrangement of crystals. The nacre was composed of alternating crystalline lamellae and films of organic material. The lamellae were formed of many polygonal crystal platelets. The growth surface of the nacre consisted mainly of stacks or towers of incomplete platelets but some areas showed a terraced form of growth in which each crystal lamella was essentially completed before the next covered it. The porcellaneous material, which is exposed to the external environment, and thus requires a greater erosion resistance, was considerably harder than the nacre. It was found that the internal shell walls showed further layers of material not present in the outermost whorl. These were a thin organic layer, which appeared as a boundary between the existing and added material, and a thick layer of nacre. This extra nacre may be useful in the shell's buoyancy control. The siphunclar tube, examined with scanning electron microscopy and polarized light microscopy, appeared in cross-section as a ring of semi-prismatic crystals outside a dark organic hoop. A bisection of the septal neck showed that this ring fits like a sleeve over the nacre of the septal neck.

Key Words: *Nautilus*, nacre, porcellaneous, prismatic, transmission electron microscopy, scanning electron microscopy, biomineralization, cephalopod, biological crystals, ultrastructure.

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Introduction

The *Nautilus* and the *Argonaut* are molluscs of the Class Cephalopoda, to which the octopus and the squid belong, and are the only members of this class which still possess external shells [1]. However, the Argonaut shell is a relatively recent introduction in evolutionary terms while the *Nautilus* and its ancestors have had shells for millions of years [23]. The *Nautilus* can be considered a living fossil with a shell little changed in millions of years, an indication of how well the structure is adapted to its environment.

The shell of the *Nautilus* consists of a hollow cone wrapped tightly around itself [22] in a smooth planispiral which, in the adult, can be up to 15 cm in diameter, and which consists of about three full turns or whorls [18]. Only the final whorl of the shell is visible since the spiral curves over itself and previous whorls are covered by the shell as it grows. The shell is usually clean and white in life [18], with orange-brown camouflage stripes over the dorsal part [18]. A black patch of organic material covers the center of the spiral, the umbilicus, and the area of the coil just above the aperture [18] where the hood of the animal sits in life and where the coiled shell enters its own aperture. A nacreous coating, or callus, covers the black patch directly over the umbilicus and extends into the aperture.

Shell growth proceeds with the deposition of material to the leading edge or aperture [18, 21]. Periodically, the animal moves forward in the shell and seals off the volume behind it with a partition known as a septum to form a series of chambers [21, 22, 24]. The final, or body, chamber of the shell, where the animal lives, occupies about 90 degrees of a whorl in a juvenile, [21] and this increases to about 145 degrees when the *Nautilus* matures [2, 18]. The only link between the animal and these chambers is a thin extension of the animal's body known as the siphuncle [21, 22], which passes through a small hole in the center of each septum [18]. The siphuncle operates as an osmotic pump which removes water from the rear chambers as the animal grows and bails out the shell against hydrostatic pressure after maturity [22]. This decreases the overall weight of the shell by balancing the mass of the shell and body against buoyancy [21] so that the animal floats easily [5, 10, 17, 18, 22].

Those chambers, which have been fully pumped out, contain only a gas similar to air [5, 18, 22], at a pressure of slightly less than one atmosphere [3, 5, 18, 24]. The hydrostatic pressure on the outside of the shell is commonly more than 30 atmospheres [24] and this will also be the pressure of the blood running through the siphuncle [22]. Thus, both the shell and the siphuncular tube must be strong enough to withstand this pressure [3]. In fact, the shell can withstand depths of about 800 m (i.e., pressures of about 80 atmospheres) before implosion occurs [30].

The wall of the *Nautilus* shell consists of three principal layers: the periostracum, the porcellaneous layer (also referred to as the spherulitic/prismatic layer [9, 12, 27]) and the nacreous layer [9, 21]; each is quite distinct in appearance and structure. A fourth thin semi-prismatic layer covering the inner surface of the body chamber in mature animals has also been reported [4, 9, 11].

The periostracum is the outermost of these layers and has a poorly understood but wholly organic structure [11, 21] related to the organic matrix, conchiolin, which envelopes the crystals of the other layers. The periostracum is not commonly found on mature specimens of most species of *Nautilus* [9] since it is usually rubbed off as the shell matures [21]. It provides nucleation sites for initial growth of the crystals which form the major part of the shell and, apart from attachment areas for the large muscles which hold the shell in place [21], is the only part of the shell in actual contact with the animal's mantle [11]. The rest of the shell is separated from the body of the *Nautilus* by a thin space filled with a fluid from which the material of the shell precipitates [11].

The porcellaneous material forms the recognisable outer surface of the shell and is deposited at the edge of the shell aperture by the specialized region of the animal's surface known as the mantle fold [18]. This layer is often referred to as the spherulitic/prismatic layer since it comprises two sub-layers [9]. The outer of these sub-layers is formed of a great many randomly oriented, polyhedral grains of aragonite, about 5-6 μm across, each of which consists of a collection of parallel spicular elements [9, 11, 18]. These grains are not truly spherical, except in the first formed material near the shell surface [9] where they are also smaller than grains further inside the shell. Toward the inner edge of the porcellaneous material, the grains are more distinct and are oriented with axes perpendicular to the surface, thus forming the second, prismatic, sub-layer [9, 11, 18]. The prismatic layer is much thinner than the spherulitic layer and the boundary between them is uneven and indistinct [11]. The small acicular crystallite components of the prisms [12] are oriented roughly perpendicular to the nacre but fan out somewhat toward it.

The nacreous layer forms most of the bulk of the shell, making up about 60% of the shell thickness [9, 18]. The nacre is deposited by the mantle within a few centimetres of the aperture [18] and consists of thin, tabular, hexagonal crystals [9, 18] in lamellae of extremely regular thickness, interlaminated with thin films of organic material [18] known as conchiolin. The conchiolin also forms organic bridges between successive films, thus separating adjoining crystal platelets within the crystalline lamellae and giving the material a brickwork appearance [18]. On the growth surface, the crystals deposit one on top of another in stacks, so that the nacre has a columnar appearance [9, 12]. Each of the crystal platelets is composed of numerous small acicular crystals perpendicular to the plane of the platelet and which appear to be continuous with the crystalline components of the prisms [12]. The crystallographic c-axis is oriented perpendicular to the face of the platelet and, in *Nautilus*, there is also rough alignment of the a- and b-axes between the individual platelets, although this is not the case with the majority of molluscs [8, 28].

The semi-prismatic layer [9, 11] covers the inside surface of the body chamber and eventually forms up to ten percent of the thickness of the mature shell [9]. It has a form similar to the prismatic part of the porcellaneous layer. A thin continuation of semi-prismatic material is also present where the septa meet the inner shell wall, and it thickens in these areas to fill the angle between septum and shell [11]. The semi-prismatic material is secreted at the back of the body chamber during shell growth, and this deposition continues for a short time after the animal reaches maturity and further extension of the shell ceases. Eventually, the entire inner surface of the mature shell is covered by a semi-prismatic layer [11].

The septa which separate the chambers from one another are almost entirely formed of nacre, although there are some thin layers of other material on their surfaces. A semi-prismatic layer covers the nacre on the concave, or body chamber, side of the septa and a thin organic film or pellicle coats both sides of all septa except for the concave side of the final septum [11, 21]. The septa join the shell wall at a very acute angle and within this angle, on the convex side of the septa, is a variable and complicated structure which is partly conchiolin and partly aragonite [18]. This material first appears as a semi-prismatic ridge which serves as an anchor for a new septum [21]. The ridge later becomes an infilling or reinforcement between the septum and the shell wall [21].

The siphuncular tube, despite major differences in structure, is similar in strength to the rest of the shell

[3], the weakest point being the septal neck where the siphuncle passes through a septum [3]. The siphunclar tube has three layers: the thin organic outer pellicle which can be compared to the periostracum; a porous chalky tube which has similarities to the porcellaneous layer; and a horny conchiolin tube which has the appearance of decalcified nacre [3, 5, 21]. These parts of the siphunclar tube are secreted by the surface of the siphuncle in a fashion similar to the secretion of the shell by the mantle.

There have been many previous studies performed on the structure of nacre, indeed, it may be the most well studied of the various configurations adopted by crystals in shells. However, it has been shown that there are differences in the nacre derived from different types of mollusc [6, 8, 26, 28], and most studies in the literature have been performed with the easily obtained shells of bivalves. Taylor and Layman [19] have examined the mechanical properties of the different crystal structures in a wide variety of bivalve shells, determining that nacre is by far the strongest, although most other structures are harder. Regions of the *Nautilus* shell have been examined with scanning electron microscopy (SEM) [9, 12, 18, 21], however, previous studies using transmission electron microscopy (TEM) have been restricted to the use of replicas [7, 18] because of the difficulty in preparing ultra-thin samples suitable for TEM. The preparation of ultra-thin samples using argon ion beam thinning allowed direct observation of the shell ultrastructure, both on the surface and in cross-section. Hence, this investigation describes the ultrastructure of the major parts of the *Nautilus* shell utilising light microscopy (LM), SEM and TEM observations.

Materials and Methods

The two *Nautilus* shells used in this study were donated by Professor W.R.A. Muntz of the Monash University Science Faculty. Both shells belonged to *N. pompilius*, the most common species of living *Nautilus*. One was a mature shell and, although it is not known whether the animal had spent any time in an aquarium prior to its death, the shell was fully grown at the time of capture. The other shell was possessed by an animal which was juvenile, or at least sub-mature, when captured and which spent a period of approximately six months in captivity before it died. A full history of these shells was not available, except that both animals were captured near Fiji. The conditions which were prevalent in the aquarium are not known.

Sections were cut from the *Nautilus* shell with a dry, diamond-edged, rotary saw and most were taken from the crest of the shell. Some sections were mechanically polished to a thickness of about 100 μm and were subject to Vickers micro-hardness tests in which a diamond pyramid was pressed into the section under various loads between 100 g and 1000 g. The diagonals of the indentation produced by the pyramid were measured to derive the hardness of the material. Each surface was tested at least three times under different loads and the results were averaged. Representative samples of the inner and outer surfaces of the shell were taken from the body chamber along with a piece of material broken from the most recent septum. These samples were powdered and examined with a Scintag PAD V X-ray diffractometer.

Selected areas of the samples were examined using a JEOL 200CX transmission electron microscope operating at an accelerating voltage of 200 kV. For this, ultrathin sections were prepared by selected area argon-ion-beam thinning [15, 16]. This method involved selecting an area of the polished sample (about 100 μm thick) by cementing a slotted copper grid onto the section. The mounted specimen was placed in the argon-ion-beam thinner where it was exposed to a beam of argon ions, incident at an angle of about 15° to the surface, until a small perforation appeared in the area of interest. One-sided thinning by a single beam was used if the immediate surface was of importance, otherwise two beams acting on opposite sides of the specimen increased the thinning speed. The areas surrounding the perforation were thin enough to be electron translucent. Prior to the TEM examination, the samples were coated with a thin film of carbon to avoid sample charging. Selected area diffraction (SAD) patterns were also taken in areas of interest on these samples in order to assess crystal orientation, alignment and identification.

Both polished and fractured samples of the shell cross-section, as well as the natural inner and outer surfaces of the shell were used in the SEM observations. The cross-sectional specimens were prepared by embedding the samples in epoxy resin and then polishing them with abrasive paper. These were etched with either 0.1 M acetic acid (CH_3COOH) for 90 seconds or 8% w/v sodium hypochlorite (NaOCl) for several hours, depending on the sample and the type and depth of etching required. The former etchant removed the mineral component to reveal the organic matrix, while the latter removed any organic material leaving the crystals. Samples of the natural surface or of fractured material were not embedded, polished or etched, but were simply removed from the shell and mounted on aluminium stubs using high conductivity silver paint. Prior to SEM examination, all samples were coated with a thin film of platinum. The samples were examined using a Hitachi S/570 scanning electron microscope operating in secondary electron mode at acceleration voltages of 5-10 kV.

Samples for LM observations were prepared by cutting a strip of material along a line which bisected the *Nautilus* shell. This strip, approximately 1 cm in width, was then placed in a plastic petri dish which was filled with epoxy resin. When the resin had dried, the plastic dish was polished away to expose the shell and the disc so formed was cut into manageable pieces. Slices about 0.6 mm thick were cut from a suitable piece, both in plane of the disc of resin and normal to it, using a diamond-edged rotary saw. This provided cross-sections both perpendicular and parallel to the plane of symmetry of the shell. The samples examined contained a region of shell from close to the center of the spiral so that all the layers of the shell wall could be seen, as well as areas of the septa and the siphunclar tube. The slices were attached to glass microscope slides and further polished to less than 100 μm. The samples were viewed between crossed polarizers with an Olympus BHA-P transmission light microscope.

Results

The planispiral shell of the *Nautilus* was white with orange-brown marking over the early part of the outer surface and a black patch where the shell emerges from the aperture (Fig. 1a). The center of the spiral, or umbilicus, was covered with a thick callus of nacre which extended inside the aperture to cover the portion of the shell which had been overgrown by the shell as it extended. Inside the shell, the spiral was separated into a number of chambers partitioned from each other by septa and joined by a fragile cord which was the remains of the siphunclar tube (Fig. 1b). The external surface had a chalky texture, while the inside was glossy and smooth. The two surfaces represented the two major layers: the outer porcellaneous layer and the inner nacreous layer; the latter being two to three times the width of the former (Fig. 1c).

The porcellaneous layer

SEM observations showed the external surface of the shell to be formed of many crystalline grains which were randomly oriented with respect to one another (Fig. 2a). All the grains were irregular in shape and about ten micrometers in length; the larger ones had a substructure of roughly aligned, acicular components about one micrometer in length (Fig. 2a). TEM observations showed that these components, or crystallites, were small in size, irregular in shape and, although aligned within each grain, were randomly oriented with respect to crystallites in other grains (Fig. 2b).

There were gaps between and within the grains which probably represent areas where the organic material had been removed during sample preparation (Fig. 2). The irregular nature of the gaps suggested that the organic material incompletely surrounded both the crystallites and the grains and that it was not regular in thickness or distribution around them so that, in many places, adjacent grains were in contact. The appearance suggested that the organic material was squeezed into the spaces between the grains as they grew.

With the SEM, the cross-section of the porcellaneous layer was seen to contain two sub-layers (Fig. 3). The wider of these, which made up the bulk of the layer, extended to the outer surface and was formed of the granular material. The general appearance of the granular material was similar at all depths within the sub-layer, although, in cross-section, it was apparent that the average size of the grains was less near the surface and greater near the second, prismatic, sub-layer. It was not possible to distinguish between sections of the granular layer cut parallel or perpendicular to the leading edge of the shell, implying that there was no preference in the crystal orientation with respect to the growth direction. Light micrographs of the shell cross-section between crossed polarizers showed no variation in the intensity of the light transmitted through the porcellaneous layer, as the sample was rotated relative to the polarisation direction, indicating that the material was isotropic at this scale. This was due to the small size of the crystallites and the randomness of their orientation.

The second sub-layer of the porcellaneous material had a prismatic structure, was about ten to twenty micrometers wide, and was present between the granular and nacreous structures. It was typified by an increase in grain size and regularity with a tendency for the alignment of crystallites to be perpendicular to the lamellae of the nacre, thus resulting in a prismatic form to the structure. The crystallite alignment within the prisms was not very good and they diverged toward the nacre, giving the material a feather-like appearance (Fig. 3a). The prisms were more regular in shape and appeared less porous than the grains but the relationship between the prismatic and granular structures was obvious.

The transition from the larger grains in the granular layer to the prisms of the prismatic layer was gradual and there was no well-defined boundary between the two sub-layers. This was in contrast to the sharp distinction between the prismatic and nacreous materials caused by the extreme differences in appearance between these two structures (Figs. 3a and 3b). At higher magnification, the transition between the prismatic and nacreous materials was observed to be accompanied by several lines which were visible in the prismatic layer, and which were parallel to the laminations of the nacre with a similar spacing (Fig. 3c).

The nacreous layer

SEM observations showed that, in cross-section, the

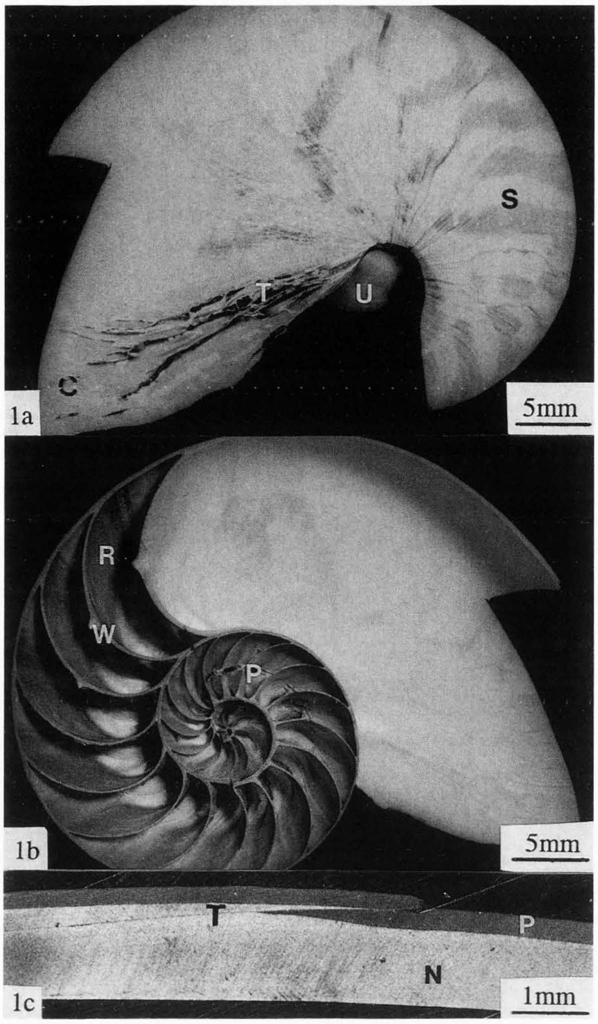


Figure 1. (**a**) A picture of the outside surface of the *Nautilus* shell showing: umbilicus (U), camouflage stripes (S), and growth during captivity (C). The discontinuities (T) in the section represent disruption to shell growth during captivity. (**b**) The inside of a bisected *Nautilus* shell showing chambers (R) separated by septa (W). A remnant of the siphuncle (P) is visible in some of the inner chambers. (**c**) Reflected light micrograph of a cross-section of shell wall etched with CH_3COOH for 60 seconds and showing the nacreous layer (N), porcellaneous layers (P) and the discontinuity (T) due to the trauma of capture.

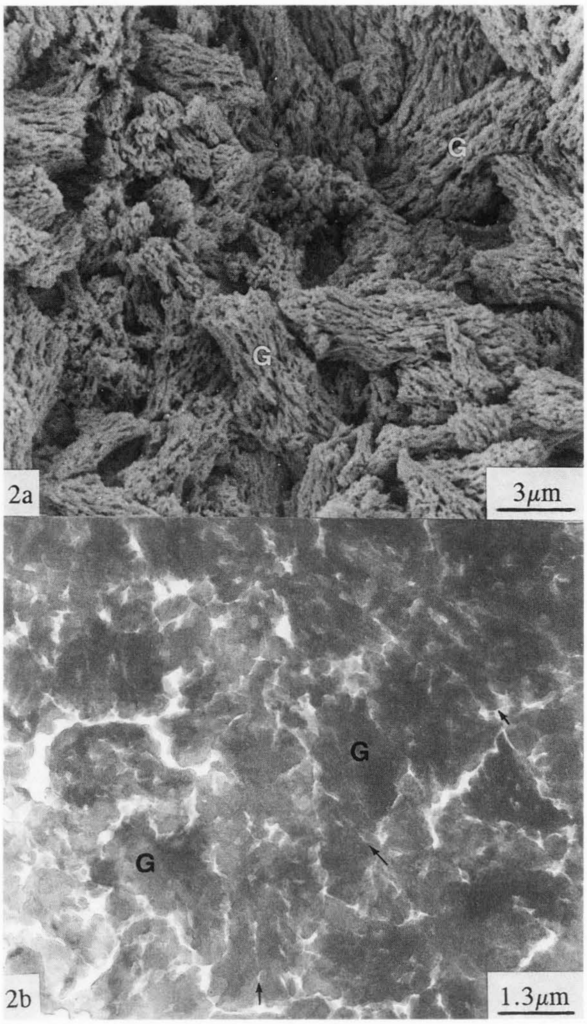
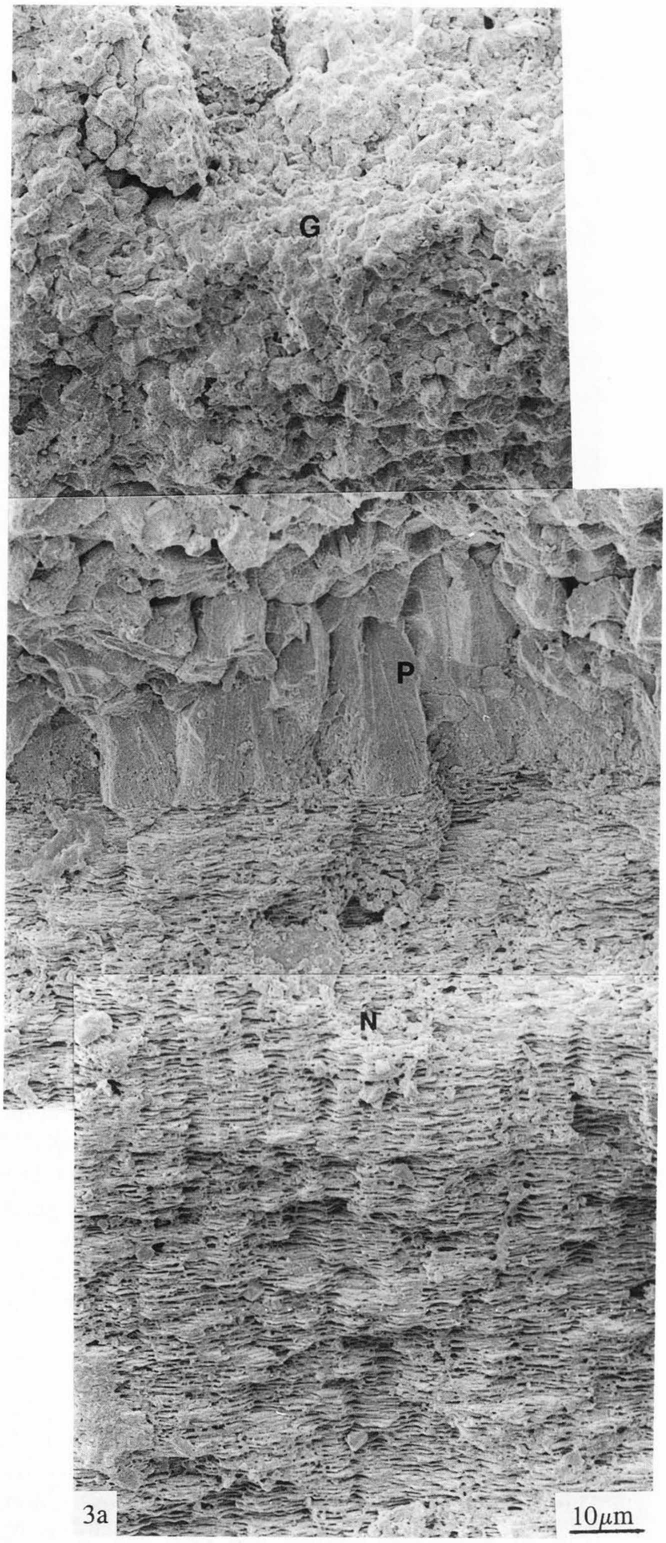
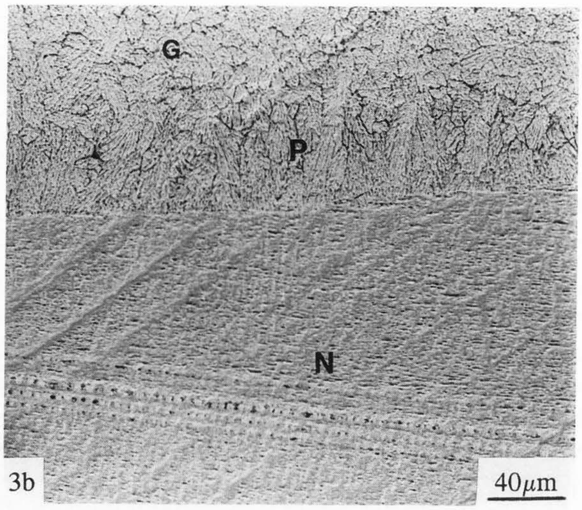


Figure 2. The external surface of the shell showing the randomness of grain size, shape and distribution in the porcellaneous material. (**a**) Scanning electron micrograph of the crystalline grains (G) showing that each grain contains crystallites which are roughly aligned. (etched for 5.5 hours in NaOCl). (b) Transmission electron micrograph in which the larger components, about 0.5 to 1.0 μm across, are the grains (G), while the finer structure (arrows) is due to the individual crystallites.

nacreous layer consisted of very regular lamellae of crystals. Over a great number of lamellae, the crystals in successive lamellae tended to form one above the other so that columns appeared perpendicular to the laminations (Fig. 4). The crystals which formed the lamellae were flat platelets and the columns were interlocked by the overlap of crystals in adjoining columns, thus giving the structure a brickwork appearance, with the crystals as the bricks and the conchiolin as the mortar. This configuration was confirmed by TEM observations (Fig. 5) which also showed that each lamella in the nacreous layer was about 0.40 μm ± 0.05 μm in width. The lamellae were separated from each other by an organic film which was apparent from the spaces that it left between laminations. The film was commonly about 50 nm thick. The plane of contact between two adjacent crystals in a lamella was shown by the TEM to often be



3a



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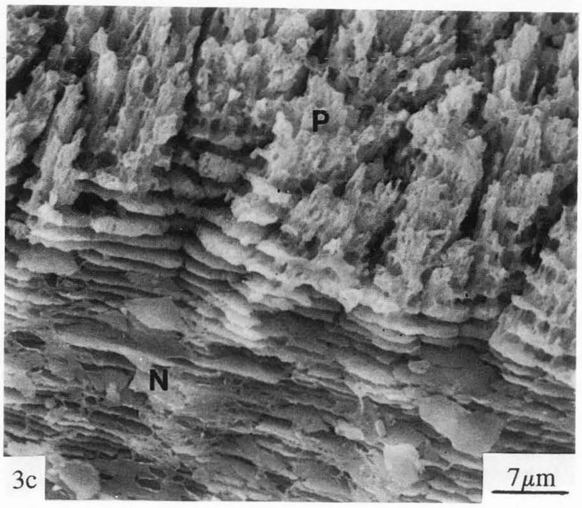


3c

Figure 3. Scanning electron micrographs of a cross-section of the shell showing the prismatic (P), granular (G) and nacreous (N) layers, and the boundaries between them. (**a**) A fractured sample etched with NaOCl (for 3 hours) to remove organic material. (**b**) A sample etched with acid (60 seconds in CH_3COOH) to remove the crystalline material. (**c**) A higher magnification view of the prismatic/nacre boundary. Notice the horizontal laminations in the prisms and some interdigitation of the two materials in the boundary region (etched with CH_3COOH for 30 seconds and NaOCl for 6 hours).

very thin as the crystals butted directly against one another, but occasionally a gap was formed by organic material trapped between the adjacent crystals preventing them from coming into full contact (Fig. 5). When the gap did contain organic material, it was commonly the same width as the organic film between the crystal lamellae or, in some places, wider, but it was never narrower than the films. Each layer of organic material formed a continuous film between crystalline lamellae despite the fact that the lamellae were formed of many crystals. Continuity was maintained in the organic film over adjoining platelets within a given lamellae. This meant that two platelets butting into each other were the

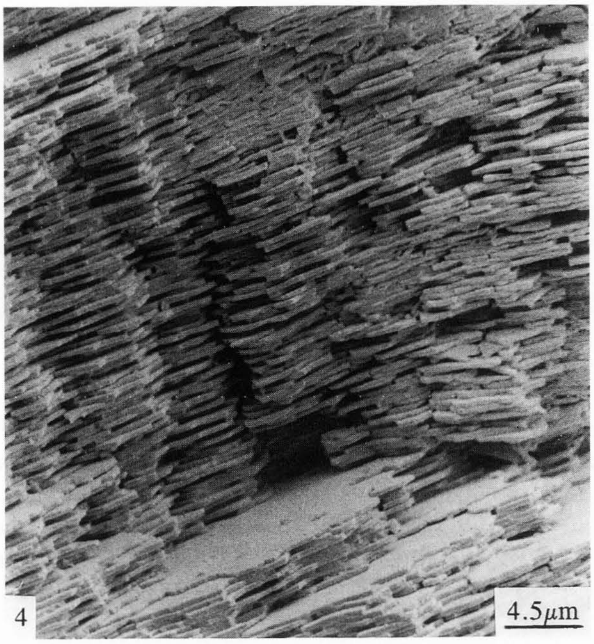


Figure 4. Scanning electron micrograph of the fracture surface showing the stacking of platelets in the nacreous layer which gives the structure a pseudo-prismatic appearance in cross-section and which results from towered growth on the deposition surface (see, e.g., Fig. 7).

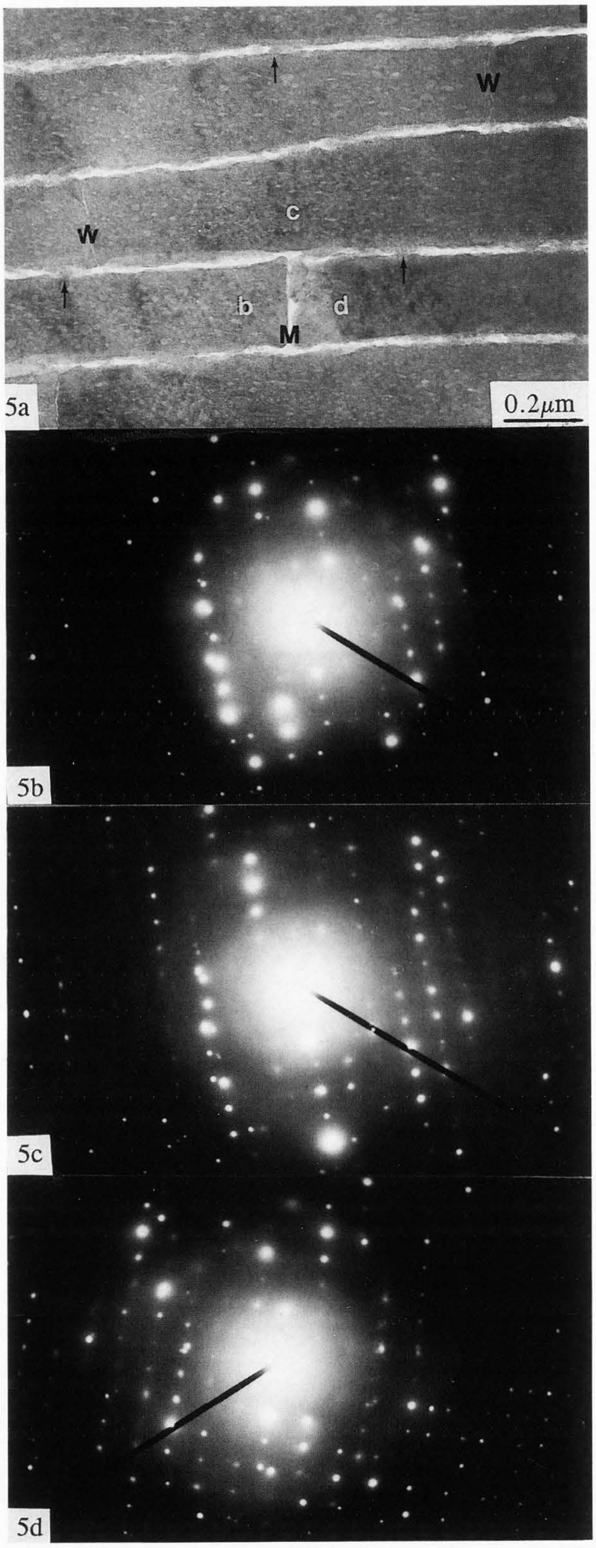
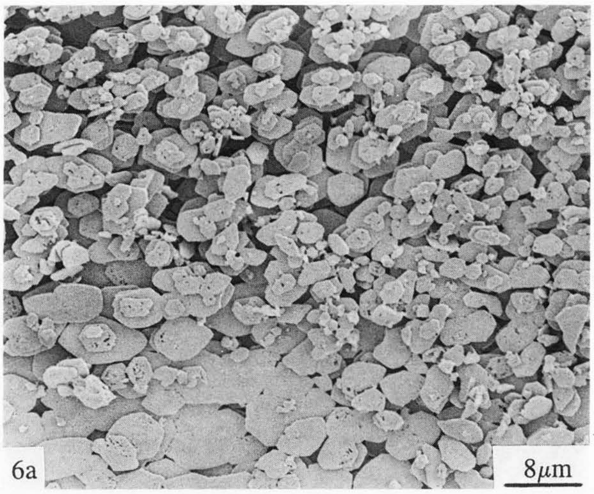


Figure 5. (**a**) Transmission electron micrograph of the nacre, showing some of the lines of contact between adjoining crystals in the same lamella, both those filled with organic matrix (M) and those without matrix (W). The continuity of the lamellae past these junctions is clear. Some connection between crystals in adjacent lamellae can be seen to occur through the organic films (arrows). (**b**, **c**, and **d**) SAD patterns of the labelled platelets showing similar single crystal diffraction patterns which indicate almost negligible crystallographic misorientation between the platelets.

same thickness at their adjacent edges, although they often varied in width elsewhere.

Selected area electron diffraction patterns of crystals, both within the same lamella and in adjoining lamellae, showed almost identical, single crystal patterns (Figs. 5b, 5c and 5d). This indicated that crystals in successive lamellae, and also those within a particular lamella, had their axes well aligned with each other. There was no obvious difference found between sections of the nacre cut parallel or perpendicular to the leading edge of the shell. The presence of a prismatic sub-structure in the platelet crystals of the nacre (as suggested by Mutvei [11]) was neither observed with TEM nor SEM.

SEM observations of the inside surface of the shell showed that the crystal platelets forming the nacre were flat polygons, usually hexagonal, up to 7 μm in length and about two thirds of this in breadth (Fig. 6a). Their width (about 0.4 μm) was the same as the width of the crystal lamellae. Conical towers of up to ten tiers in



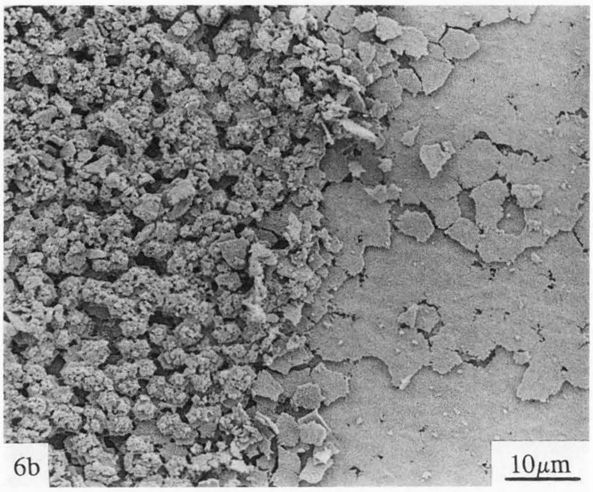


Figure 6. Scanning electron micrographs of the growth surface of the nacre in the body chamber in two similar regions of the shell (samples etched for 5.5 hours in NaOCl). (**a**) In one region, the crystal tablets which form on the nacre growth surface are nucleated on top of one another to form towers. Several layers are seen here to be forming at once. (**b**) The other region shows towered growth on the left of the field of view and terraced growth on the right.

height were created on the inner, growing, surface of the shell, by platelets forming one on top of another to produce the observed columnar structure (Fig. 6a). This was the most common configuration assumed by platelets on the inner surfaces of the *Nautilus* shell, however, there were areas of the inner shell surface where no towers were found. In these areas, a terrace effect was produced as each layer was almost completed before the next lamella started to cover it (Fig. 6b). There was no obvious pattern in the distribution of these two types of growth; the transition between them was abrupt (Fig. 6b) and the boundary did not form a smooth curve over the sample surface.

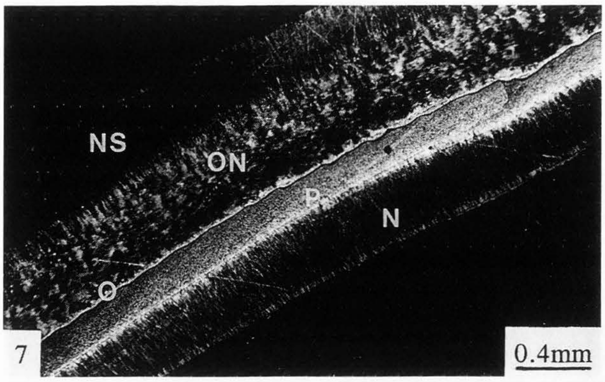
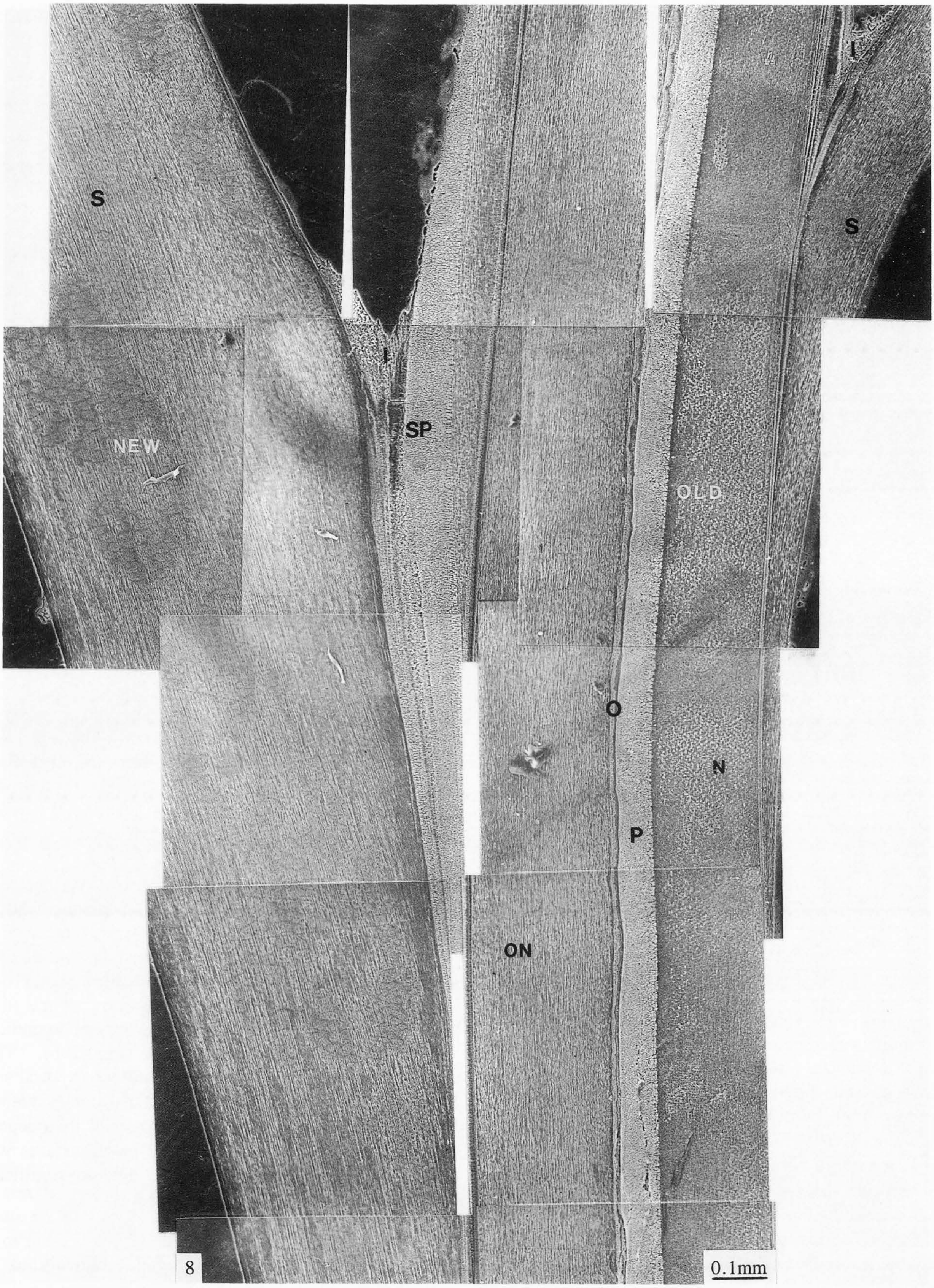


Figure 7. Light micrograph of a section, cut perpendicular to the aperture, of one of the internal shell walls where the shell has been overgrown by the most recent whorl. The layers visible, from bottom to top, are: the original nacre (N), the porcellaneous material (P), a dark organic layer (O), the overlaid nacre (ON) and a new, almost-separated septum (NS).

Figure 8 (on the facing page at right). SEM montage of the overgrown shell wall showing the various layers and septal branchings from both the concave or inner side (OLD) and the convex or outer side (NEW). A semi-prismatic layer (SP) exists between the septum (S) and the shell wall at the joint, but is distinct from the infilling (I). The three main layers of the shell wall are; the original nacre (N), the porcellaneous layer (P) and the overlaid nacre (ON) which is deposited as the shell spirals over itself, with a thin organic layer (O) which lies between the latter two of these. (Sample was etched 30 seconds in CH_3COOH and 6 hours in NaOCl.)

Chambers and septa

The two major layers of the shell, the porcellaneous and the nacreous, formed the basis for the entire shell but were the sole components only in the body chamber. In the other, evacuated chambers of the outermost whorl a thin, flaky coating of organic material was observed covering the inner surface (Fig. 1b). In addition, all whorls except the outermost showed further layers of material covering the originally external porcellaneous material. These extra layers originated near the point where the coil of the shell re-entered its own aperture. The first new layer was a thin coating of organic material which, in life, formed the black patch behind the animal's hood and over the umbilicus in the center of the spiral (Fig. 1a). A nacreous coating, which was a continuation of the umbilical callus, covered the black organic material just within the aperture of the shell. This new growth was thickened inside the shell to about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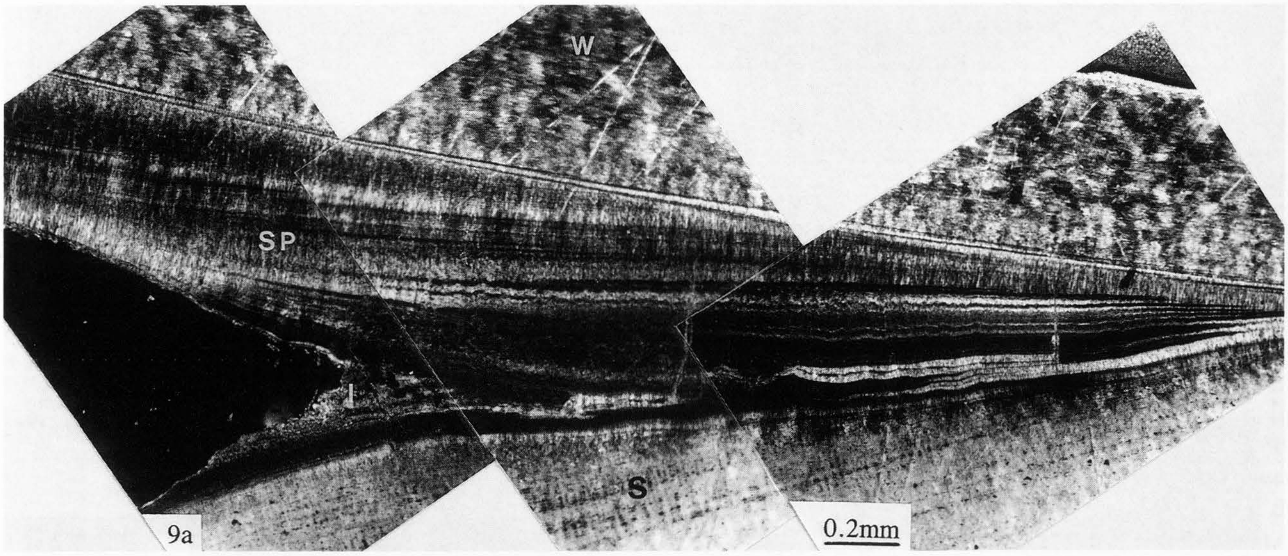

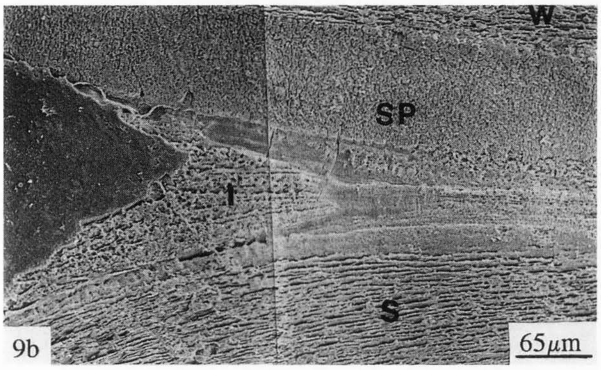

Figure 9. The infilling (I) and semi-prismatic material (SP) between the shell wall (W) and a septum (S) in two different sections. (**a**) Polarized light micrograph of section perpendicular to the branching showing the numerous layers of variable crystallinity which make up the infilling. (**b**) A scanning electron micrograph showing a branching with a layer of some semi-prismatic material.

the same thickness as the entire shell wall that it covered (Fig. 7). Thus, in cross-section, the shell wall of overgrown whorls showed an inner nacreous layer and a normal porcellaneous layer which were covered by a thin organic coating and a second nacreous layer. This second nacreous layer was significantly thicker than the original (Figs. 7 and 8). The outer shell wall of the outermost whorl possessed only the initial nacreous and porcellaneous layers.

Covering the internal shell walls in the regions where a septum branched away from the shell proper was a further distinct layer (Fig. 8). Between the nacre of the wall and the nacre of the septum, a thin strip of material existed which was just wide enough that the two structures could be distinguished with the SEM. This strip widened into a semi-prismatic layer of up to 0.1 mm thickness in the immediate vicinity of the septal branching and reduced back to a thin layer over the surface of the wall nacre on the far side where it was covered by the beginnings of the next septum. The semi-prismatic material was thicker at branchings where the septum curves outwards from the wall, rather than in towards the center of the shell (Fig. 8). On the inner side of the shell wall, the semi-prismatic material was not thicker near the septal branch and was not obvious as any more than the thin line separating the septal and shell wall nacreous layers. Apart from this structured material, the acute angle between shell and septum was also filled with a small quantity of disordered crystals which contained a high proportion of organic material (Fig. 9).

The septa themselves were almost entirely nacreous with a columnar structure similar to the main shell. They had a thin coating of semi-prismatic material on the convex, or dorsal, side which was identifiable by a bright fringe visible under polarized light (Fig. 10a). SEM showed the semi-prismatic material to be thin, about 90 μm in width, with prisms oriented perpendicular to the laminations of the nacre (Fig. 10b). This fringe was also observed on the concave or ventral side of some septa, but in the majority of samples viewed (which were taken from near the center of their septa), the organic pellicle which covered these surfaces was laid down directly onto the nacre and no significant semi-prismatic layer existed (Fig. 10c).

The siphunclar tube

The siphunclar tube, when cut obliquely to its length, produced a cross-section which revealed a disordered arrangement of crystals around the circumference

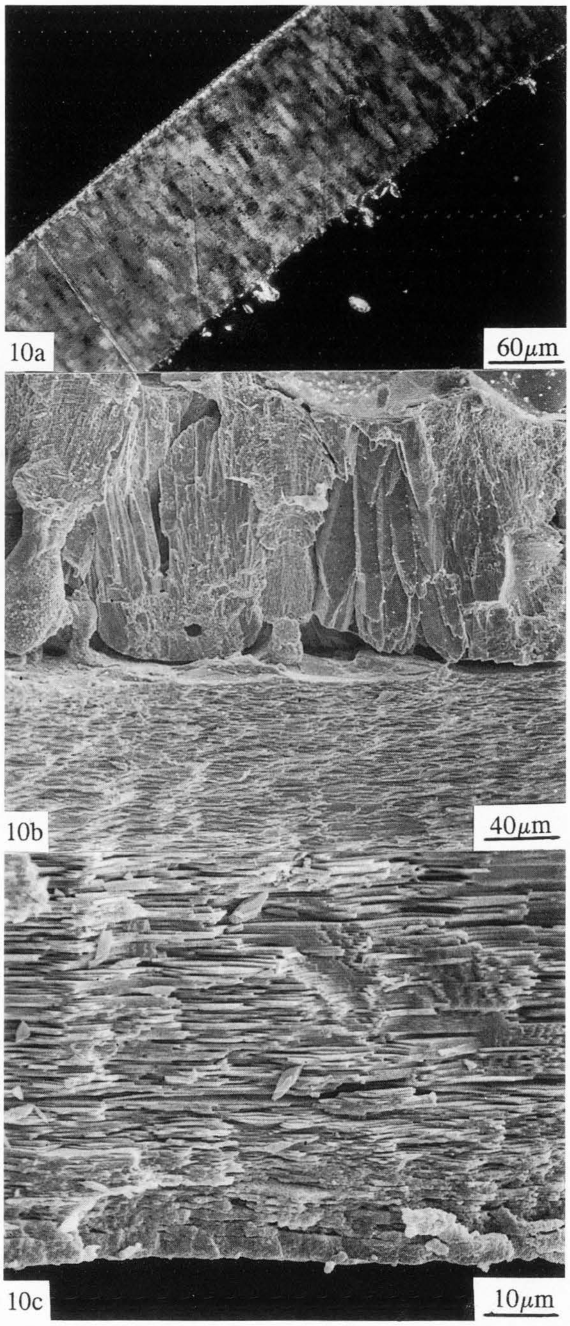

Figure 10. (**a**) Light micrograph, using polarized light, of a septum showing a prismatic appearance of the nacre caused by the columnar arrangement of the nacre tablets. The bright fringe on the upper side in this view is due to the overlying thin semi-prismatic layer. (**b** and **c**) Scanning electron micrographs of fracture surfaces of the convex (**b**) and concave (**c**) sides of a septum. In Figure **10b**, the semi-prismatic material, which is at the top of the field of view, overlies the lamellae of the nacre, and is about 90 μm thick; this layer does not appear to be continuous with the nacre. Figure **10c** shows that the nacre has become less coherent in the last 5 μm.

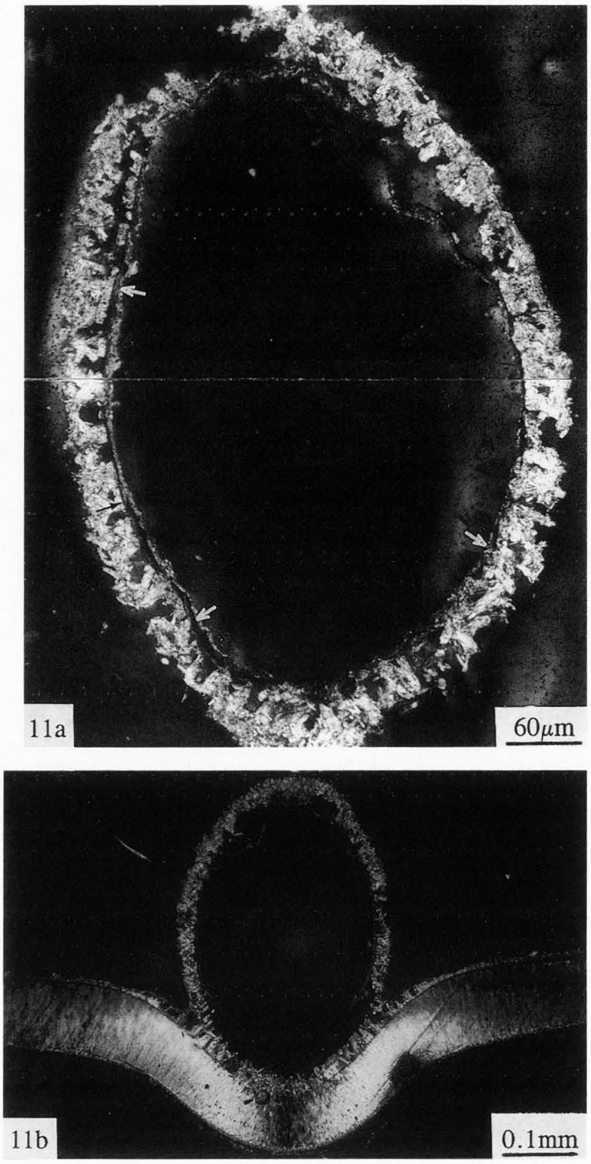

Figure 11. (**a**) Light micrograph of the siphunclar tube in cross-section showing the disordered arrangement of the crystals that surround it. A faint ring of organic material (arrows) is visible along the inner circumference of the tube. (**b**) Light micrograph of siphuncle near the point where it passes through a septum. The curvature of the nacreous crystals of the septum, in the region of the septal neck, is the cause of the intensity variations in the (polarized) transmitted light.

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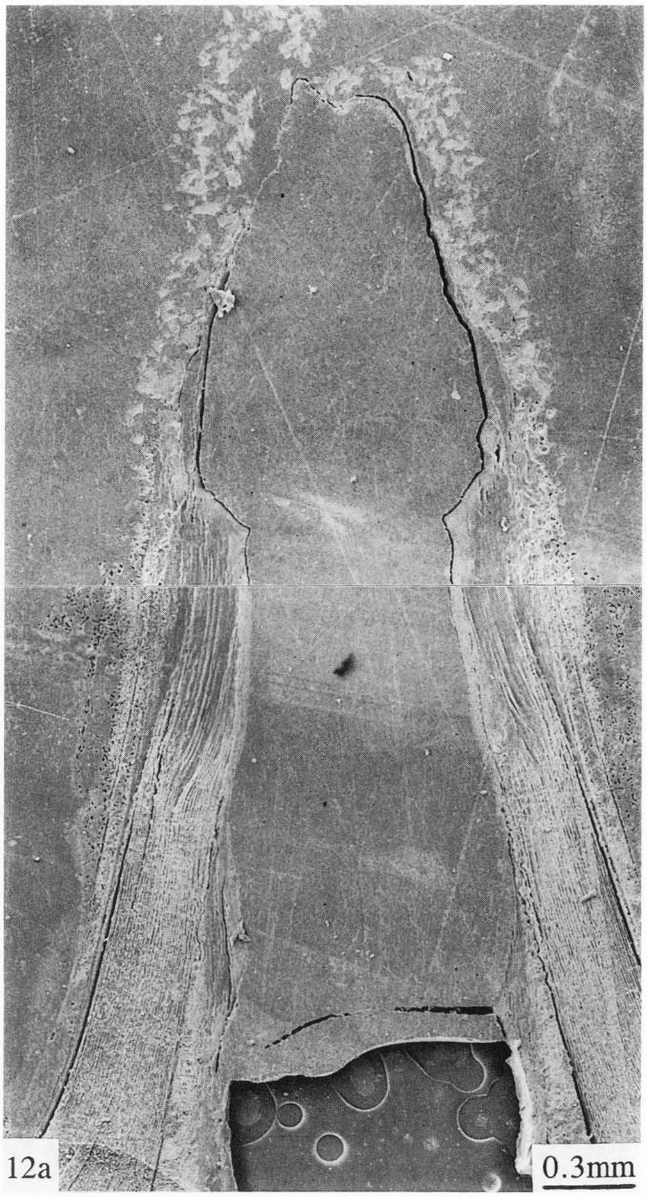
of the tube without any obvious pattern to their distribution (Fig. 11). The crystals were randomly oriented and very loosely packed, suggesting that the tube had a high porosity (there was no etching of the LM samples and so no part of the structure was removed in this manner). A faint dark line, possibly due to the horny



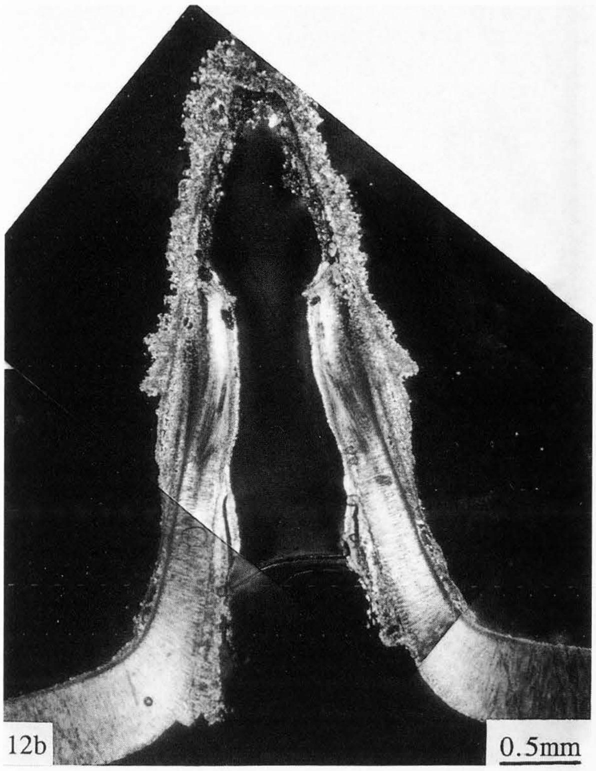


Figure 12. Micrographs of the siphunclar tube as it passes through a septum showing the septal neck thus created. In the middle of the septal neck, nacre curves up into the siphunclar tube and becomes more contaminated by organic material. The semi-prismatic crystals lining the tube extend down the sides of the septal neck past the termination of the nacre and almost to the main part of the septum. (**a**) SEM view of the same area (etched 30 seconds CH_3COOH and 6 hours NaOCl). (**b**) LM view of sample between crossed polarizers.

organic component of the tube, defined the inner limits of the tube and was clearly visible when viewed with normal light under the LM. The line was indistinct against the dark background when it was viewed between crossed polarizers, suggesting that the material was not birefringent, and thus, not crystalline (Fig. 11a).

The septal neck, formed in the center of each septum where the siphuncle passes through it, was created by curvature in the nacre toward the convex side of the septum (Fig. 11b). This curvature continued into the septal neck until, at the contact between tube and septum, the laminations were almost perpendicular to the septum proper (Fig. 12). The laminations of the nacre were less regular in this area and the darker appearance of the material when viewed between crossed polarizers (Fig. 12) suggests local distortions and strains, the most likely explanation being an increase in the amount of organic material. At the end of the septal neck, the nacre became even more distorted until it ceased altogether, creating the hole in the septum through which the siphunclar tube passed. The nacre did not merge with the semi-prismatic material of the siphunclar tube, but rather the latter fitted over the septal neck like a sleeve. The semi-prismatic material of the tube reached almost to the base of the septal neck, and the nacre forming the neck extended for about 1.7 mm into the tube. The crystals of the siphunclar tube were more tightly packed in this area, so that there was some alignment with the semi-prismatic layer coating the convex surface of the septum.

Hardness tests

Table 1. Vickers micro-hardness values for various structures in the *Nautilus* shell.

Material	Hardness
Nacre Surface	126 ± 7
Nacre Cross-section	166 ± 6
Porcellaneous Layer	310 ± 16
Porcellaneous Cross-section	246 ± 24
Septum Cross-section	166 ± 34
Aragonite (Mineral)	307 ± 10

Vickers micro-hardness tests on the shell showed that the porcellaneous material was far harder than the nacre, both in cross-section and on the surface (Table 1). This was also shown in a less rigorous way, to the extent that hardness can be correlated with erosion of the samples, by the reaction of the two materials to their treatment during the preparation of electron microscope samples. TEM samples prepared of the porcellaneous material consistently took over twice as long to create a suitable specimen in the argon-ion beam thinner as the nacre. Although the thickness of samples placed in the ion beam thinner was not closely monitored, it is unlikely that the former were always significantly thicker than the latter. The septum, being almost totally nacreous, was the same hardness as the nacre. The nacre was shown to be slightly harder in cross-section than on its surface, while the porcellaneous material was softer in cross-section. The comparison with the hardness of mineral aragonite shows that the porcellaneous material is as hard as the inorganically formed mineral. The mineral sample tested was polycrystalline and although the micro-hardness was taken of a single crystal region of this, it was not possible to determine the crystallographic plane on which the measurement was taken.

Discussion

The observations reported in this study confirmed that the porcellaneous and nacreous layers were the main structures of the shell walls and the septa. The porcellaneous outer surface of the shell with its granular structure was found to differ greatly from the nacreous inner surface with its more organized laminations of crystal platelets. Both SEM and TEM observations confirmed the ordered nature of the nacre and the disordered arrangement of crystals in the granular layer. However, the designation of the outer sub-layer of the porcellaneous material as "spherulitic" [9, 12, 27] was not supported by the ultrastructure of the region, since the crystals did not show any evidence of radiating growth. The rough alignment of the crystallites within irregular clusters and the random arrangement of these clusters suggested that the basic units of this layer were grains, and therefore, that this region could be better described as granular.

The extra layers which appear in the walls of the internal whorls of the shell have not previously been reported in the literature. These layers, in particular the added layer of nacre, add significantly to the thickness of these walls, commonly doubling the original thickness. It is unlikely that the purpose of this increase in thickness is to strengthen the structure since these are internal walls and, except in the body chamber, are not subject to the same pressure differentials as the external walls. It is possible that the extra material is a consequence of the nacre deposition occurring on the outer side of the body chamber with the entire mantle secreting on all surfaces indiscriminately. However, the control demonstrated by the animal over material deposition, for example, in the sudden shift in structure between the porcellaneous and nacreous layers, makes this unlikely. A more plausible explanation is that the extra mass that this material represents is useful to the animal as part of its buoyancy control mechanism. The mass may assist in balancing the increase in buoyancy of the shell when a chamber is emptied, if the deposition of material to the leading edge of the shell is insufficient. It may also be that this material represents a reservoir of calcium carbonate which can be resorbed and excreted as an aid to buoyancy control in the adult animal.

TEM did not reveal the acicular components of the platelets described by Mutvei [12] nor the four-part structure revealed in some bivalve nacre [13]. Although TEM micrographs showed that some substructure existed within the platelets, the appearance of this substructure suggested impurities and organic inclusions within the material. The possible presence of any parallel crystallites was not revealed by the micrographs in this study.

A thin semi-prismatic layer was found on the inner, convex surface of the septa and on the walls in the evacuated chambers but was not observed to cover the entire inner surface of the body chamber in the manner described by Meenakshi *et al.* [9] and Mutvei [11]. This may be due to the fact that the semi-prismatic material only extends to cover the full surface of the body chamber as part of the final steps toward maturity and none of the shells used in this study were fully mature.

There are two different views in the literature on the cause of the very regular thickness of the crystalline lamellae in nacre [6, 20, 26]. One view [29] holds that the organic matrix is deposited first, creating cells which are filled in by the crystals as they grow. This requires that there be no interference between the deposition of the organic and crystalline substances since the two materials are formed concurrently. The other view [25] is that either the crystals and conchiolin, usually the latter, are deposited periodically. Intermittent deposition of organic material would lead to the interruption of crystal growth and would cover the tops of half-formed platelets on the nacre growth surface.

Our observations have shown that the plane of contact between two adjacent crystals within a lamella occasionally contained organic material but in general was difficult to detect. In other words, there commonly was little or no organic material between the crystal platelets within a given lamella. Periodic deposition of organic material would mean that matrix which is laid over incomplete stacks in unfinished layers would collect in the spaces between the crystals, and thus, would create many and wider organic bridges between successive organic films. Also, if the organic material is periodically deposited but crystal deposition continues uninterrupted, then it would be expected that some crystalline material would be present in the organic films between successive lamellae. The TEM observations reported here have shown that, while there were some connections between the crystals in adjacent lamellae, in general there was very little crystalline material included in the organic films. Hence, our observations support the view that the organic matrix was pre-formed in layers and the crystals filled the gaps between the layers. This method of nacre formation would produce crystalline lamellae with relatively constant widths and films which are continuous over adjoining crystals; both have been observed in this study.

Previous reports describe the deposition of nacre in *Nautilus* only in the form of towers or stacks with crystals formed one on top of another on the growth surface [7, 8, 9, 14, 26, 27]. However, this study showed that, although towered growth may be the dominant method, a terraced form of growth, previously described as only occurring in bivalves [6, 13, 26], was also present in the *Nautilus*. The suddenness and irregularity of the transition between these two forms of growth suggests that there are only minor differences in the conditions of growth; any major differences would manifest with a more gradual change in the growth pattern. The influence of the organic matrix in this context cannot be ignored. The nearly identical orientation of the crystal platelets in successive lamellae, as shown by the SAD patterns, also suggest that the crystal growth was influenced by some consistent factor, which could be the previous crystalline lamellae or the organic conchiolin. The preformed matrix view provides an explanation for these observations. There are pores present in the matrix layer [8] which could provide a channel through which crystals can extend from one lamella to another, effectively resulting in the platelets forming epitaxially on top of one another. This would result in the formation of towers or stacks of crystals. In regions where the organic matrix was thicker than normal or contained fewer pores to provide for growth of crystals into the new lamella, the underlying lamella would be more complete before the new layer progresses very far. This would lead to the flat or terraced growth.

On the Vickers micro-hardness scale, the porcellaneous material of the outer surface of the shell was almost twice as hard as the nacre from which the bulk of the shell structure is formed; in fact, it almost reached the hardness of inorganically formed mineral aragonite. A greater resistance to sodium hypochlorite etching of the porcellaneous layer suggested less organic material in its composition, or, at least, that the organic material was attacked less by the etchant, and this was also compatible with a harder outer surface. The major differences between the granular material and the nacre is the smaller average size of the grains and the ordered arrangement of crystal platelets in the latter. The mechanical hardness of the porcellaneous layer is, as expected, due to the random polycrystalline nature of this material. However, the polycrystalline nature of the granular material would give it very little flexibility since the individual crystallites cannot easily move past each other. In the case of the nacre, each of the crystal lamellae is separated by an organic film which will absorb any applied stress, giving the material more strength and flexibility than the porcellaneous structure, albeit, at the cost of mechanical hardness.

A break in the shell is a situation to be avoided as much as possible because it would create a fairly major hazard to the *Nautilus*, especially considering the enormous pressure differential across the shell. The hard exterior, as indicated by the indentation hardness test used here, has a greater resistance to puncture and wear than the softer, but stronger, nacre. Thus, the part of the shell exposed to the harsh conditions of the external environment has a greater wear resistance than the inner surface, which is only exposed to the more sheltered conditions within the shell. Since the wear resistance is only required on the immediate surface of the shell, the hard porcellaneous layer need not be very thick. The nacre which makes up the major portion of the shell, with its brick wall structure, provides strength as well as a degree of flexibility. The strength of the nacre, along with the general shape of the shell, provides necessary resistance to the immense hydrostatic pressure (up to 80 atmospheres) found in the animal's natural environment. The laminated form of the nacreous structure with its lamellae of hard, strong crystal separated by a small amount of softer material, also provides an excellent crack-stopping mechanism. The major stresses on the shell are perpendicular to the nacre lamellae, so that if a crack started in one lamella, it would get diverted into the soft organic material between the lamellae, parallel to the stresses, and would not continue into the next lamella. Thus, the damage is localized.

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Discussion with Reviewers

A.C. Smillie: The authors have made the suggestion that the extra material (from layers in the walls of the internal whorls of the shell) might be a reservoir of calcium carbonate which can be resorbed and excreted as an aid to buoyancy control. How do the authors see the mineral being resorbed and excreted?

Authors: This paper merely attempts to point out the existence of these layers and does not intend to provide a full explanation; the possibilities given were just conjecture. It is understood that the control over the extrapallial fluid must be very good in order to dissolve the mineral instead of depositing it, not to mention some method of the animal removing these ions from the fluid into its bloodstream. The authors observations cannot determine what mechanism may be behind this process.

S.W. Wise: Coiling, as in the *Nautilus*, is thought to have eliminated the need for counter-weighting devices such as the cameral layers common in many straight-shelled nautiliods of the Paleozoic. Could the presence of extra layers you observed be more of a relic or vestigial structure from a previous adaptation no longer needed in this organism, rather than a necessary and functional part of this modern shell?

Authors: This is possible. However, it must be noted that, even if the coiling of the shell removes the need for such counter-weighting devices, these extra layers still exist and their weight must be balanced for the shell to maintain neutral buoyancy. It may be that the layers are no longer necessary but by their very existence they act as defacto ballast anyway and the chambers must be larger than they would otherwise be in order to counteract the extra weight.