Function of calcium phosphate renal concrements in extant *Nautilus*: a paradigm for Cambrian-relict short-term mineral reserve equivalent to vertebrate bone

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\textbf{A B S T R A C T}

Renal uroliths (concrements) of calcium phosphate have long been known to exist in both growing and mature (non-growing) *Nautilus* specimens, but to date no evidence-based explanation for their existence has been available. The currently favored speculation is that they function as a calcium reserve for shell and septal calcification. Here we present new observational and experimental data that are consistent with the hypothesis that they serve as a mineral/ionic reserve, allowing short-term (<1 day) addition of ionized calcium and phosphorus to blood and other body fluids, in a way analogous to that of vertebrate bone. In both in-ocean experiments and during long-term observation of captive nautiluses, concrements disappear during two different, energy-intensive activities involving removal of anions and cations from newly secreted cameral liquid in the chamber formation cycle, and during dives to depths requiring high osmotic pressures within the canaliculi of the siphuncular epithelium to keep previously emptied chambers from flooding due to suddenly increased ambient hydrostatic pressure. New concrements reappear at other points in the chamber formation cycle and when normal living depth is restored. The use of concrements as an ion reserve and the Cambrian ancestry of nautiloids indicate that *Nautilus* may exemplify a solution to the problem of energy supply in newly evolved swimmers of the Cambrian radiation independent of that seen in fish.

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1. Introduction

From early in the Paleozoic era until the modern day, vertebrates and cephalopods have remained the two dominant, larger, actively mobile carnivores in the sea, and as such have a long history of both competition and evolutionary convergence arising from the two groups sharing and partitioning the same adaptive zones and oceanic habitats (Packard, 1972; O’Dor and Webber, 1986; Chamberlain, 1990, 1991, 1993). Among the many recognized convergences are the streamlined shape, form and function of sensory organs, and high rates of blood flow through the body. In this paper we identify an additional, previously unrecognized convergence, one that may have arisen early in the histories of both fishes and cephalopods – the formation and use of mineral reserves where various mineral salts can be rapidly transferred from a solid state to dissolved ions.

In fishes, Ruben and Bennett (1980, 1981, 1987) proposed that the major structural material of the internal skeleton, bone, was first evolved not for skeletonization, but as a mineral reserve of phosphorus that, because it was calcium phosphate rather than calcium carbonate, would not be affected by the increases in extracellular fluid acidity that are produced by high metabolic activity, such as muscular swimming (Ruben and Bennett, 1980, 1981, 1987). An added advantage would have been biologically accessible phosphorus that would have improved metabolic output in these first swimming vertebrates (an energetically expensive new
lifestyle), especially in environments where biologically accessible phosphate was in low abundance. This pioneering hypothesis thus regarded calcium phosphate deposits not only as reserves of ionic calcium, but also as a physiologically important source of phosphorus, something that swimming animals emerging from the “Cambrian explosion” would have required (Marshall, 2006). Vertebrates still use a system in which daily breakdown of amorphous, inorganic calcium phosphate to ionized (activated) Ca\(^{2+}\) and (PO\(_4\))\(^{3-}\) produces ions that can be dissolved in blood or other extracellular fluids as the need arises, irrespective of blood or body fluid acidity (Ruben and Bennett, 1987 and references therein).

1.1. Renal concretions in Nautilus

While vertebrate bone is the most voluminous of the known mineral storage sites, analogous (but not homologous) volumetrically important mineral accumulations are also found in the two other extant members of the other two of three larger groups of swimmers of the later Cambrian explosion: marine arthropods and phragmocone-bearing cephalopods. Both CaCO\(_3\) and Ca\(_3\)(PO\(_4\))\(_2\) concretions are found in the gills and other body regions in larger, active crustaceans such as crabs and lobsters (Watabe, 1989), and in the renal complex of extant nautilid cephalopods (Keferstein, 1866; Willey, 1902). While the nautilid renal complex is completely different in evolutionary origin and ultimate anatomical position compared to all other motile animals, evidence presented here indicates that the renal concretion reserve in extant Nautilus (Schipp and Martin, 1981) is used in ways highly similar to the calcium phosphate reserve complex in fish (as well as other vertebrates). As we argue here, Nautilus exemplifies a typically Cambrian approach to the problem of energy production.

Renal concretions, or uroliths, of Nautilus consist of as much as 5 g of submillimeter-sized, concentric bodies (Fig. 1) of amorphous (non-crystalline) calcium phosphate and proteoglycan (McConnell and Ward, 1978), and lesser but varying amounts of magnesium oxalate occurring both as concentrically layered magnesium oxalate concretions and as outer magnesium oxalate coatings on calcium phosphate concretions (Crick et al., 1985) in the renal appendages of the renal system (Fig. 2). Concretions that are structurally and mineralogically similar to those found in the kidney complex in Nautilus also occur in the only other extant, phragmocone-bearing cephalopod genera, the sepiids (including Sepia and Sepiella) and Spirula (Schipp and von Boletzky, 1975). The unique association of concretions and phragmocones suggests a relationship to the neutral buoyancy organs in these cephalopods.

1.2. Hypotheses for concretion function

There is now detailed understanding of the sites of concretion formation and accumulation in the renal sacs of Nautilus (Schipp et al., 1985), and while concretions are known to occur in both immature and mature animals, to date there are no data on either their frequency of occurrence or abundance in different Nautilus growth stages. Most importantly, the function of such large volumes of mineralized concretions, chemically distinct from nautilid skeletal material (where the shell and septa are composed of crystalline aragonite (CaCO\(_3\)) interbedded in a conchiolin matrix), has remained enigmatic since their first discovery (Keferstein, 1866; Haller, 1895) more than 150 years ago – or even if they serve any function at all.

Three hypotheses have been proposed to explain the presence of such relatively large volumes of renal concretions in Nautilus. The first (the mineral waste hypothesis) is that they are simply waste products (Griffin, 1897; Willey, 1902; Ward and Wicksten, 1980), perhaps resulting from the large amount of calcium phosphate consumed in the largely crustacean diet of Nautilus (Martin, 1975). A prediction of this hypothesis is that concretion abundance would be tied to food abundance or kind (amount, if any, of phosphate-rich

Fig. 1. Concrements in the renal appendages of Nautilus pompilius. Morphologies vary from single, concentric spheres to chains. Scanning electron micrograph, magnification = 200×.

Fig. 2. (A) Radiograph and (B) tomographic image of two different Nautilus pompilius specimens, showing the siphuncle and positions of concretion-filled renal appendages when imaged from side and front (renal appendages photographically enhanced). The siphuncle appears in (A) as the white structure extending from the midpoint of the septa through each chamber. The siphuncle is composed of an outer concholin tubule, permeable to water (Collins and Minton, 1967; Chamberlain, 1978), inside of which is a lining of epithelial cells which houses the ion pump that removes fluid from the chambers to blood vessels at the center of the tube (Denton and Gilpin-Brown, 1966; Greenwald et al., 1980; Greenwald and Ward, 1987). In the side view (A) the upper two (dorsal) renal enclosures are distinguishable as a pair of partly overlapped white shadows but the images of the lower (ventral) ones overlap completely. The ventral renal appendages are resolved as an elongate mass using anterior imaging as in (B). For all concrement measurements in this paper, only the areas of the ventral pair of renal appendages were used. Abbreviations: R. part of the retractor muscles; CR, crop; S, stomach.

Tomographic image courtesy of B. Westermann, used with permission.
crustacean carapace) but not to cycles of shell or septal formation. The second view (the calcium reserve hypothesis) is that the animals keep a mineral reserve of calcium for exclusive use during shell and septal formation (Schipp and Martin, 1987; Ward, 1987). If correct, concrements should disappear during higher rates of shell formation, especially during the period of most rapid calcification, i.e., the formation of new septa. Finally, it has been proposed that the removal of magnesium from body fluids (where it is then precipitated in the renal complex as magnesium oxalate) increases the rate of shell and septal formation (Mann, 1992). This can be termed the calcification enhancement hypothesis. It should show just the opposite pattern to that of the calcium reserve hypothesis. Concrement volume should increase during shell and septal calcification, but concrements appearing during this interval should be dominantly magnesium oxalate, rather than calcium phosphate.

To this list we add a new hypothesis: that the renal appendages of extant Nautilus and Allonautilus, organs apparently evolved in the earliest phases of cephalopod phylogeny but still retained in these modern, yet anatomically primitive, shell-bearing cephalopods, serve not as a mineral storage for shell calcification, but as a reservoir explicitly evolved to hold calcium and phosphate in such a way that these ions can be rapidly moved into blood or pallial fluid during times of greater physiological work in a manner analogous to vertebrate bone, by providing physiologically labile Ca²⁺ and P⁴⁺ ions. In the nautiloids (and potentially ammonoids as well) this would occur during two different times of maximal ATP utilization, both involving active transport of Na⁺ and Cl⁻ ions against a high hydrostatic pressure gradient: during a short interval of the chamber formation cycle (Ward et al., 1981; Saunders and Ward, 1987) and during deep diving. We call this the phosphate storage hypothesis.

A prediction arising from the phosphate storage hypothesis is that calcium phosphate concrements would disappear during the time of greatest metabolic work. In immature animals (both captive and in the wild) at least, this would be the interval during which a newly formed chamber, totally filled with cameral fluid of seawater osmolarity, is emptied of this fluid by the siphuncular epithelium actively transporting salt ions out of the chamber and inducing an osmotic gradient across the siphuncular wall favorable to fluid removal as described by Denton and Gilpin-Brown (1966), Ward (1979), Greenwald et al. (1980), and Greenwald and Ward (1987). This is a crucial process because maintaining neutral buoyancy in growing (immature) individuals involves a delicate balance of overall density increase, brought about by enlarging the shell and tissue mass, with an equivalent density decrease due to the concomitant removal of cameral liquid from the chambers (Ward et al., 1981; Saunders and Ward, 1987). New chambers are originally filled with a body filtrate of seawater density, and this can only be removed via the siphuncle by active transport of Na⁺ and Cl⁻ ions out of the cameral fluid. Liquid can only be removed if the osmotic pressure created by the active transport exceeds ambient pressure as transmitted through the liquid-filled body of the animal, including the tissue within the siphuncular strand. It is in this energetically costly phase of the chamber formation cycle that concrement ions may become a crucial resource for immature animals.

A second, analogous prediction of the phosphate reserve hypothesis is that concrements would also be utilized during dives greater than about 300 m, the deepest depths that nautiluses have experimentally been observed to remove cameral liquid against ambient pressure (Ward et al., 1981; Saunders and Ward, 1987). To prevent flooding of chambers during dives greater than this, mobilization of concrement ions would be needed to help drive the siphuncular osmotic pump at the high rates needed to oppose the high hydrostatic pressure at these depths. This would mean that concrements should disappear during deep dives.

2. Materials and methods

Concrements occur in four liquid-filled lumens in the kidney complex. These lumens contain many thousands to perhaps hundreds of thousands of individual particles (Fig. 1). When animals are X-rayed from the side these concrement-filled lumens are observed radiometrically as a white area within the body chamber (Fig. 2). Almost always, as in the case illustrated in Fig. 2, the four concrement-filled lumens appear as upper and lower composites, rather than as four separate masses, because the lumens in each pair at least partly, and often nearly completely, overlap in the line of sight of the X-rays. Generally, the lower composite reservoir is larger, contains a higher volume of concrements, and is more readily distinguished in X-rays. For these reasons, we used the area of the side view of the lower reservoir as an estimator of overall concrement volume. This strategy is enhanced by the observation that where both upper and lower reservoir images were readily traceable in a radiographic time-series of the same animal, change in the upper reservoir always tracked change in the lower one.

To test the hypotheses outlined above we performed three separate experiments.

2.1. Concrements in freshly caught animals

Thirty-two individuals from four populations of Nautilus (N. macromphalus, New Caledonia; N. belauensis, Palau; N. pompilius, Great Barrier Reef, Australia, and N. pompilius, Bohol Island, Philippine Islands) were caught in overnight box traps lowered to normal Nautilus living depths. The animals were radiographed within a few hours of capture. Following radiography the animals were marked with identifying labels and released. Concrement abundance was assessed as described above.

2.2. Monitoring concrement abundance during growth and chamber formation

We tracked both size increase of the body chamber (shell growth at the aperture as well as soft tissue growth) and phragmocone growth (new septal and siphuncular formation and cameral liquid removal) using daily to weekly radiography of laboratory aquarium maintained specimens of Nautilus pompilius that were fed at a constant rate. This methodology was adapted from our earlier radiographic work on Nautilus (Ward and Chamberlain, 1983) because it allowed quantitative estimation of concrement abundance during various stages of the chamber formation cycle.

Four immature Nautilus pompilius were obtained from the Philippine Islands and were each observed for over a year, with weekly to bi-weekly radiography. From the radiographs, we computed rates of apertural shell growth, septal shell growth, concrement abundance, and shell and septal thickness. Ratios of shell material being produced in the body chamber (including aperture) compared to the newly forming chamber were obtained by dissecting and weighing shells similar to those of the test animals.

2.3. Deep-water testing of mature animals

We used deep-water (high-pressure) experimentation to test for concrement and cameral liquid volume change under various pressure conditions. In-water experiments were conducted off Panganbangan, Bohol Island, Philippine Islands, and Pago-Pago, American Samoa. For these experiments, freshly caught mature nautiluses were removed from overnight traps, transported to land in cooled seawater (<20 °C) where they were radiographed and then replaced in the sea in closed cages attached by a line to a buoy on the sea surface. Time spent at the surface was no more than 3 h between capture and return to the sea. Three different experiments were
conducted: four Nautilus were held at 500 m for 6 days; four at 250 m for 2 days, and two at 25 m for 2 days. No specimens were used in more than a single test. Depths were ascertained by echo sounding (Furuno FCV-585 deep-water transducer, 1 kW power; depth error less than 1% of recorded value; Furuno Electric Co., Nishinomiya, Japan). Depths were verified upon retrieval of the traps to ensure that movement during the experiments had not taken place. Upon retrieval, the specimens were returned to land, re-radiographed, marked, and then taken back to sea and released at their original point of capture. No nautiluses were killed in these experiments, and one marked experimental animal was subsequently recaptured.

3. Results

Expressed in terms of area of lower concrement mass relative to area of body chamber, the average relative abundances of concrements in mature and immature specimens radiographically imaged in this study are 0.0095 and 0.0030, respectively (a ratio of 0.01 is equivalent to a concrement mass of 1 g). However, there was a strikingly wide range in concrement abundance among the animals studied. Mature animals showed a range from nearly 0 to a maximum value of 0.0370 (i.e., nearly 4 g of concrements), while immatures ranged from 0 to 0.0240. In addition, matures showed fewer individuals with few or no concrements. These observations, particularly the wide range in concrement abundance, are explained by the behaviors discussed below.

3.1. Concrement abundance through the chamber formation cycle in immatures

The area of the lower concrement mass in three different, captive nautiluses maintained in surface aquaria and radiographed at 1–2 week intervals for at least 300 days each is shown in Fig. 3. The striking result demonstrated here is the regular appearance and disappearance of concrements during different phases of the chamber formation cycle. An additional juvenile was radiographed at more frequent intervals; these results are shown in Fig. 4. Nautiluses held in surface aquaria and not radiographed were used as controls; there was no difference in overall growth rate, indicating that the frequent radiography had no observable effects on growth rate.

The rate and timing of concrement appearances and disappearances during the various phases of the chamber formation cycle allowed testing of the concrement function hypotheses. In all four of the long-term N. pompilius specimens, concrement abundance varied significantly over time and was tightly correlated to specific stages of the chamber formation cycle as shown in Figs. 3 and 4. These results indicate that concrements are typically near their minimal numbers or are completely absent during forward movement of the mantle, the time when fluid is excreted into the developing new chamber through the septal mantle, or newly growing sipuncular strand (or both). Further, concrement abundance is still low as the new septum begins to calcify. As septal calcification proceeds, and especially with the first appearance of gas in the camera, concrement abundance rapidly rises, even though septal calcification is taking place. That this occurs while the septum is being calcified is inconsistent with the calcification reserve hypothesis. Concrement abundance remains high until the decoupled phase of cameral liquid removal, and then dramatically declines as concrements rapidly (but steadily, rather than suddenly) disappear. It would appear from these observations that concrement abundance is low and then increases during septal calcification, and that it decreases primarily during decoupled fluid removal.

Fig. 4 provides additional data on concrement variation during the chamber formation cycle. It is evident from the middle panel of Fig. 4 that apertural calcification continues at a constant rate independent of the phases of chamber formation, and thus is unaffected by the periodicity of concrement abundance, septal calcification, cameral fluid secretion, or cameral fluid removal. The lower panel in Fig. 4 shows the change in cameral liquid volume in the last two chambers of the shell. The inception of the plot for the last-formed chamber at about day 55 corresponds to the filling of this chamber with fluid, and the initial calcification of the last-formed septum. We can see that the penultimate chamber was being emptied of fluid by decoupled pumping during the entire time that the new chamber was forming. These findings are consistent with predictions of the phosphate reserve hypothesis, but not the others.
3.2. Concrement abundance in nautiluses during deep dives

A further prediction of the phosphate reserve hypothesis concerns concrement abundance at depths greater than those (300 m) where osmotic cameral liquid emptying has been observed to take place. The deep-water testing protocol described above provides data relevant to interpreting this issue.

All test animals used in this experiment had concrements at the start of the experiments. This was true for the 6 specimens held at 500 m depth; the 4 specimens held at 250 m; and the 2 specimens held at 25 m. There was no observable change in concrement abundance or cameral liquid levels in the nautiluses held at 25 m or 300 m. However, results for the four nautiluses maintained at 500 m were strikingly different. All four of the specimens showed a reduction of concrements observable by radiography (Fig. 5), with three of the animals showing a complete loss of concrements in these six-day experiments. These three animals had no observable chamber flooding at the conclusion of the experiment. The fourth animal, however, whose concrements had not completely disappeared, returned to the surface with more cameral liquid in its chambers than it had when it went down.

4. Discussion

The results reported here demonstrate that concrement abundance is intimately related to the metabolically costly process of active transport by the siphuncular epithelium, both during new chamber formation as well as during dives to depths of more than 300 m. Prevention of cameral flooding below 300 m and removal of cameral fluid during the chamber formation process are tasks requiring considerable outlays of metabolic energy. The flooding problem has been little appreciated. We believe, in fact, that much of the morphologies in both hard and soft parts in the phragmocone are adaptations to inhibit the refilling of emptied chambers.
Just as about 10 g of shell loss causes ascent, as little as 10 ml of refilled cameral liquid sinks a nautilus (Ward, 1987). Uncompensated refilling is probably a common danger in the day-to-day life of a deep-diving nautilus, and we propose here that these episodes require large, but short-term energy expenditures made possible by the renal reservoir system.

Fluid transport out of a chamber is controlled by the action of the ion pump housed in the epithelial cells of the siphuncle. To remove cameral fluid, Na\(^+\) and Cl\(^-\) ions contained in the fluid, which initially has an osmolarity isotonic to both nautilus blood and seawater, must be removed by the ion pump to start the flow of water out of the new chamber and into the blood stream via the siphuncle. That same process prevents re-filling of a chamber. Because the siphuncular tube is not plugged anywhere along its length, re-filling of all chambers in the phragmocone is subject to the effects of ambient pressure. Preventing re-flooding and maintaining buoyancy mean that the epithelial cells of the entire siphuncular strand, from first to last chamber, require a continuous supply of metabolic energy to run the ion pump, especially when the cells have to work harder as ambient hydrostatic pressure increases during a dive. Thus, renal concrements would seem to represent a crucial phosphate reservoir on which an animal can repeatedly rely in times of metabolic stress.

Modern animals typically enhance their mitochondrial energy output by increasing the rate of ATP–ADP cycling via increased rates of oxygen consumption. But, many vertebrates can temporarily augment cellular stocks of ATP by mobilizing bone phosphate. We advocate that _Nautilus_ employs a similar strategy: one relying not on its carbonate skeleton, but rather on its phosphatic concrements. In view of the low oxygen physiology of _Nautilus_ (O’Dor et al., 1990; Wells et al., 1992), this strategy may be particularly important to the overall energy budget of _Nautilus_. We suggest that _Nautilus_ is a living fossil in more than the Paleozoic design of its skeleton; it is also a living fossil in terms of its Paleozoic energy production system.

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