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Amorphous calcium phosphate in the stylets produced by a marine worm (Nemertea)

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Summary. Electron microprobe analyses of the calcified stylets produced by the nemertean worm *Amphiporus formidabilis* reveal large calcium and phosphorus peaks. IR spectroscopy and X-ray diffraction indicate that the calcium and phosphorus in stylets constitute an amorphous calcium phosphate rather than a crystalline mineral phase.
Key words. Amorphous calcium phosphate; calcification; nemerteans; Rhynchocoela; *Amphiporus formidabilis*.

Comparative studies conducted over the past two decades have greatly expanded our knowledge of the various minerals that are produced by organisms[3-6]. Biologically derived calcium phosphates, for example, had been previously considered almost exclusive products of vertebrates, but now it is known that many marine invertebrates form structures containing calcium phosphate[3]. Although crystalline types of calcium phosphate occasionally occur in invertebrate skeletal elements, the crystallographically amorphous form of this mineral appears to be more widespread[3].

As part of an ongoing effort to document the diversity of biologically produced minerals, we have examined the calcified stylets of a marine worm belonging to the phylum Nemertea[7]. Nemertean stylets are nail-shaped stabbing devices that are used in prey capture (figs 1 and 2). Each fully formed stylet measures 50–300 µm long and consists of a relatively thin organic core that is surrounded by an inorganic cortex[8-11]. Preliminary observations reported in the form of an abstract indicate that the inorganic fraction of stylets contains large amounts of calcium and phosphorus, as well as lesser quantities of several other elements[12]. In this paper, we show by means of electron microprobe analysis, IR spectroscopy, and X-ray diffraction that amorphous calcium phosphate (ACP) constitutes the mineral phase of the stylets produced by the nemertean worm *Amphiporus formidabilis*.

Materials and methods. Adult specimens of *Amphiporus formidabilis* Griffin, 1898 (order Hoplonemertea) were collected intertidally on San Juan Island, Washington, USA. Regions of the proboscidial organs that contain stylets were removed from $MgCl_2$-relaxed specimens and subsequently digested in Clorox bleach[10]. The elemental composition of isolated stylets was analyzed with a JEOL JSM-35CF scanning electron microscope equipped with a Tracor Northern TN-2000 microprobe system[13].

About 50 Clorox-digested stylets were also ground into a fine powder with an agate mortar and pestle. The powder was subsequently homogenized in 7 mg of KBr and pressed into a 3-mm diameter pellet. IR spectra were obtained from the stylet-KBr pellet using a Nicolet MX-1 Fourier Transform Infrared spectrometer.

For X-ray diffraction, three or four stylets were glued to the end of a glass fiber and mounted in a Debye-Scherrer powder camera. The sample was continuously rotated for 20 h while an exposure was taken with nickel-filtered copper radiation. Alternatively, a few stylets were placed in a glass crucible and heated to 500 °C for 18 h before being analyzed by X-ray diffraction as described above.

Results. Whole stylets isolated from adult worms display several identifiable peaks of X-rays when examined qualitatively by electron microprobe analysis (fig. 3). Comparatively large calcium and phosphorus peaks are routinely observed along with smaller peaks of barium, strontium, and potassium. The relative heights of the calcium and phosphorus peaks vary considerably depending on the orientation of the X-ray detector to the region of the stylet under analysis. Some specimens also produce a peak corresponding in energy to the K_α X-rays of chlorine; the zinc peak shown in figure 3, however, arises from brass components of the microscope. Contrary to a previous report[12], titanium is not detected in whole stylets or in ground sections analyzed with

energy dispersive spectrometry (EDS) or wavelength dispersive spectrometry (WDS), respectively.

An IR spectrum of unheated stylets is shown in figure 4. Note especially the lack of splitting of the triply degenerate PO_4 antisymmetric bending mode (maximum absorption at 547 cm^{-1}) and the absence of absorption bands at 1415 cm^{-1}.

No sharp reflections are visible in X-ray diffraction patterns of non-incinerated stylets. Instead, two rather diffuse and broad reflections are obtained at approximately 10 Å and 5 Å. The X-ray diffraction patterns of incinerated stylets, on the other hand, show five discrete lines, the most intense of which occurs at 3.42 Å. The other reflections produced by heated stylets are very weak and difficult to interpret. The overall pattern obtained from incinerated stylets is consistent with any one of several oriented calcium phosphate minerals.

Discussion. The IR spectroscopy data presented here indicate that amorphous calcium phosphate occurs in the stylets of *Amphiporus formidabilis*. In particular, the lack of splitting of the PO_4 absorption at 547 cm^{-1} is characteristic of ACP. In more crystalline material, this absorption is divided into two bands of unequal intensity by the apatite crystal field[14]. The absence of absorption bands around 1415 cm^{-1} indicates that carbonate ions are lacking, or present in small amounts[15].

Results obtained from X-ray diffraction studies also suggest that stylets contain an amorphous mineral phase, as discrete reflections indicative of crystalline material are only apparent after the specimens are heated to 500 °C. The two diffuse bands observed in diffraction patterns of non-incinerated stylets may be due to relatively small amounts of organic matrix substances within the stylet, or even to the glue used to mount these very small objects. The identification of ACP is further supported by the ultrastructure of stylets in high magnification scanning electron micrographs[10]. No crystalline structures are visible at the surface of the stylet or along fracture planes[10]. Moreover, the globular texture that is often displayed by the inorganic cortex[10] is characteristic of ACP[16].

Additional mineral phases have not been detected by the methods employed in this study. Thus, the divalent barium and strontium cations observed in X-ray spectra of stylets probably substitute for calcium within the ACP. The significance of the monovalent elements identified in analyses of stylets remains unclear. The findings presented here fail to support a previous deduction based on polarization microscopy that the mineral phase in nemertean stylets is crystalline[10]. Although we infer ACP constitutes the major fraction of nemertean stylets, we do not suggest that the entire inorganic portion of stylets necessarily consists of a glass-like phase that lacks short range order. In fact, the birefringence displayed by stylets under crossed nicols indicates that some orientation occurs in the inorganic cortex of stylets[10]. Whether this birefringence arises from a small amount of ordered inorganic material within the mineral phase, or from repeated arrays of organic matrix substances, is not clear.

In addition to being found in nemertean stylets, amorphous calcium phosphate is reported to occur in a variety of calcified structures that are produced by members of the following phyla: Annelida, Arthropoda, Chordata, Echinodermata, Ectoprocta, Mollusca, and Platyhelminthes[3,6]. These ACP's are typically present in the form of a hydrogel that contains substantial amounts of water[3]. Similarly, fully developed stylets of *A. formidabilis* weigh about 0.4 µg[10], and water accounts for at least 30% of the total weight, judging from weight losses recorded after overnight incubation at 80 °C.

Amorphous calcium phosphate is unstable and will crystallize within a matter of hours under in vitro conditions[17], or within days in vivo[16]. Thus, nemertean stylets and other tissues with non-transient forms of ACP presumably have some methods of stabilizing the amorphous phase. In the case of calcified concretions occurring in the blue crab *Callinectes sapidus*, it has been suggested that magnesium, ATP, and ADP serve this function[18]. The mechanisms by which ACP is stabilized in nemertean stylets have not been investigated. It is clear from X-ray spectra, however, that stylets contain little if any magnesium. Hence, the ACP in stylets is apparently re-inforced by some other means. Calcium and phosphorus containing crystals that are produced by marine invertebrates include brushite[6], dahllite[3,19], francolite[20,21], and hydroxyapatite[22]. It is not known if the ACP in nemertean stylets provides any functional advantage over a mineral phase composed of these crystalline calcium phosphates. Crystalline forms of calcium phosphate usually occur in structures that are subjected to considerable abrasion or mechanical stress[3]. The gizzard plates of certain marine gastropod molluscs represent an exception, in that they can crush the shells of ingested prey even though the plates seem to contain only ACP[3].

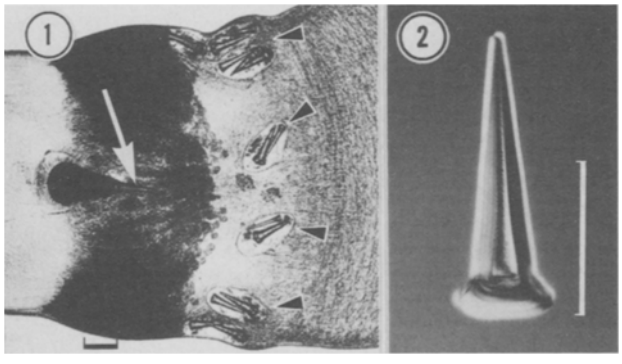

Figure 1. Whole mount of the stylet apparatus in a living proboscis removed from an adult *Amphiporus formidabilis*. The arrow marks the central stylet that is used to stab prey. Reserve stylets, which are formed intracellularly in reserve stylet sacs (arrowheads), replace the central stylet when it becomes lost or damaged. Scale bar, 200 µm.

Figure 2. Photomicrograph of an isolated stylet. Scale bar, 100 µm.

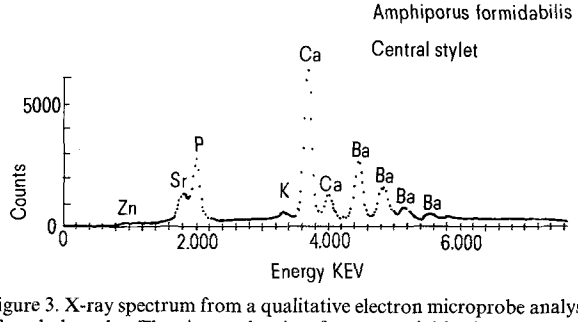

Figure 3. X-ray spectrum from a qualitative electron microprobe analysis of a whole stylet. The zinc peak arises from material in the instrument. Counts = total counts detected during a 10 min analysis. Energy = energy of X-ray.

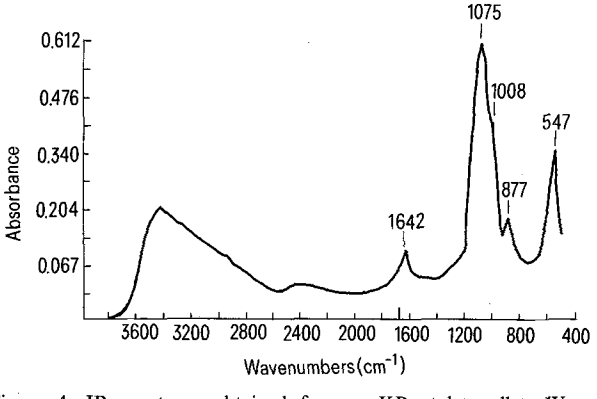

Figure 4. IR spectrum obtained from a KBr-stylet pellet. Wavenumbers = cm^{-1}.

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Biliverdin as an electron transfer catalyst for superoxide ion in aqueous medium

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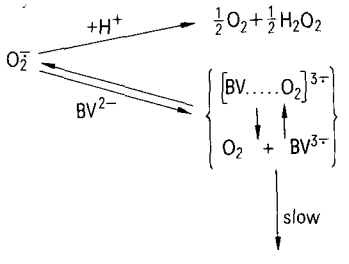
Summary. Stopped flow experiments gave evidence of the formation of a biliverdin-superoxide complex and/or a biliverdin radical anion by reaction of aqueous O_2^- with biliverdin. Such transient species are likely intermediates both in the bleaching of biliverdin, during exposure to the aerobic xanthine oxidase reaction, and in the reduction of ferricytochrome *c* under the same conditions.
Key words. Biliverdin; superoxide ion; cytochrome *c*; stopped flow technique; xanthine oxidase; superoxide dismutase.

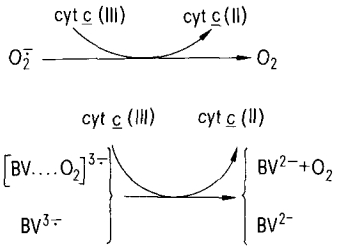
Although the bleaching of biliverdin (BV)[1] during exposure to the aerobic xanthine oxidase reaction has been reported by Fridovich[2], the chemical processes involved remain to be clarified. Recently we found that BV (and its dimethyl ester) interacts rapidly with KO_2 in DMSO, giving rise to a reversible 1:1 adduct[3]. This prompted us to investigate whether a similar charge-transfer complex could be the actual intermediate in the reaction of BV with enzymatically generated superoxide[4]. In this paper we give spectroscopic evidence of the formation of radical anions, such as the complex $[BV\cdots O_2]^{3-}$ or BV^{3-} or both, as likely transient intermediates in the bleaching of BV by aqueous O_2^- and in the BV-catalyzed reduction of ferricytochrome *c* by the same reagent. In studying the system BV/KO_2 in DMSO a thermodynamic approach (i.e. chemical equilibrium determination) was followed[3]. Such a method, however, appeared to be unsuitable in the case of aqueous media since the superoxide ion undergoes a rapid dismutation (to O_2 and H_2O_2) in protic solvents[5]. For this reason a kinetic approach, based on a stopped flow technique, was chosen. When BV[6], dissolved in an oxygenated solution of xanthine, was mixed with an oxygenated solution of xanthine oxidase in stopped flow apparatus, a transient species A could be detected showing an electronic absorption maximum at 730 nm (figs 1 and 2).

Equal volumes of two solutions containing xanthine-oxidase in phosphate buffer pH 7.6 and a mixture of xanthine and biliverdin in DMSO were mixed in a stopped flow apparatus (Nortech Laboratories Limited, England, model FPX-1; mixing time 5 ms) according to the conditions described in the captions of the figures. The transient signals were recorded by means of a Tektronix model 5115 oscilloscope and a Polaroid camera. The base line of the starting spectrum was practically restored after the decay of A (within 200 ms), indicating that the BV bleaching occurs on a completely different time scale (cf. Robertson Jr and Fridovich[2]). Formation of A was not observed when deoxygenated solutions were used, and the concentration of A was lowered to zero by the addition of increasing amounts of bovine erythrocyte superoxide dismutase. Thus, figure 2 can be regarded as a pre-steady state of the reaction of BV with O_2^- generated by the system xanthine/xanthine oxidase/oxygen.

It must be pointed out that the reported spectrum of the BV radical anion at basic pH (BV^{3-})[7] fits the spectrum of A nearly exactly[8]. However, one cannot rule out that A is a charge-trans-



Scheme 1. bleaching of BV



Scheme 2.