Reports

Transformation of Amorphous Calcium Phosphate to Crystalline Dahllite in the Radular Teeth of Chitons

Abstract. *A comparison of infrared spectra from individual teeth along the radula of a chiton (Polyplacophora, Mollusca) shows that the first-formed calcium phosphate mineral is amorphous. Over a period of weeks the mineral transforms to dahllite. The* c *axes of the dahllite crystals are aligned approximately perpendicular to the tooth surface.*

To understand the processes by which organisms form minerals, the first-formed mineral deposit must be identified and characterized. In some cases the mature mineral may well be identical to the initial precipitate, but this is not always so (*1*). One example is the formation of magnetite in the teeth of polyplacophoran mollusks (*2*), where the first-formed mineral is ferrihydrite. In other cases, exemplified by the calcium phosphate minerals commonly found in vertebrate skeletons and teeth, the mature mineral and the initial phase are reportedly different (*3*). However, because of the transient nature of this process it has not been possible to unequivocally isolate and characterize these first-formed precipitates.

The problem is partially resolved in some continuously growing mineralized tissues where the initially formed minerals are well separated in space and time from the subsequently formed deposits. Among the best studied examples of this kind are the radulae of some polyplacophoran mollusks. In species of the family Chitonidae the teeth are mineralized by magnetite ($FeO{\cdot}Fe_2O_3$), with its ferrihydrite ($5Fe_2O_3{\cdot}9H_2O$) precursor, lepidocrocite (γ-FeO(OH)), and a carbonate apatite mineral (*2*). In the case where the carbonate apatite mineral is dahllite ($Ca_{10}(PO_4,CO_3)_6(OH)_2$), we show that the first-formed mineral phase of the dahllite is amorphous calcium phosphate (ACP) using fourier transform infrared spectroscopy. Furthermore, the transformation to a crystalline product occurs over a period of weeks, and the newly formed crystals have their *c* axes aligned approximately perpendicular to the surface of the tooth.

In the radula of the chiton *Acanthopleura haddoni* (Windworth, 1927), the teeth of the first 18 rows are composed of a mineral-free organic structural framework (the organic matrix). The major organic constituents of the *A. haddoni* teeth are assumed to be those commonly found in all mollusk radulae, namely proteins and α-chitin (*4*). The x-ray diffraction patterns of the first five rows of teeth become progressively sharper, indicating that the α-chitin polymers assemble into well-aligned structures (*5*). The first mineral deposited is ferrihydrite. The tooth row in which sufficient amounts of this mineral occur to give it an intense ochre color, is for convenience called row 0. By row 4 the ferrihydrite has converted en masse to magnetite. The lepidocrocite starts to form in row 8. The final stage of tooth mineralization that we examined in detail involves the infilling of the remaining part of the tooth with dahllite and its precursor stage. The three mature minerals in the tooth are located in discrete microarchitectural units and are all intimately associated with an organic matrix framework. We do not know the rate of formation of new tooth rows for this species. In other chitons the rate varies between half to two rows per day (*6*).

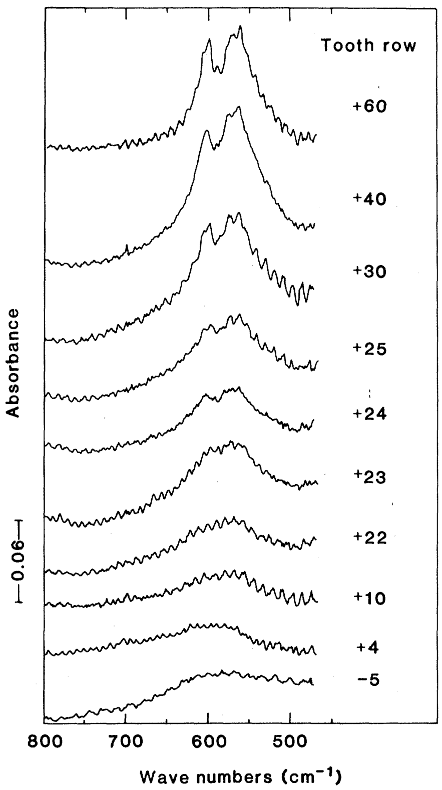

Fig. 1. Infrared spectra of individual teeth in the region between 800 and 475 cm^{-1}. Row 0 is defined as the first tooth row that has an intense ochre color due to the presence of ferrihydrite.

Radulae were dissected from frozen chiton specimens collected in Elat, Gulf of Aqaba. After extraction, the radulae were washed with distilled water and maintained wet while individual teeth were dissected for analysis. The average weight of a tooth is 35 μg. It is not possible to pool teeth from different radulae as the tooth developmental processes are not always synchronous. The infrared spectra were each obtained from an individual tooth after the stylus had been removed as close to the tooth base as possible (*7*). For scanning electron microscopy (SEM) and x-ray dispersive analysis, the corresponding teeth from the opposing row were used (*8*).

The infrared spectra consist primarily of absorptions due to the organic matrix, the stylus remnant, and the dahllite. The iron oxide minerals do not have any significant absorption bands in the normal infrared spectral range. The region between 800 and 475 cm^{-1} contains essentially no absorptions due to the organic constituents but does include an important absorption band of the dahllite. This is the band attributed to the triply degenerate antisymmetric bending mode at about 550 to 600 cm^{-1}. In ACP this absorption appears as a single broad band, whereas in more crystalline material this degenerate mode is split into bands of unequal intensity. Thus, the extent of splitting provides an approximation of the degree of crystallinity of the calcium phosphate mineral (*9*).

We measured the infrared spectra of 20 individual teeth from one radula. Figure 1 shows a portion of each of ten spectra between 800 and 475 cm^{-1}; these show the key changes in this calcium phosphate absorption band with increasing row number. The tooth from row 5 consists only of organic matrix and shows no appreciable absorption in this region. The tooth from row 4 contains organic matrix, magnetite, and a small amount of ferrihydrite. It too shows no appreciable absorption in this region. The first indication on the infrared spectrum of the presence of a calcium phosphate mineral is in row 10, although this

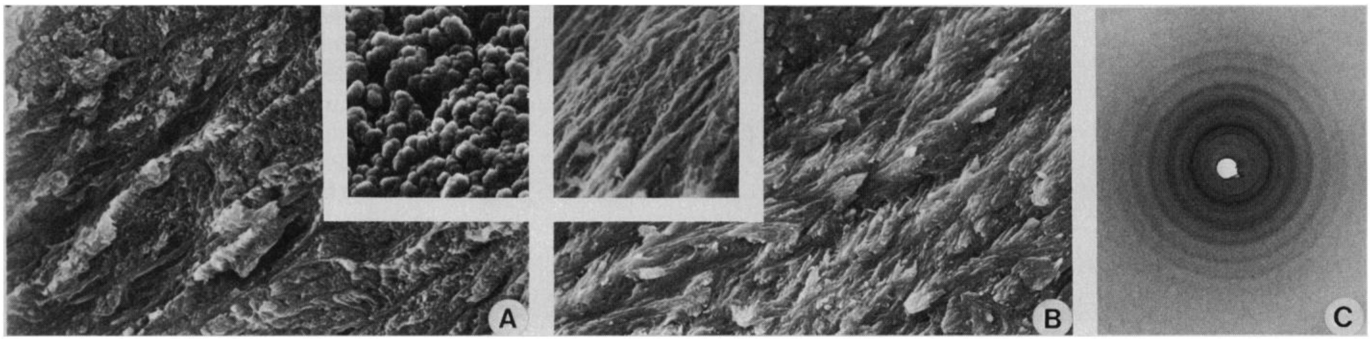

Fig. 2. (A) Amorphous calcium phosphate (ACP) fabric in a partially mineralized tooth (×9,500). Inset shows spherical shape of the ACP components (×25,500). (B) Dahllite crystal fabric in mature tooth (×6,000). Inset illustrates lamellar aggregation fabric of crystals (×10,000). (C) The x-ray diffraction patterns of the tooth from row 52. The inner oriented arc is the 002 reflection of dahllite. The magnetite reflections show no indication of having a preferred orientation.

may be due to the presence of small amounts of mineral in the stylus remnants included in the infrared analyses (*10*). The first unequivocal identification of a calcium phosphate mineral based on x-ray dispersive analysis is in row 19, and it probably begins to accumulate by row 17 (*11*). All the infrared spectra between rows 10 and 22 show no evidence of splitting and hence by this criterion, are composed of ACP. The first indication of splitting occurs in row 23 and is clearly observable in row 24. The degree of splitting of this absorption band increases progressively right through to the most mature tooth in row 60. The teeth in rows 61 to 66 show evidence of abrasion and hence must have been engaged in substrate scraping for food. Thus the initially formed ACP crystallizes over a period of a few weeks to a few months depending on the rate of tooth formation in this species. A comparison of the degree of splitting of the absorption band from the tooth in row 60 with hydroxyapatite from mature rat tooth enamel suggests that the chiton tooth dahllite is not as crystalline as the mature enamel.

An ultrastructural examination of the ACP-dahllite transition by SEM shows that the ACP consists of spherical to near-spherical bodies (Fig. 2A) characteristic of many amorphous minerals. The spherules are irregular and have a wide range of sizes (approximately 70 to 150 nm). Some of the larger ones are probably composed of clusters of three or more fused spherules. The more mature teeth have a thin layer of spherules on the growing surface. The internal portions, however, are more massive and the spherular bodies are apparently replaced by platy crystal aggregates (Fig. 2B). The crystals appear to be needle shaped. There is also a prominent lamellar lineation parallel to the median line of the tooth and approximately perpendicular to its surface. This is primarily due to the organic matrix. In specimens that were fixed, etched, and dried at the critical point, smooth ridges can be seen that are part of the matrix framework. Inspection of the mineral phase itself also shows a weak lineation in the same direction. X-ray diffraction of a mature tooth from row 52 clearly shows that the dahllite crystals have their *c* axes aligned (±40°) in a preferred direction parallel to the median line of the tooth (Fig. 2C) (*12*).

We have identified the nature of the initial precipitate of a biologically formed calcium phosphate mineral; in addition, we have also shown that an amorphous mineral converts to a crystalline phase in which the crystals are oriented in a specific manner relative to the overall ultrastructure of the mineralized tissue. Clearly this sequence of mineral formation events involves parameters over and above those that are responsible for the nonbiologic transformation of amorphous minerals to crystalline ones. Because of the relation between the crystallite orientations and the tooth ultrastructure, it is likely that the crystallization process is mediated somehow by the organic matrix. It is not known whether this is a solid-solid transition or a dissolution-recrystallization process, although in vitro ACP transforms to hydroxyapatite by the latter (*13*). One scenario for the organic matrix involvement is that during the time of ACP formation, calcium or phosphate ions, or both, also bind to the matrix. This relatively ordered matrix surface then acts as the site from which the maturation process begins. A second possibility is that the matrix macromolecules bind to the surface of the formed ACP granules and induce locally ordered areas which then propagate through the structure.

A number of invertebrates are known to form stable ACP as part of their mature mineralized hard parts (*14*). It is possible that these animals have deliberately stabilized this thermodynamically unstable phase to prevent it from crystallizing (*15*). Relatively few invertebrates actually form crystalline calcium phosphate minerals (*14*). In the vertebrates on the other hand, crystalline carbonate apatite is common, and is known to mature with age (*3*). The first-formed deposits in the vertebrate tissues have yet to be identified unequivocally and to date a number of different mineral types have been proposed, including ACP (*16*).

The transformation of ACP to dahllite occurs in a biological mineralization process. Information of this type is essential for understanding crystal growth in organisms and in particular the roles of the organic matrix macromolecules that regulate these processes.

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5. Single teeth were mounted in a Norelco Chesley microcamera and irradiated with nickel-filtered copper radiation. The patterns confirm the presence of α-chitin but show no indication of ordered protein being present.
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7. The tooth was dried for less than a minute under a heat lamp, crushed, and then mixed with 7 mg of KBr (Merk, Darmstadt). Pellets 3 mm in diameter were pressed and then examined in a Nicolet MX-10 fourier infrared spectrometer. The KBr spectrum was not subtracted from the sample spectrum.
8. For SEM and x-ray dispersive analysis, the organic constituents of the teeth were removed by oxidation in 5.5 percent (by volume) sodium

hypochlorite solution for 24 hours. After thoroughly washing with distilled water, the teeth were mounted on a platform and coated with gold palladium for SEM observation or carbon for x-ray dispersion analysis. For observations of the organic components by SEM, the teeth were fixed and etched according to the method of M. A. Crenshaw and H. Ristedt [in *The Mechanisms of Mineralization in the Invertebrates and Plants*, N. Watabe and K. M. Wilbur, Eds. (Univ. of South Carolina Press, Columbia, 1976), p. 355.

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10. The x-ray diffraction studies, infrared spectra, and electron microprobe analysis of tooth styli from other species of Chitonidae showed that ACP is present and when heated to 500°C converts to dahllite.
11. Combined SEM and x-ray dispersive analyses show that infilling by ACP of the phosphatic subunit of the tooth caps occurs from the periphery toward the center.
12. The x-ray diffraction patterns also confirm an earlier observation that the magnetite crystals show no preferred orientation [K. M. Towe and H. A. Lowenstam, *J. Ultrastruct. Res.* **17**, 1 (1967)].
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17. We thank J. Erez, Marine Laboratory, Hebrew University, Elat, for the chiton samples and H. Hüttemann, Tübingen University, for x-ray dispersion data and one of the SEM micrographs. This work was carried out while H.A.L. was a visiting fellow at the Weizmann Institute. Supported by U.S.–Israel Binational Science Foundation and Minerva Foundation grants to S.W.

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Amazon Rain-Forest Fires

Abstract. *Charcoal is common in the soils of mature rain forests within 75 kilometers of San Carlos de Rio Negro in the north central Amazon Basin. Carbon-14 dates of soil charcoal from this region indicate that numerous fires have occurred since the mid-Holocene epoch. Charcoal is most common in tierra firme forest Oxisols and Ultisols and less common in caatinga and igapo forest soils. Climatic changes or human activities, or both, have caused rain-forest fires.*

Natural and human-caused fires have altered the Holocene vegetation of North America (*1*). In this report we demonstrate that South American lowland rain forests also have experienced fires within the past 6000 years. Moderate-level disturbance has been proposed as a mechanism for the maintenance of high diversity in tropical forests (*2*). We propose that fire be considered a moderate-level disturbance for tropical rain forests when it occurs repeatedly, infrequently, and at low intensity.

Currently, forest fires occur widely in some areas of the tropics (*3*). In the Neotropics, it is grassland and savanna areas with lengthy dry seasons that burn most often. Lowland rain forest has generally been thought to be immune to burning (*4*). Although the fire history of the region has not, to our knowledge, been investigated, charcoal has been found in the soils of several South American rain-forest sites (*5*). Because charcoal has been found in association with ceramic artifacts, its presence is frequently attributed to human occupation.

We present evidence here that mature rain forests of the upper Rio Negro region of Venezuela have been repeatedly disturbed by fire during the last six millennia. Evidence of mid- to late-Holocene fires occurs not only in areas of known human settlement but also in the soils of several types of primary rain forest. We know of no previous effort to quantify or date Amazon charcoal from primary rain forests that have no associated artifactual evidence of human occupancy. Earlier studies have been confined to sites where the presence of ceramic pieces or "terra preta" Anthrosols, or both, confirmed human occupation (*6*).

The upper Rio Negro region of Venezuela (Fig. 1) is a relatively wet portion of the Amazon Basin. At San Carlos de Rio Negro (1°56′N, 67°03′W), the mean annual precipitation is 3530 mm. An average of more than 200 mm of rain falls in all months of the year. Of the three generally recognized forest types (*7*) (Fig. 2), only the tierra firme forests (which cover about 30 percent of the land area) are cut and burned for agricultural purposes (*8*). Disturbance of the forest by shifting cultivators is currently small in scale and largely restricted to the area bordering major rivers where the very sparse (±0.05 person per square kilometer) human population is concentrated. Because of the oligotrophic nature of the region's soils and the scarcity of faunal resources, human settlement was presumably very low (by Amazon standards) in pre-Columbian periods (*9*).

Near San Carlos (within 20 km) we sampled at eight mature tierra firme forest sites to a depth of 1 m with a total of 27 0.25-m^2 pits and 32 cores 8 cm in diameter (Fig. 1). At three mature caatinga forest sites near San Carlos we sampled with a total of 13 pits. We found charcoal in seven of eight tierra firme sites and one of three caatinga forest sites.

In areas more distant from San Carlos,

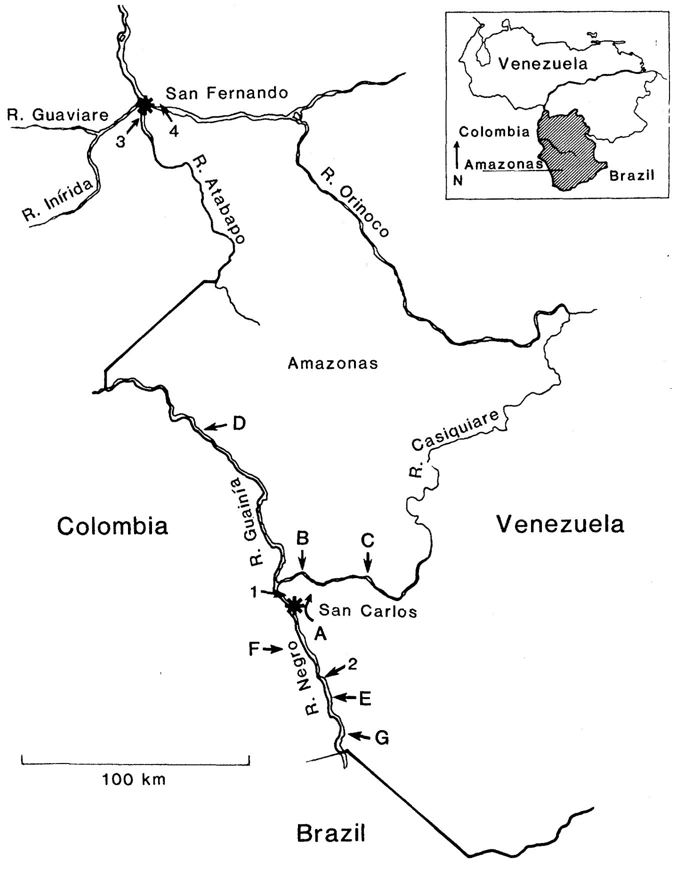

Fig. 1. Regional map and sample locations: A, tierra firme and caatinga forests within 20 km of San Carlos; B, Anthrosols along the lower Casiquiare River; C, 6-km transect near Guispero; D, 3-km forest transect near Galito; E, 10.5-km forest transect near Guanabana; F, tierra firme forests along Cano Mayabo, within 20 km of San Carlos; G, 7-km forest transect near Cocuy; 1, wildfire in igapo forest near Guarinuma, January 1982; 2, wildfire in tierra firme forest near Manare, 1983; 3 and 4, wildfires in several mature forests near San Fernando, 1983.