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Tetracycline Labeling Studies of Calcification in Nemertean Worms

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Abstract. Calcification of the stylet apparatus in nemertean worms was investigated by fluorescence microscopy following incubation of living specimens in seawater solutions of tetracycline. The synthesis of nail-shaped stylets that contain calcium phosphate, and the composition of the granular basis that anchors the central stylet to the worm's proboscis, were examined in five species of nemerteans belonging to the order Hoplonemertea. After a two-week treatment with either tetracycline-HCl or chlortetracycline, the basis appeared intensely fluorescent in all specimens. Such observations, coupled with results from electron microprobe analyses, indicate that the basis is calcified. None of the developing stylets, however, exhibited fluorescence after incubation in tetracycline. Hypotheses accounting for the lack of tetracycline labeling by stylets are discussed.

Since the pioneering studies of André (1956) and Milch et al. (1957) on the incorporation of tetracycline into bones and teeth, numerous workers have utilized the characteristic fluorescence of this antibiotic in ultraviolet light to identify active sites of calcification in vertebrate tissues (e.g., Bevelander, 1965; Frost, 1968, 1969; Melsen & Mosekilde, 1978; Simpson, 1981; Steendijk, 1964; Tam & Anderson, 1980; Yen & Shaw, 1974). Relatively few investigators, however, have used tetracycline labeling techniques to examine the wide variety of calcified structures that are produced by invertebrates (Lowenstam, 1981). Examples of tetracycline labeling studies conducted on invertebrates include investigations on skeletogenesis in echinoid echinoderms (Bevelander et al., 1960; Kobayashi & Taki, 1969; Pease & Pease, 1975) and analyses of tetracycline incorporation into developing bivalve shells (Bevelander, 1963) and jellyfish statoliths (Spangenberg & Beck, 1972).

In the small group of predominantly marine worms that constitute the phylum Nemertea (Gibson, 1972, 1982), members of the order Hoplonemertea capture prey by using an eversible proboscis that is armed with calcified stylets. The nail-shaped stylets measure 10–300 μm long and are composed of calcium phosphate (Wourms, 1976). Styletogenesis takes place intracellularly within membrane-bound vacuoles occurring in large epithelial cells (or, "styletocytes") that fill the lumina of the so-called reserve stylet sacs (Stricker & Cloney, 1981, 1982). In addition to the reserve stylet sacs, each hoplonemertean proboscis typically contains a central stylet that is anchored to the pre-capturing organ by an extracellular mass of granules referred to as the basis (Stricker & Cloney, 1981, 1982). The central stylet is used to stab the prey following

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eversion of the proboscis, and reserve stylets formed in the reserve stylet sacs replace the central stylet when it is lost or damaged (Stricker & Cloney, 1982).

Stylet formation in nemerteans has been examined by conventional light microscopy (Stricker & Cloney, 1982), polarization microscopy (Stricker, 1983), as well as scanning (Stricker, 1983) and transmission electron microscopy (Stricker, 1981, 1984, 1985). In this study, developing stylets and associated tissues are examined by fluorescence microscopy following a two-week incubation in seawater solutions of tetracycline-HCl or chlortetracycline. The significance of the fluorescence exhibited by the basis is discussed, and supplemental data from electron microprobe analyses are presented to verify that the basis is calcified. The fact that developing stylets within the reserve stylet sacs fail to incorporate tetracycline is discussed in terms of the intracellular location of styletogenesis and the nature of the calcium phosphates in stylets.

**Materials and Methods**

Adult specimens of *Amphiporus formidabilis* Griffin, 1898, *A. imparispinosus* Griffin, 1898, *Emplectonema gracile* (Johnston) Coe, 1901, *Paranemertes peregrina* Coe, 1901, and *Tetraestemma* sp. were collected intertidally from the vicinity of San Juan Island, Washington, U.S.A. Five to ten conspecific worms were subsequently placed in covered jars containing approximately 400 ml of a seawater solution of tetracycline-HCl (Sigma Chemical Co., St. Louis, Missouri). Based on the findings of Bevelander et al. (1960) and Spangenberg & Beck (1972) that tetracycline at concentrations greater than 15 mg/100 ml tends to inhibit calcification, the following concentrations were used in this study: 0.1 mg/100 ml, 1 mg/100 ml, and 10 mg/100 ml. In addition, specimens of *A. formidabilis* were incubated in a 1-mg/100 ml solution of chlortetracycline (Sigma Chemical Co.) in seawater, or a 0.01-mg/100 ml seawater mixture of alizarin red S. The pH of the incubating solutions was raised to 7.5 with 1 N NaOH, since worms became moribund in concentrated solutions whose pH had not been adjusted. Experimental specimens, and controls placed in covered jars containing only seawater, were maintained at 9–12°C in constant darkness. Fresh preparations of incubating solutions were made every other day. Experiments were conducted in the summer of 1981 and repeated in the winter of 1983.

According to previous studies (Stricker & Cloney, 1982), stylets reach full length within several weeks. Thus, an incubation period of two weeks was chosen in order to allow considerable calcification to take place. Tetracycline-induced fluorescence also was checked after four and seven days of incubation.

In order to monitor the amount of calcification that occurred during the two-week period of incubation, the numbers and shapes of the stylets produced by adults of *A. formidabilis* were noted prior to and following treatment with tetracycline. Stylets were viewed *in situ* in worms with relatively translucent bodies by compressing MgCl₂-relaxed specimens between glass slides.

Following incubation, the proboscides were removed and examined under ultraviolet light, using either a Zeiss photomicroscope equipped with a BG3 excitor filter and a 41/65 barrier filter, or a Nikon Optiphot photomicroscope
Fig. 1. The stylet apparatus of an adult *Amphiporus formidabilis* following two weeks of incubation in 1 mg/100 ml chlortetracycline in seawater: A, bright-field microscopy; B, fluorescence microscopy. Scale bars each represent 200 μm. Fig. 2. The stylet apparatus of *Emplectonema gracile* following a two-week treatment with tetracycline-HCl (1 mg/100 ml in seawater). A, bright-field microscopy; B, fluorescence microscopy. Scale bars each represent 200 μm. Fig. 3. The stylet apparatus of an adult *Amphiporus imparispinosus* following two weeks of incubation in 10 mg/100 ml tetracycline-HCl in seawater. A, bright-field microscopy; B, fluorescence microscopy. The double arrowheads indicate a mass of fluorescent material that was forced into the proboscial lumen around the central stylet following application of pressure to the overlying coverslip. Scale bars each represent 100 μm. Fig. 4. The stylet apparatus of an adult *Tetraestemma* sp. following two weeks of incubation in 1 mg/100 ml tetracycline-HCl in seawater. A, bright-field microscopy; B, fluorescence microscopy. Scale bars each represent 50 μm. ba, basis; bg, basis glands; cs, central stylet; rss, reserve stylet sac.
equipped with an ultraviolet filter cube and epifluorescence illumination. Proboscides from control worms also were checked for autofluorescence in the absence of tetracycline.

For electron microprobe analyses, the basis and central stylet were removed from MgCl$_2$-relaxed proboscides of *A. formidabilis*, *A. imparispinosus*, *E. gracile*, and *P. peregrina* using fine forceps. The central stylet/basis complexes were transferred to distilled water, and the surrounding tissues were teased away before the preparations were allowed to dry in air. Dried specimens were attached to carbon stubs (Ladd Research Industries, Inc., Burlington, Vermont), coated with carbon, and examined with a JEOL JSM-35CF scanning electron microscope. The microscope was equipped with a Tracor Northern TN-2000 electron microprobe system employing energy dispersive spectrometry (EDS). Aluminum and copper standards were used for calibration, and accelerating voltages were adjusted from 20 to 30 kV in order to keep dead-time counting levels between 10% and 20%. Count rates varied from 400–1,200 cps, and X-ray spectra were accumulated for approximately 10 min. Qualitative identifications of the elements present were made by computer software designed to distinguish peaks according to differences in X-ray energies (Ident program, Tracor Northern, Inc., Middleton, Wisconsin). Spectra were obtained from the middle to posterior parts of the basis in order to avoid accumulating X-rays emitted by the central stylet. Background X-rays were identified by analyzing the spectra produced by the mounting media (carbon paint from Ladd Research Industries, Inc. and double-stick tape).

Results

Nearly all specimens immersed in tetracycline survived the two-week period of incubation without displaying any overt signs of damage. The intensity of fluorescence was about the same in the three concentrations of tetracycline-HCl tested, and the overall pattern of fluorescence in specimens treated with chlortetracycline was similar to that found in worms incubated in tetracycline-HCl. The fluorescence exhibited at four and seven days appeared less intense than that at two weeks, but there was no major difference in the localization of the fluorescence within the proboscis. No labeling was evident in the worms treated with alizarin red S at the concentration used in this study, or in the control specimens that were immersed in seawater.

In specimens of *Amphiporus formidabilis* that were checked prior to and after incubation, stylet formation appeared to proceed at approximately equal rates in all three concentrations of tetracycline-HCl used. By the end of the incubation period, many of the worms added one or two nascent stylets, and some of their older stylets appeared markedly thicker.

The mid-proboscs region of all tetracycline-labeled specimens showed the characteristic yellowish fluorescence of tetracycline in ultraviolet light. No other regions of the proboscis exhibited yellow fluorescence, but a faint bluish fluorescence of unknown significance occurred in some of the glandular epithelial cells lining the anterior chamber of the proboscis. Unless stated other-
Fig. 5. The stylet apparatus of a tetracycline-treated specimen of *Amphiporus formidabilis*. The basis and central stylet were removed from the proboscis, using fine forceps, and subsequently viewed by bright-field (A), and fluorescence microscopy (B). Note that there is no fluorescence visible in the central stylet. Scale bars each represent 100 μm. Fig. 6. *Emplectonema gracile*. The central stylet (cs) and basis (ba) isolated from the proboscis. A, bright-field microscopy. B, fluorescence microscopy. The double arrowheads indicate tetracycline-induced fluorescent material that occurs outside the central stylet (single arrow, A). Such material gives the erroneous impression that fluorescence actually occurs within the central stylet when the basis and central stylet are viewed in situ within the proboscis. Scale bars each represent 100 μm. Fig. 7. Two reserve stylet sacs (rss) of *Amphiporus imparispinosus*, showing irregular clusters of fluorescent material within the reserve stylet sacs. A, bright-field microscopy. B, fluorescence microscopy. The arrowhead indicates weakly fluorescent material in the basis glands. Scale bars each represent 75 μm. ba, basis; cs, central stylet; rss, reserve stylet sac.

wise, the term fluorescence will hereafter refer to tetracycline-induced yellow fluorescence.

The greatest intensity of fluorescence was invariably located in the vicinity of the basis (Figs. 1–4). In the case of tetracycline-HCl treatments, the basis
appeared fluorescent throughout (Figs. 3, 4); however, in some of the specimens of *A. formidabilis* immersed in chlorotetracycline, the anterior one-half of the basis was more fluorescent than the posterior end (Fig. 1). Following application of pressure to the coverslip situated above each whole-mounted proboscis, fluorescent material would become squeezed into the proboscidial lumen enveloping the central stylet and anterior part of the basis (Figs. 1, 3). This material may have been derived from the basis glands or basis sheath that surround the basis (Stricker & Cloney, 1981), since some of the granules in these components of the middle proboscidial region also exhibited a faint golden fluorescence (Fig. 7).

Although the central stylet occasionally appeared fluorescent when viewed *in situ* on the basis, no fluorescent material could be detected in the central stylet after the basis and stylet were removed from the proboscis (Figs. 5, 6). Such isolated preparations demonstrated that the fluorescence occurring in the vicinity of the central stylet was due to surrounding substances rather than to material actually within the stylet.

In almost all worms, practically no fluorescence occurred in the reserve stylet sacs where stylet formation takes place (Figs. 1, 2). In order to determine if there may have been a weak fluorescence in the reserve stylets that was quenched by surrounding tissues in proboscidial whole mounts, some of the tetracycline-labeled preparations were soaked in sodium hypochlorite (Clorox) for 1 h. In such cases, the stylets isolated from surrounding tissues also failed to fluoresce. A few specimens of *Amphiporus impartispinosus* displayed some tetracycline labeling in the cytoplasm of their styletocytes (Fig. 7). The fluorescence, however, was not localized within the developing stylets, but was scattered irregularly throughout the cytoplasm.

Electron microprobe analyses revealed that calcium occurs in both the stylet and the basis (Figs. 8, 9). Relatively large numbers of calcium counts and comparably high counts of phosphorus were obtained from stylets. Stylets also produced identifiable peaks for barium, potassium, chlorine, and in most cases, strontium. The height of the calcium peaks arising from the basis was invariably much lower than that displayed by stylets. Nevertheless, the basis always exhibited Kα X-rays characteristic of calcium. In addition to calcium, chlorine and magnesium normally were identified in the basis, although some of these counts may have arisen from the MgCl₂ anesthetic that was absorbed by the basis during relaxation of the worms. Some specimens also produced a small peak of X-rays with energies of about 2.015 keV, corresponding to the Kα X-rays of phosphorus. This peak was identified as arising from phosphorus, after prolonged counting at high accelerating voltages.

**DISCUSSION**

The intense fluorescence observed in the basis of all specimens treated with tetracycline indicates that this structure contains calcium. The hypothesis that the basis is calcified also is supported by: (1) observations made with polarization microscopy (Stricker, 1985); (2) studies using the chelating agent EDTA (Stricker, 1985); and (3) results from the electron microprobe analyses reported in this paper.
Fig. 8. X-ray spectrum from a qualitative electron microprobe analysis of a central stylet of *Emplectonema gracile*. BA, barium; CA, calcium; CL, chlorine; K, potassium; P, phosphorus. The iron (FE) and zinc (ZN) peaks probably are due to non-stylet materials within the instrument; COUNTS, total counts detected during a 10-min analysis; KEV, energy of X-ray. Fig. 9. X-ray spectrum from the basis of *Emplectonema gracile*, analyzed under similar conditions as the central stylet. Only calcium (CA) and chlorine (CL) can be identified in the basis. The copper peak (CU) arises from material within the microscope, and the few magnesium (MG) counts may be the result of the MgCl₂ anesthetic that was used during removal of the central stylet and basis. Note that there are not enough phosphorus counts to be identified; COUNTS, total counts during a 10-min analysis; KEV, energy of X-ray.

Calcification probably increases the rigidity of the basis and thus may help to keep the central stylet in place during prey attack. In addition to providing structural support, calcium salts in the basis may constitute a cationic depot that can be mobilized during styletogenesis. Whether or not calcium actually
is transported to the styletocytes from the basis, or the glands that produce the basis, is not known. However, ultrastructural similarities between the electron-dense granules observed in the styletocyte and some of the granules found in the basis glands point to a connection between the basis complex and the reserve stylet sacs where stylets are formed (Stricker, 1985). Moreover, the fact that the basis is invariably labeled by tetracycline after only short periods of incubation indicates that a highly active process of calcification occurs in the vicinity of the basis; this, in turn, may reflect a high turnover rate of calcium salts, with some of the calcium being transported to the reserve stylet sacs.

Incorporation of tetracycline into either reserve or central stylets was not observed in this study, even though signs of calcification were detected in whole mounts of intact specimens of *Amphiporus formidabilis*. Such indications of calcification included the appearance of nascent stylets that were not present before incubation, and the fact that some stylets were markedly thicker following incubation.

Several hypotheses can be proposed to explain why tetracycline is not incorporated into stylets while active calcification is occurring. One possibility is that the concentrations used during this study were less than the minimum needed for a successful labeling of stylets. This seems unlikely, however, since the basis appears highly fluorescent in all tetracycline-labeled specimens.

Another possible explanation concerns the mineralogy of the calcium phosphate in nemertean stylets. Spangenberg & Beck (1972) have noted that a 5-mg/100 ml solution of tetracycline fails to combine with either gypsum (CaSO₄·2H₂O) or brushite (CaHPO₄·2H₂O), but tetracycline at such concentrations readily labels calcite (CaCO₃) and hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂]. Preliminary analyses utilizing infrared spectroscopy and X-ray diffraction (Stricker & Weiner, unpublished observations) suggest that the mineral phase of the stylets produced by *A. formidabilis* consists of amorphous calcium phosphate (ACP). Other workers have noted that tetracycline is incorporated readily into ACP (Kashiwa & Sigman, 1966; West & Storey, 1972). Thus, it seems unlikely that the lack of labeling is due to the type of calcium phosphate in stylets.

Alternatively, tetracycline may not be able to penetrate into the intracellular site where styletogenesis occurs. Such an hypothesis is consistent with the observation that the intracellularly formed statoliths of jellyfish fail to incorporate tetracycline (Spangenberg & Beck, 1972). The spicules of echinoid larvae, however, are thought to develop intracellularly (Gibbins et al., 1969), and these structures display strong fluorescence following incubation in tetracycline (Bevelander et al., 1960). Moreover, chlortetracycline is known to bind to calcium within the cytoplasm of several types of cells (Gawlitta et al., 1980; Saling & Storey, 1979), but none of the stylets of *A. formidabilis* incubated in chlortetracycline exhibited fluorescence. We cannot preclude the possibility, however, that tetracycline passes through the plasmalemma of the styletocyte but fails to reach the developing stylet, owing to peculiar properties of the membrane that surrounds the stylet vacuole.

The fact that the basis differs from stylets in displaying a strong tetracycline-
induced fluorescence simply may be due to the extracellular location of the anchoring device. Alternatively, the mineralogy of the basis may be unlike that of the stylets, an hypothesis that is supported by the notable difference in the phosphorus peaks produced by these two structures in electron microprobe analyses.

Finally, it remains unclear why many of the specimens of *A. imparispinosus* exhibited irregular clusters of fluorescent material in their styletocytes, while other specimens failed to show such fluorescence. Several authors (e.g., Malek et al., 1962; Tapp et al., 1965) have noted that tetracyclines also may localize in non-calcified tissues that are necrotic. Large concretions of material often were visible in the reserve stylet sacs of *A. imparispinosus*, but such structures normally are not seen during styletogenesis (Stricker, 1984). Thus, it is possible that the specimens of *A. imparispinosus* were more sensitive to the incubating medium than were other worms tested and consequently became moribund during the testing period.

**LITERATURE CITED**


