Reference: *Biol. Bull.* **192:** 1–16. (February, 1997)

Coelenterate Cnidae Capsules: Disulfide Linkages Revealed by Silver Cytochemistry and Their Differential Responses to Thiol Reagents

WALTER M. GOLDBERG AND GEORGE T. TAYLOR

Electron Microscopy Laboratory, Department of Biological Sciences, Florida International University, University Park, Miami, Florida 33199

Abstract. The sulfur cytochemistry of cnidae from the Portuguese man-of-war *Physalia physalis,* the scyphozoan *Cassiopeia xamachana,* and the black coral *Cirrhipathes luetkeni* was evaluated on the basis of electron microscopy, X-ray microanalysis, amino acid analysis, and response to disulfide reducing agents. The cnidae examined included large and small holotrichous isorhizas in *P. physalis,* another small isorhiza in *C. xamachana,* and both spirocysts and microbasic mastigophore nematocysts in *C. leutkeni.* A strong reaction with methenamine-silver reagent was characteristic of all cnidae capsules, but the pattern and extent of that argentophilia was dependent upon the type of cnida and its state of maturity. The large isorhizas of *P. physalis* reacted primarily in the outermost capsule layers, but in *C. xamachana* isorhizas, silver stained the entire capsule with the exception of the outermost region. The small isorhizas of *P. physalis* and the mastigophore capsules of *C. leutkeni* stained throughout, whereas the spirocyst capsules were outlined by silver, clearly delineating the inner and outer layers. All of these reactions were abolished with alkylation, but only after treatment with disulfide reducing agents; alkylation alone diminished silver staining only slightly, indicating that the argentophilic response was due primarily to disulfide linkages. The cystine content of these cnidae varied from 4.1 to 4.7 mole percent for a given species, but amino acid analyses did not separate components of the cnidom.

Cnidae, both within and among species, exhibited differential responses to the disulfide reducing agent dithiothreitol (DTT). Isolated, unfixed, large isorhizas of *P. physalis* discharged and appeared to dissolve rapidly in the presence of this reagent, whereas small isorhizas from both *P. physalis* and *C. xamachana* discharged, but dissolved slowly if at all. The discharge and solution responses of the capsule coincided with the complete development of the tubule. Cnidae containing an undeveloped or partially developed tubule were resistant to DTT, displayed a weak capsular argentophilia, and contained background levels of sulfur; these results suggest that formation of disulfide linkages is one of the final steps in capsular maturation. In contrast, mature nematocyst and spirocyst capsules in *C. leutkeni* tentacles were resistant to DTT among other reagents, despite the presence of disulfides. This suggests that other types of covalent, intermolecular linkages could play a prominent role in the development of capsular stability in this species.

Received 17 July 1996; accepted 16 October 1996.

Introduction

Coelenterate cnidae are among the most complex intracellular secretion products known (Gupta and Hall, 1984). Each is composed of a double-walled capsule containing an inverted tubule. During eversion the tubule discharges explosively, completing the process within 3 ms, one of the fastest mechanical events known in the biological sciences (Holstein and Tardent, 1984). More than 30 types of cnidae have been described, and they are classified into three groups: nematocysts, spirocysts, and ptychocysts (*e.g.,* Mariscal, 1974, 1984). Hydrozoans have the greatest variety of nematocysts, and scyphozoans have the least, but only anthozoans produce all three types. Ptychocysts occur only in cerianthid anemones and are the most phylogenetically restricted of the cnidae. Spirocysts are also limited in their distribution,

occurring only in the tentacles of various anthozoans; but spirocysts are also quite common, often outnumbering tentacular nematocysts (Mariscal and McLean, 1976; Goldberg and Taylor, 1989).

Nematocysts in particular have been examined closely, yielding details of structure and function relationships. For example, upon discharge, the capsule must withstand internal pressures of up to 140 bar during eversion (Lubbock and Amos, 1981; Tardent, 1988), a feat requiring enormous tensile strength. Recent work has shown that disulfide-linked, woven mini-collagens composing the internal wall of the nematocyst capsule (Kurz *et al.*, 1991) may be the key structural element in the resistance of these cnidae to such pressures. The tensile strength derived from this capsule structure is estimated to be nearly as high as that of steel (Holstein *et al.*, 1994).

The occurrence of disulfide-linked collagens in nematocysts was first established by Blanquet and Lenhoff (1966) after the suggestion by Brown (1950), and subsequently by Yanagita and Wada (1954), that since coelenterate cnidae are soluble in disulfide reducing agents, they might be composed of keratins. Hamon (1955) confirmed the earlier observations of disulfide linkages by demonstrating the presence of cystine histochemically. However, Blanquet and Lenhoff clearly showed that cystine is responsible for stabilizing collagenous proteins, the dominant components of the nematocyst capsule. They also showed that the cnidae at their disposal dissolved in a number of disulfide reducing agents including dithiothreitol, sodium thioglycolate, and mercaptoethanol.

Disulfide linkages have since been shown to be widespread in nematocyst capsules judging from amino acid composition (Fishman and Levy, 1967; Blanquet, 1988; Brand *et al.*, 1993) and X-ray microanalysis revealing the presence of sulfur (Mariscal, 1980, 1984, 1988). However, although the nematocysts from a variety of cnidarians dissolve quickly in disulfide reducing agents as might be predicted, some nematocysts and spirocysts appear to be resistant to such treatment (Mariscal and Lenhoff, 1969; Mariscal, 1971). Despite a number of subsequent chemical studies (see review by Blanquet, 1988; Brand *et al.*, 1993), little progress has been made in answering the questions raised by Mariscal and Lenhoff's original observations. To this end, we have examined the chemistry of cnidae capsules in three coelenterates, each representing a class within the phylum. The sulfur cytochemistry of each type of cnida is confirmed by X-ray microanalysis and correlated with amino acid composition, degree of maturity, and response to disulfide reducing agents.

Materials and Methods

Microscopy

Three species were collected for this study, including *Physalia physalis* Lamarck (Hydrozoa: Siphonophora) from various localities in southeast Florida; *Cassiopeia xamachana* Bigelow (Scyphozoa: Rhizostomae) from several nearshore locations in the Florida Keys, and *Cirrhipathes luetkeni* Brook (Anthozoa: Antipatharia) from a depth of 25 m off Hollywood, Florida.

Nematocyst batteries separated from fixed tentacles of *P. physalis*, individual tentacles isolated from *C. luetkeni* polyps, and oral vesicles—the baglike, oval structures associated with the oral arms (see Bigelow, 1900)—from *C. xamachana* were dissected using iridectomy scissors under low-power microscopy. All of these cnidae-containing structures, referred to as tentacles or tentacular tissues, were prepared for transmission electron microscopy (TEM) by fixation at room temperature for 2–4 h in an artificial seawater solution containing 2.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1 *M* cacodylate buffer at pH 8.0. The tissue was then stored in 0.1 *M* cacodylate buffer at 4°C. Post-fixation with osmium is incompatible with silver staining (*e.g.*, Hayat, 1993) and was omitted. Tentacular tissues were dehydrated through ethanols and embedded in Spurr resin. Thick sections examined in the light microscope were contrasted with 0.1% toluidine blue in 1% borax. For TEM we used a Philips EM 300 electron microscope operated at 60 kv. No contrast agents other than silver were employed in transmission microscopy.

Cnidae fixed as above were also examined by scanning electron microscopy (SEM). Aldehyde fixation was followed by osmication using 1% OsO_4 in 0.1 *M* cacodylate buffer, pH 8.0, for 1 h at room temperature. Dehydration in ethanols was followed by critical point drying (CPD) with CO_2 as the transitional fluid, or by cryofracture from 100% ethanol in liquid nitrogen prior to CPD. Tentacles were then sputter-coated with Au-Pd and examined in an ISI Super 3A scanning electron microscope operated at 10 or 15 kv.

Silver staining for disulfide groups

The methenamine-silver stain (Rambourg 1967; Locke and Krishnan, 1971) was employed for general electron contrast and sulfur cytochemistry. All reagents were made fresh daily using double-distilled, deionized (ddd) water, and all steps employed constant agitation on an orbital rotator. Fixed tentacular tissues were rinsed several times in ddd-water, then treated with 5% sodium metabisulfite for 10 min to block pre-existing aldehyde groups or those introduced by the fixative. After washing in three additional changes of ddd-water, tissues were immersed in methenamine-silver reagent and placed in closed 1.5-ml polypropylene microcentrifuge tubes in a 70°C oven for 30 min. After a brief ddd-water rinse, all tissues were treated with 5% sodium thiosulfate to remove unreduced silver deposits, then rinsed with ddd-water again before dehydration and embedment.

Cytochemical control procedures

Before silver staining, additional aldehyde-fixed tentacles were treated with the disulfide reducing agent dithiothreitol (=DTT, Cleland's reagent) 0.2 *M* in 0.05 *M* phosphate buffer pH 8.0 at 37°C for 90 min. Some samples were stained with methenamine-silver and examined without further treatment; others were treated with iodoacetic acid to alkylate naturally occurring sulfhydryl groups as well as those produced by disulfide reduction (alkylation blockade). Iodoacetic acid (0.1 *M*) was prepared with 0.2 *M* boric acid in 50% *N*-propanol according to the method of Swift (1968). Because we used tissue rather than sections, the reaction time for this blockade was extended from 4 h at room temperature to 18 h. Blockaded tissues were rinsed first with 20% *N*-propanol, then several times with ddd-water, and treated with methenamine-silver as above. Samples treated with iodoacetic acid alone prepared as above, or with the boric acid–propanol solvent alone, were also examined both with and without silver treatment, as were tissues treated with DTT alone. Parallel experiments employed alkaline 0.3 *M* thioglycolic acid prepared and used under the same conditions as DTT for disulfide reduction, and 0.1 *M N*-ethyl maleimide prepared according to Kiernan (1990) to block sulfhydryl groups. These sequential control treatments (disulfide reduction followed by alkyl bockade) will be referred to as post-reduction alkylation.

Elemental composition

Sections about 100-nm thick (dark gold interference color) from tissues treated with methenamine-silver were mounted on carbon-coated copper grids and examined in a Philips EM 300 transmission electron microscope operating at 100 kv. X-ray microanalysis employed a Link Analytical EDS system. The goniometer was tilted at 36° and counts were obtained using a spot size of 250 nm over an interval of 600 s real time. Count rates were 1800–2500 cps; dead time varied from 20% to 25%. No fewer than four undischarged cnidae of each type (described below) were scanned, including two types of nematocysts from *P. physalis* tentacle, one from *C. xamachana,* and both a nematocyst and a spirocyst from *C. luetkeni.* In all but *C. luetkeni,* the spot was moved every 50–60 s to another location on the same nematocyst capsule. In the latter species, the spot was moved every 30–50 s and changed to a new cnida every 150 s due to the small size of the capsules. Thus each 600-s count for each type of cnida in *C. luetkeni* was a composite of four nematocysts and four spirocysts; four such 600-s counts were taken from a total of 16 cnidae in this species. X-ray spectra of Spurr resin (blank sections) served as a means of determining background. A composite peak showing maximum, minimum, and mean net counts was constructed for each type of cnida

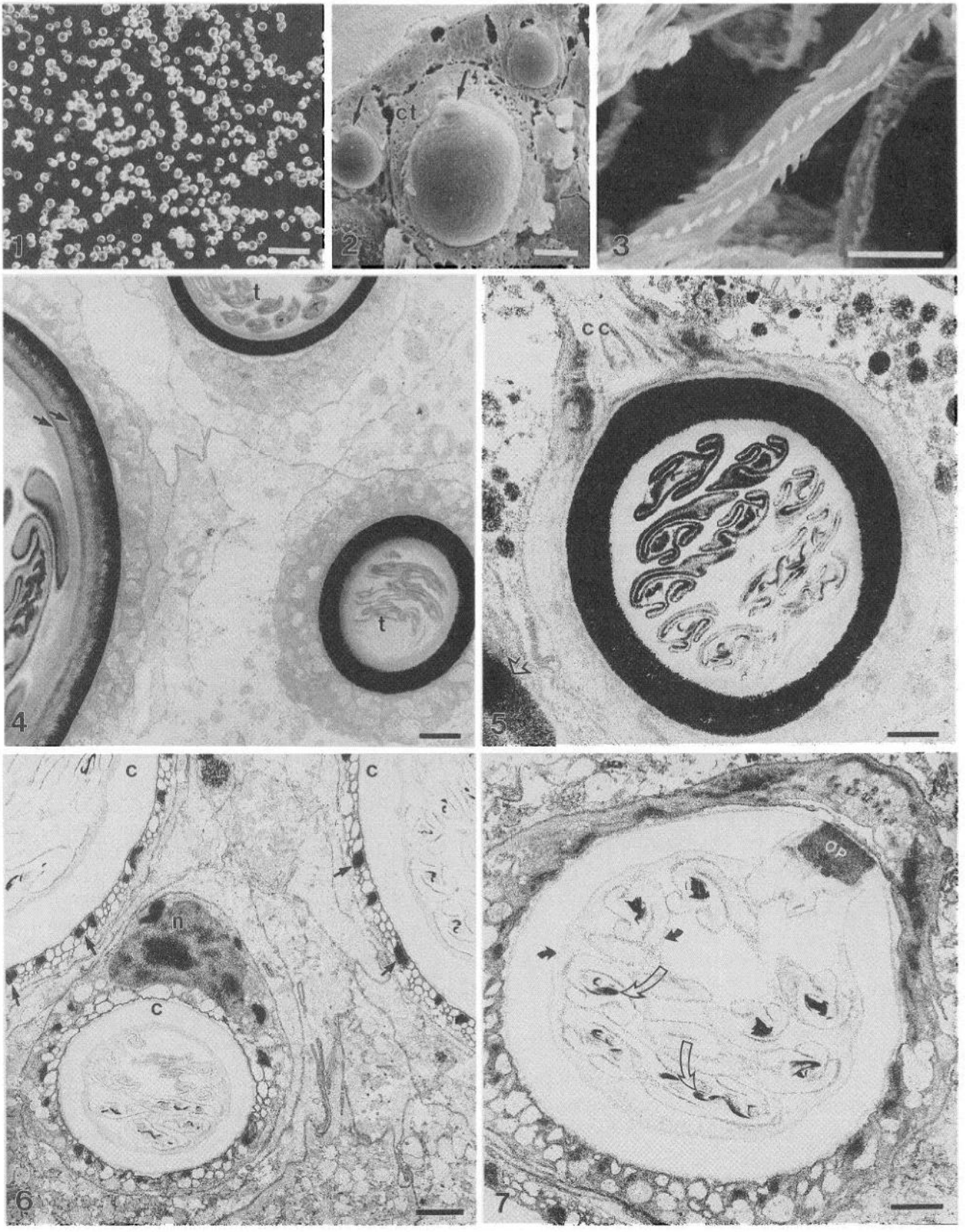
Isolation of cnidae and amino acid analysis

Freshly collected *P. physalis* tentacles, *C. xamachana* oral vesicles, and whole segments of *C. luetkeni* colonies were allowed to autolyze in seawater at 4°C over a period of 2–7 days. Debris was removed by filtration through a coarse nylon mesh, and the filtrate was treated briefly with a sonicator probe to disperse the remaining material. Recovery of undischarged cnidae from *P. physalis* required an initial low-speed centrifugation (200 × *g*) for 4 h over concentrated sucrose (approximately 2.5 *M*). The sucrose layer was diluted and cnidae were concentrated by centrifugation, then resuspended in a small volume of 0.1 *M* cacodylate buffer, pH 8.0. The crude, buffered autolysate was layered over 2.0 *M* sucrose and centrifuged at 10 × *g* for an additional 60 min, or allowed to settle at 4° overnight. A clean nematocyst preparation was obtained after repeating this process. The filtered *C. xamachana* autolysate was separated from debris by centrifugation into 1.25 *M* sucrose for 1 h at 1000 × *g*. Cnidae in the sucrose layer were concentrated, resuspended in cacodylate buffer, and briefly sonicated after addition of 1 μl ml^{-1} Triton X-100. The crude cnidae were filtered through a 10-μm nylon mesh, layered onto a discontinuous gradient of 1.75, 2.0, 2.25, and 2.5 *M* sucrose, and spun for 1 h with a swinging bucket rotor at 2000 × *g*. Cnidae were in the 2.25 and 2.5 *M* layers. This process was repeated 2–3 times. The *C. luetkeni* autolysate contained a considerable amount of mucus and was treated with 0.5% cetylpyridinium chloride in addition to Triton X-100. The treatment and procedure was otherwise the same as for *C. xamachana.*

Clean, isolated cnidae from each species were taken from buffer to distilled water and disrupted with a sonicator probe. The fragments were recovered by brief centrifugation, rinsed with distilled water, then dehydrated in ethanols and oven-dried. Cnidae fragments yielding 1–2 μg of protein were oxidized in 95% performic acid for 30 min to convert cystine into cysteic acid and were subsequently hydrolyzed by microwave digestion in 6 *N* HCl for 18 min under an atmosphere of nitrogen. Amino acids were derivitized with Edman's reagent (phenylisothiocyanate—see Heinrickson and Meredith, 1984) and quantitated by reverse-phase high-performance liquid chromatography at Florida State University Analytical Laboratories, using a detection wavelength of 254 nm. Analyses of each species were performed in triplicate, with the cnidae of each replicate representing a single individual or colony.

Effects of DTT, thioglycolic acid, and collagenase

Isolated cnidae stored in 0.1 *M* cacodylate buffer were taken to distilled water. The effects of disulfide reduction



Figures 1–7. Cnidae from *Physalia physalis.*

were examined for 15 min after placing a 10-μl drop containing several hundred cnidae on a slide, followed by 25 μl of 0.2 *M* DTT or 0.3 *M* thioglycolic acid in 0.2 *M* bicarbonate buffer, pH 10.3 (Brand *et al.*, 1993). Isolated cnidae were also tested with collagenase (Type 1A: 320 units mg^{-1} and 1.7 units mixed protease, Sigma Chemical Co., St. Louis, MO) by exposing them to 1500 enzyme units in TES buffer, pH 7.5 (10:1 enz:cnidae vol), for 5 h at 37°C. Cnidae were recovered by centrifugation, washed in distilled water, and again exposed to DTT or thioglycolic acid as above.

Results

Isolated cnidae and electron microscopy

Physalia physalis. Clean, largely undischarged nematocyst preparations were obtained from *P. physalis* (Fig. 1). Cnidae isolated in sucrose were remarkably difficult to discharge and, in contrast to results reported by Lane (1960), were often unresponsive to centrifugation or to immersion in distilled water, 50 m*M* sodium citrate, *N*-HCl or *N*-NaOH, despite capsular permeability to toluidine blue. Two size classes (diameters 8–12 μm and 20–35 μm) were obtained from tentacles (Fig. 2). These ranges are in general agreement with the measurements obtained by Lane and Dodge (1958), Hulet *et al.* (1974), and Cormier and Hessinger (1980). The larger of the two was characterized by a uniform tubule diameter of about 2.5 μm, with spines of about equal size (0.9–1.2 μm), uniformly distributed along its length in three rows (Fig. 3). The smaller cnidae contained similar tubules and spination, but these were not examined in the discharged condition. The designation of these nematocysts as holotrichous isorhizas seems appropriate and is consistent with identifications made previously (Mariscal, 1974; Brand *et al.*, 1993).

Cnidae from *P. physalis* tissues treated with methenamine-silver demonstrated a clear and consistent pattern of argentophilia. The large isorhizas were always strongly silver-positive, but only in the outermost portion of the capsule (Fig. 4). Deeper regions, delineated by what appeared to be fibrous annuli, were only lightly stained. These electron-opaque annuli subdivided the capsule into as many as five distinct layers. In contrast, the small isorhiza capsules were most often completely blackened with metallic silver (Figs. 4, 5). In less intensely stained capsules, one or two electron-opaque annuli could be distinguished, but in small isorhizas these layers did not react differentially with silver. The annuli were present in isorhizas that were untreated except for aldehyde fixation, and were therefore unrelated to the silver stain.

In *P. physalis* and all other species examined, the background stain varied in intensity, probably due in part to variations in temperature during the 30-min interval at 70°C. Figure 4 shows a low-background preparation in which some generalized staining of membranes is evident. However, nothing in the tissue compares to the intensity of the capsule stain. Figure 5 shows a small isorhiza with typically strong capsular argentophilia but with higher background staining of membranes and small vesicles. Nuclei and mucus cell secretory inclusions were also stained. The nematocyst tubule was quite variably electron-opaque (*cf.* Figs. 4 and 5), and silver deposits were often observed on the tubule periphery. Al-

Figure 1. Large and small isorhizas isolated from tentacular tissue in sucrose. Darkfield microscopy. Scale bar = 100 μm.

Figure 2. Freeze-fracture SEM of nematocyst battery cross-section showing large isorhiza and surrounding cnidocyte (ct) flanked by two small isorhizas. The opercula (arrows) are visible at the top of each; battery surface is at the upper left. Scale bar = 5 μm.

Figure 3. Everted tubule of large isorhiza showing arrangement of three spiral rows of spines. Scale bar = 5 μm.

Figure 4. Low-magnification overview of methenamine-silver preparation showing two small isorhizas (top and right) with surrounding tissue, and a section of a large isorhiza (left). Note that small cnidae capsules are completely invested with electron-opaque silver deposits, whereas the large isorhiza is primarily reactive in the outer capsule; annuli marking inner capsule layers are noted by arrows. The tubule (t) of small isorhizas displays little reactivity toward silver. Scale bar = 2 μm.

Figure 5. Detail of methenamine-silver reaction on small isorhiza and surrounding cnidocyte. The capsule is clearly delineated from surrounding cellular material by the degree of argentophilia. A tangential section through the cnidocil complex (cc) is shown at the upper left. Extracellular vesicles (upper right) are silver-positive, as is mucus secretory material (clear arrow). Tissue was treated with iodoacetic acid, but not with disulfide reducing agent. Scale bar = 1 μm.

Figure 6. Low-magnification overview of tentacular tissue after DTT-iodoacetic acid blockade of methenamine silver reaction. Note that nucleus (n), numerous cnidocyte vesicles (arrows), and membranes are reactive, but the capsular (c) response is all but completely blocked by alkylation. Scale bar = 2 μm.

Figure 7. Detail of blockade on small isorhiza. Note that the operculum (op), the periphery of the tubule (solid, curved arrows), and the cnidocyte cytoplasm are reactive, but the capsule is not. Electron-opaque spines visible in the tubule center (clear, curved arrows) are not argentophilic. Scale bar = 1 μm.

though the opacity of the spines in the center of the tubule could be confused with silver stain, they were present in unstained controls (see also micrographs in Hulet *et al.*, 1974, and Hessinger and Ford, 1988) and were therefore unrelated to argentophilia.

Control procedures using DTT or thioglycolic acid followed by iodoacetate or ethyl maleimide alkylation essentially eliminated capsular argentophilia, while background staining remained on nuclei, cnidocyte membranes, and many extracapsular vesicles (Fig. 6). At higher magnification some nonspecific silver was still visible around the tubule periphery, and the operculum was also stained (Fig. 7), suggesting that these structures may have a different chemical composition from that of the capsule. Tissues treated with DTT or thioglycolic acid alone exhibited a somewhat less intense capsular argentophilia and a higher background, but were otherwise not significantly different from untreated tissues. Treatment with disulfide reducing agents alone, however, removed the electron-opaque annuli that subdivided the capsule, resulting in the uniformly electron-lucent appearance of capsules in Figures 6 and 7. Control tissues treated with propanol and boric acid, or iodoacetic acid (as in Fig. 4), or ethyl maleimide alone were indistinguishable from experimental groups.

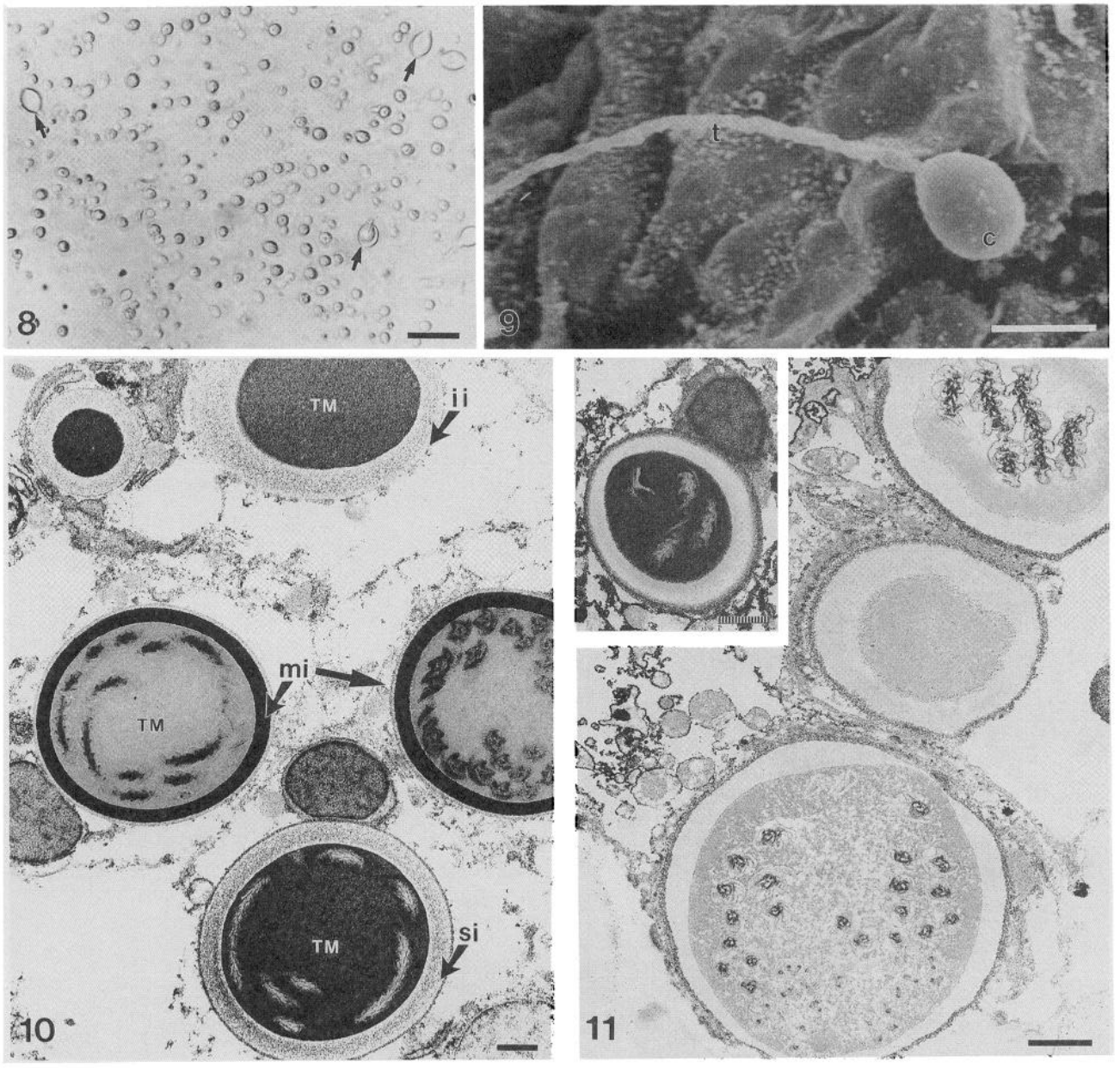
Cassiopeia xamachana. The nematocysts in the tentacles of this scyphozoan were divided into two distinct regions. The free edges of the oral arms were white and digitate (called "digitella" by Bigelow, 1900; "tentacles" by Smith, 1937) and contained primarily ovoid nematocysts 12–15 μm long, tentatively identified as euryteles. The oral vesicles, on the other hand, contained primarily small (6–9 μm), round cnidae. Clean nematocysts obtained from *C. xamachana* oral vesicles are shown in Figure 8. The smaller cnidae contained a tubule about 1 μm in diameter narrowing gradually (Fig. 9). Spines could not be measured in our SEM material, but appeared uniform in TEM preparations. These cnidae were identified by Mariscal and Bigger (1976) as holotrichous isorhizas, and this diagnosis is consistent with our observations. A smaller number of the eurytele nematocysts were also present in these preparations and were not separated by our isolation procedure. In addition, the oral vesicles contained developmental stages of these cnidae, some of which were included in our samples.

The capsules of mature isorhizas from *C. xamachana* tentacular tissue were 0.4–0.5 μm thick and were strongly argentophilic throughout, except for the ~0.1 μm thick outermost region (Fig. 10). As in the small isorhizas of *P. physalis,* one or two electron-opaque annuli could be distinguished, but these were obscured unless the silver stain was omitted. The tubule of mature isorhizas was electron-opaque in part due to nonspecific silver deposits and in part due to naturally occurring electron-opaque material in the tubule center.

Mature isorhizas were often closely accompanied by various stages of their development. The most common of these were capsules containing an electron-opaque matrix in which the tubule had not yet developed, or in which an incipient tubule could be observed (Fig. 10). We refer to the former as immature cnidae and the latter as submature. In both cases, the capsule was substantially less argentophilic than the mature cnidae. The immature stages reacted variably with silver; reactions ranged from diffuse and nonlocalized deposits to no reaction at all. The submature cnidae also exhibited variation in capsular argentophilia, which ranged from diffuse deposits to more concentrated metallic silver, but was always less than in the mature cnidae. In addition to acquiring argentophilia during the transition from submature to the mature condition, the capsule exhibited a 20%–50% decrease in thickness. Moreover, the electron-lucent tubule of submature cnidae and their contrasting electron-opaque matrix (Figs. 10, 11 inset) changed to an electron-opaque tubule in an electron-gray matrix during maturation. The latter change was the most consistent indicator of capsules most likely to exhibit a strong argentophilic reaction. The background reactivity of tissue from the oral vesicles was similar to that of *P. physalis* tentacle and included generalized membrane, nucleus, and vesicle staining.

Post-reduction alkylation of mature and submature nematocyst capsules resulted in a virtually complete silver blockade, except in the outermost capsule layer at the cnidocyte membrane interface (Fig. 11) and in the operculum. Immature nematocysts in blockaded tissues continued to exhibit a weak response to methenamine-silver. This response could not be distinguished from the same weak reactivity in unblocked tissues, suggesting that it may not be due to sulfur. The response of submature cnidae to capsular silver was variable. In some cases, argentophilia was like that of the immature cnidae; in others, a greater amount of silver was deposited. In the latter case, silver was effectively blocked by post-reduction alkylation (Fig. 11 inset), although the silver at the capsule periphery could not be blocked. Mature nematocyst capsules treated with disulfide reducing agents alone or with iodoacetate or ethyl maleimide alone were still strongly argentophilic, but they were less intensely blackened and were accompanied by greater amounts of background silver.

Cirrhipathes luetkeni. Clean cnidae preparations from whole colonies (Fig. 12) yielded spindle-shaped nematocysts and cylindrical spirocysts, both of which were 15–18 μm long and about 3–4 μm at the widest diameter. A small number of nematocysts measuring about 22 × 5 μm were also present. Nematocysts outnumbered spirocysts about 2:1 because whole colonies were used to ob-



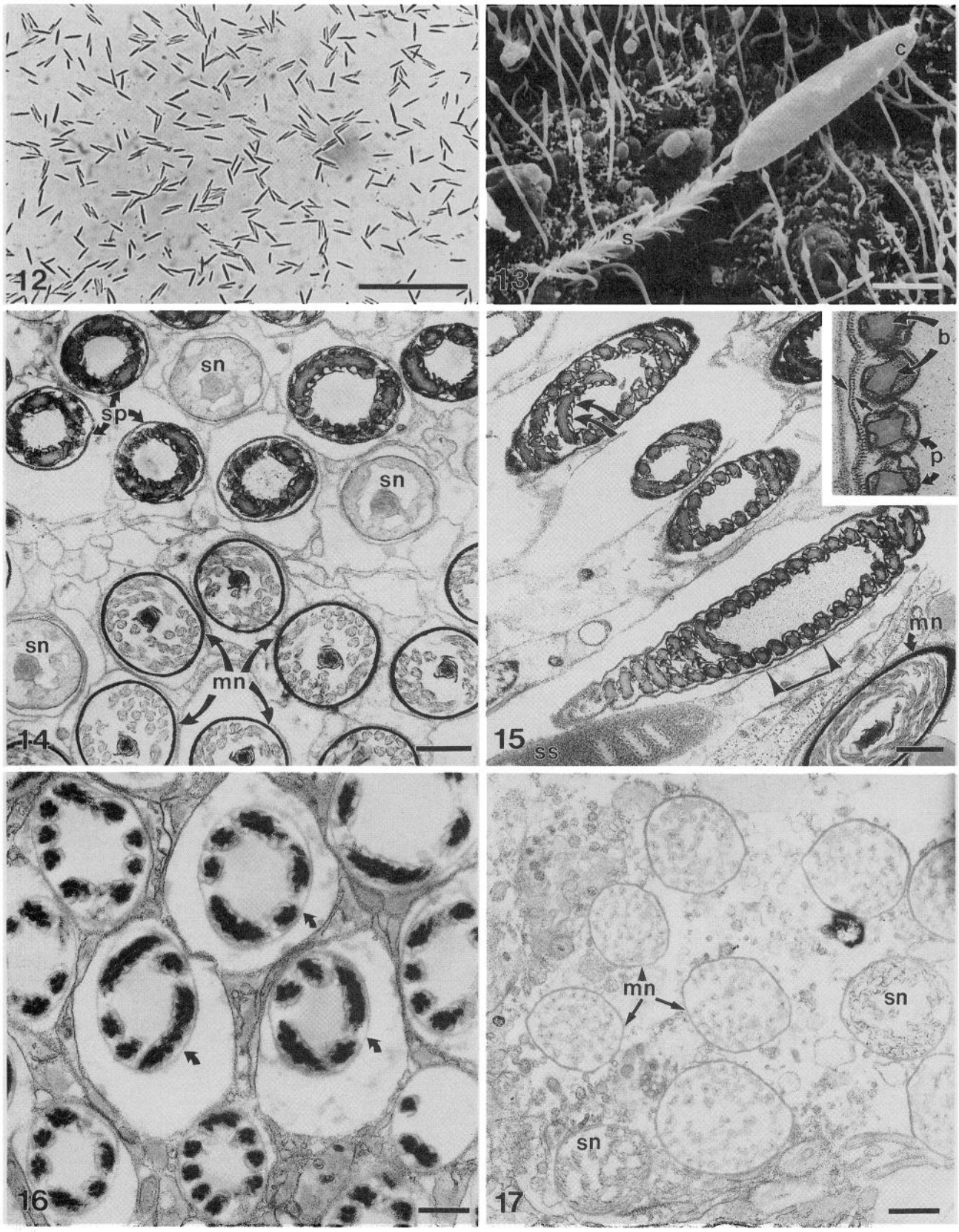
Figures 8–11. Cnidae from *Cassiopeia xamachana.*

Figure 8. Cnidae isolated from oral vesicles are primarily small isorhizas with a small percentage of euryteles (arrows). Scale bar = 25 μm.

Figure 9. SEM preparation of small isorhiza capsule (c) and everted tubule (t). Spines are appressed to the tubule and are not visible. Scale bar = 5 μm.

Figure 10. Small isorhizas in various stages of development. Immature isorhizas (ii) at top, mature isorhizas (mi) in center, and submature isorhiza (si) at bottom. Note diffuse silver deposits on capsules of immature isorhizas and the electron-opaque tubule matrix (TM) in their centers. Central electron-opaque matrix of (si) surrounds electron-lucent tubule, whereas in (mi) the tubule is electron-opaque and the matrix (TM) is electron-gray. Note differences in capsular argentophilia: ii = diffuse, si = slightly greater, mi = intense. Scale bar = 1 μm.

Figure 11. DTT-iodoacetic acid blockade of mature isorhizas blocks capsular argentophilia; cytoplasm is still reactive. Inset: submature capsular silver is also occasionally blocked except for the outer capsule layer. Scale bar = 1 μm.



Figures 12–17. Cnidae from *Cirrhipathes luetkeni*.

tain cnide; tentacles alone would have yielded a greater proportion of spirocysts. Nematocysts and spirocysts could not be separated and were included in the final product employed for observation and chemical analysis. Many but not all spirocysts were recovered without capsules. Isolated nematocysts were resistant to discharge, and unless they were disintegrated by probe sonication, most were left intact by the isolation procedure. Those found in the discharged state (Fig. 13) were consistent with the description of microbasic b-mastigophores (Mariscal, 1974).

The capsules of both nematocysts and spirocysts in fixed material were strongly argentophilic. Tentacular cross-sections at low magnification were quite striking when the silver-blackened outlines of the cnidae were compared to the rest of the tissue (Fig. 14). The inner and outer surfaces of the spirocyst capsule were separately outlined by silver deposits (Fig. 15 and inset). The tubule wall in *C. luetkeni* spirocysts was thin, folded at intervals into pleats, and strongly argentophilic (Fig. 15 and inset). The tubule interior was essentially solid, and thus typical of the antipatharians examined by Goldberg and Taylor (1996). The unstained *C. luetkeni* tubule contained four helically arranged bundles of electron-gray material. In cross-section, the bundles were separated by electron-lucent, cruciform partitions, but in contrast to the tubule wall and its pleats, none of the material within the tubule (bundles or partitions) was argentophilic (Fig. 15 inset).

Unlike spirocysts, the nematocyst capsules were strongly silver-positive across the entire capsule cross-section (Fig. 14). Nematocyst tubules, on the other hand, displayed an inconsistent pattern of silver deposition. Often in the same section, the entire tubule was outlined with silver in one area, but in other areas only the shaft was argentophilic, or the tubule failed to stain at all.

The silver reactivity of the cnidae capsules in *C. luetkeni* is persistent. We have tested this species (as well as four other antipatharian species of the genus *Antipathes*—see Goldberg and Taylor, 1996) using specimens that had been fixed and stored in ethanol for years, and they display essentially the same reactivity as freshly collected material.

Post-reduction alkylation treatment of tissue stored in cacodylate buffer eliminated all silver reactivity from the nematocyst capsule, and most but not all reactivity from the spirocysts. Unfortunately, the background staining after blockade treatment increased considerably, particularly on the cell membranes. However, when we employed fixed material stored in ethanol rather than in cacodylate, the membrane background was substantially reduced. No capsular silver was deposited in the spirocyst controls (Fig. 16), but electron-opaque deposits (Ag?) formed within the tubules. Since tubule precipitates were absent in all other preparations, we suspect that they are artifacts in this case. The nematocysts were completely free of silver deposits after blockade treatment (Fig. 17). In *C. luetkeni* cnidae as in those of *C. xamachana,* treatment with disulfide reducing agents (DTT or thioglycolic acid) or sulfhydryl blocking agents (iodoacetate or ethyl maleimide) alone did not significantly reduce argentophilia cnidae, but increased background silver deposits.

Argentophilia of immature cnidae

The relationship between argentophilia and maturity was evident in *C. xamachana* nematocysts because of the

Figure 12. Isolated cnidae from whole colonies are a mixture of nematocysts and spirocysts, with many more of the former. Scale bar = 100 μm.

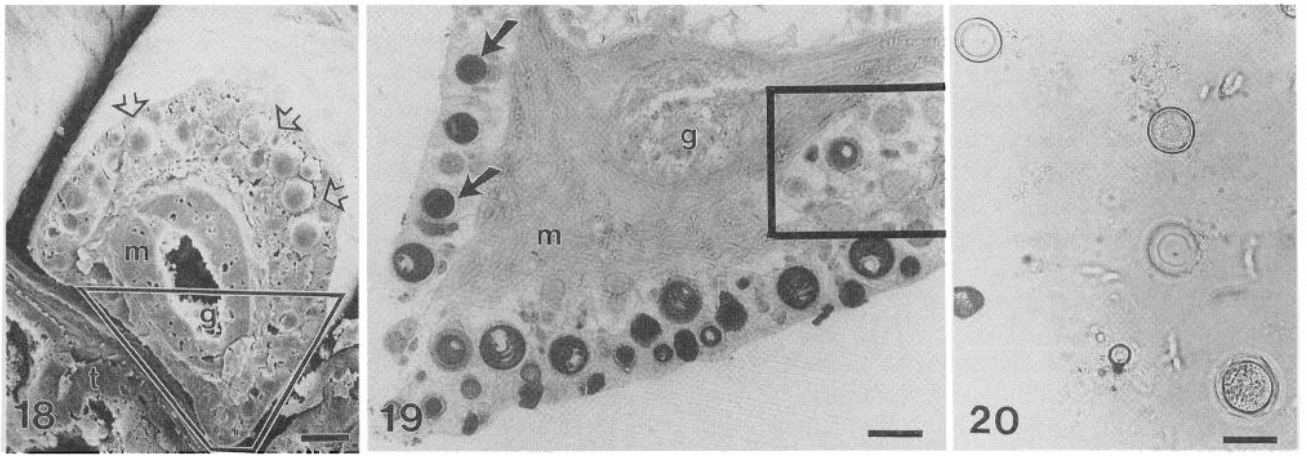
Figure 13. Discharged microbasic b-mastigophore nematocyst on tentacular surface showing shaft (s) about the same length of capsule (c) with gradual transition to tubule at lower left. Tentacular cilia are bulbous with pointed tips, possibly artifacts. Scale bars = 5 μm.

Figure 14. Methenamine-silver response of spirocyst capsules (sp) and mature microbasic mastigophore nematocyst capsules (mn) in tentacular cross-section. Note that capsules in submature nematocysts (sn) are weakly argentophilic and the tubules within are surrounded by an electron-gray matrix. Mature nematocyst tubules are matrix-free; their capsules are strongly argentophilic. Scale bar = 2 μm.

Figure 15. Tangential view of spirocysts treated as above showing uniform silver reactivity of capsule wall. The tubule and pleats (curved arrows) are also silver-positive; internal portions of the tubule are not. Note submature spirocyst (ss), lower left, with diffuse silver over electron-opaque matrix; capsule wall is indistinct and is not argentophilic. Mature nematocyst (mn) is shown at lower right. Scale bar = 2 μm. **Inset:** Detail of argentophilia in tubule wall and pleats from area of bar-connected arrowheads in Fig. 15. Note distinct staining of inner and outer layers of the capsule (straight arrows). The tubule wall is continuous with the pleats (p), and both are silver-positive. The tubule interior is not argentophilic and contains four bundles (b) of electron-gray material partitioned in an electron-lucent, cruciform pattern. Scale = 3.0 × Fig. 15 scale bar.

Figure 16. DTT-iodoacetic acid blockade of mature spirocysts blocks capsular argentophilia (arrows); pleats are also blocked; electron-opaque deposits in tubule are most likely artifacts. Scale bar = 1 μm.

Figure 17. Treatment as above showing complete silver blockade of mature nematocysts (mn) and submature nematocysts (sn). Scale bar = 1 μm.



Figures 18–20. Immature nematocysts in *Physalia physalis*.

Figure 18. SEM cross-section of freeze-fractured nematocyst battery showing mature cnidae at the outer face (clear arrows). Immature cnidae are formed in the deeper layers of the battery. Mesoglea (m) supports the inner battery; gastroderm (g) lines the center of the tentacle (t) and extends into each battery. Scale bar = 25 μm.

Figure 19. Toluidine blue-stained thick section taken in plane of trapezoid (Fig. 18) with corresponding locations of mesoglea (m) and gastroderm (g). Mature cnidae at the periphery of the battery contain a tubule with no matrix. These occur along with capsules containing a basophilic matrix (arrows), possibly representing submature isorhizas. Immature cnidae (boxed area) are only weakly basophilic. Scale bar = 25 μm.

Figure 20. Large, immature or submature isorhizas treated with 0.2 *M* DTT, pH 10.3, are resistant to depolymerization in contrast to mature stages. Scale bar = 25 μm.

proximity of various stages of development. As noted above, silver deposition in the capsule corresponded with the extent of tubule development. In *P. physalis* almost all of the cnidae on the outer face of the battery were mature (Fig. 18). Immature isorhizas, located deeper within the battery, were only weakly reactive toward toluidine blue and were virtually unresponsive to methenamine-silver reagent. However, with the development of the submature stage, the tubule matrix and capsule became strongly basophilic (Fig. 19). Because the tubule matrix apparently was not rendered electron-opaque with aldehyde fixation alone, we were unable to determine whether the basophilia coincided with silver deposition. Thus, in the electron microscope, the submature cnidae could not be distinguished with certainty from mature isorhizas.

Developing spirocysts in *C. luetkeni* occurred just below the outermost, mature cysts. Submature spirocysts were filled with a granular, naturally electron-opaque matrix surrounding the nascent tubule. The matrix also contained a diffuse silver-reaction product. The spirocyst capsule was not clearly developed as a double-walled structure at this stage (Fig. 15), and it exhibited affinity for silver only when the electron-opaque tubule matrix had almost completely disappeared.

Submature nematocyst capsules were most often located within clusters of mature mastigophores and were readily distinguished from mature nematocysts by their weak argentophilia. In every case, the weak staining of the capsules was associated with the presence of some remaining electron-gray matrix surrounding the tubule (Fig. 14). This matrix did not occur in mature nematocysts, suggesting that strong argentophilia in the capsule of these cnidae occurs coincidentally with the complete maturation of the tubule.

Response of unfixed cnidae to DTT

The large isorhizas of *P. physalis* discharged within about 10–15 s of exposure to DTT. After discharge the capsule dissolved rapidly, followed by the tubule. The time from discharge to complete solution was generally 30–60 s. Brand *et al.* (1993) also observed rapid solution in DTT, and found that *P. physalis* capsules and tubules dissolved almost simultaneously. It should be noted, however, that these authors employed discharged cnidae in their study. Our results contrast with those of Mariscal and Lenhoff (1969), who noted that disulfide reducing agents solubilize only fully discharged nematocysts. Solubility in DTT was typical of the mature isorhizas only. Those with clearly developed cysts but lacking a developed tubule failed to dissolve in this reagent. Most of these immature, DTT-resistant cysts in our *P. physalis* samples were the larger isorhizas (Fig. 20), but our isola-

tion procedure favored the larger cnidae. We also found that some of the large, mature isorhizas with an apparently well-developed tubule were resistant to DTT. However, these constituted <0.5% of that population, and we cannot be certain from light microscopic observations that they were completely mature.

The smaller isorhizas of both *P. physalis* and *C. xamachana* required 30–60 s for discharge after DTT treatment. Upon discharge, the tubules dissolved slowly over a period of several minutes, and in some cases failed to dissolve at all. Many of the small isorhiza capsules were only partially dissolved and could still be recognized after 15 min. In contrast, the euryteles in *C. xamachana* tentacles discharged and began to dissolve immediately on exposure to DTT; the tubule dissolved within 30 s. Small, immature isorhizas in both species were as resistant to DTT as the larger, immature ones in *P. physalis.*

Unlike the other cnidae in this study, isolated, undischarged mastigophores and spirocysts in *C. luetkeni* failed to respond to DTT: they did not discharge and the capsule did not dissolve. Efforts to dissolve these cnidae in collagenase were unsuccessful, although it should be noted that all cnidae in this study were collagenase-resistant, consistent with observations made by Blanquet and Lenhoff (1966). We followed our collagenase treatment with DTT, again without effect. These cnidae also failed to discharge or disintegrate after 30 min at room temperature in the presence of 0.5 *M* thioglycolic acid, 3 *M* HCl, 1 *M* NaOH, or 0.2 *M* borohydride.

X-ray microanalysis

The spectra presented in Figure 21 range from 1.2 to 5.0 keV. The zinc peak at 1.0 keV and the copper and zinc peaks above 5.0 keV originated from the grids and were eliminated from the analysis, as were traces of chromium and iron that originated from the microscope pole-pieces. We found no elements in these energy ranges that were natural constituents of nematocysts or spirocysts. Elements that were consistently present are depicted in the figure as a solid peak followed by a dashed line. Mean counts are indicated by a bar at the apex of the solid peak. The highest and lowest counts are indicated by the highest and lowest bars. Elements not consistently present are shown as peaks without bars or dashes.

Small amounts of silicon (1.7 keV) were found in all cnidae except *C. xamachana* nematocysts, and small amounts of chlorine (2.8 keV) were found in all but *C. luetkeni* nematocysts. In addition, trace amounts of aluminum (1.5 keV) were found both in spirocysts and in nematocysts of *C. luetkeni.* Sulfur (2.3 keV), on the other hand, was present in all cnidae, and was the dominant naturally occurring element. Nonetheless, since sulfur counts were often 1 per s or less, we employed samples that had been treated with methenamine-silver (3.1 and 3.3 keV) in case a consistent multiple of silver to sulfur could be demonstrated. However, silver-to-sulfur ratios were often inconsistent among individual cnidae. The ratios were generally in the range of 3:1 for all but spirocysts, which were closer to 2:1. The large isorhizas from *P. physalis* and the small isorhizas of *C. xamachana* had the highest sulfur and silver counts. The immediately adjacent submature cnidae (those without completely differentiated tubules) exhibited only background levels of sulfur, corresponding to the cytochemical results. The second-highest levels of sulfur were found in mature, large *P. physalis* isorhizas. Submature capsules could not be distinguished for separate X-ray examination. Surprisingly, the lowest sulfur counts were found in mature, small *P. physalis* isorhizas, followed closely by the microbasic mastigophores of *C. luetkeni.* Spirocyst sulfur counts were unexpectedly higher than those of the small nematocysts. However, since the spot size was wider than the diameter of the spirocyst capsule, sulfur counts were unavoidably collected from both the capsule and the tubule. This was also the case with the mastigophores, but the tubule alone appeared to contribute little sulfur to the spectrum. Immature cnidae of both types from *C. luetkeni* displayed only background sulfur counts.

Amino acid analysis

The amino acid analysis included a mixture of cnidae types, along with various stages of maturity that were unavoidable with the procedure employed. These results (Table I), while not ideal, provide a general impression of capsule chemistry and are instructive where they corroborate information obtained by cytochemistry and X-ray microanalysis.

In *P. physalis* nematocysts, levels of glycine, proline, and hydroxyproline were consistently high among the three colonies examined. Cysteic acid was the dominant sulfur amino acid. In *C. xamachana* nematocysts, proline and hydroxyproline were substantial constituents, although levels of these amino acids were not as high as those of *P. physalis.* The high levels of sulfur found in X-ray microanalysis were reflected in the total S-amino acids found in this species (6.84% ± 1.30%) but about 45% of these occurred as metsulfone. When cysteic acid and the small amount of residual cystine alone were considered, the total was not significantly different from that of *C. xamachana* cnidae.

Levels of proline and hydroxyproline in *C. luetkeni* cnidae were similar to those found in *C. xamachana,* and the total S-amino acid was similar to totals found in *P. physalis.* Cysteic acid residues were highest (3.00 ±

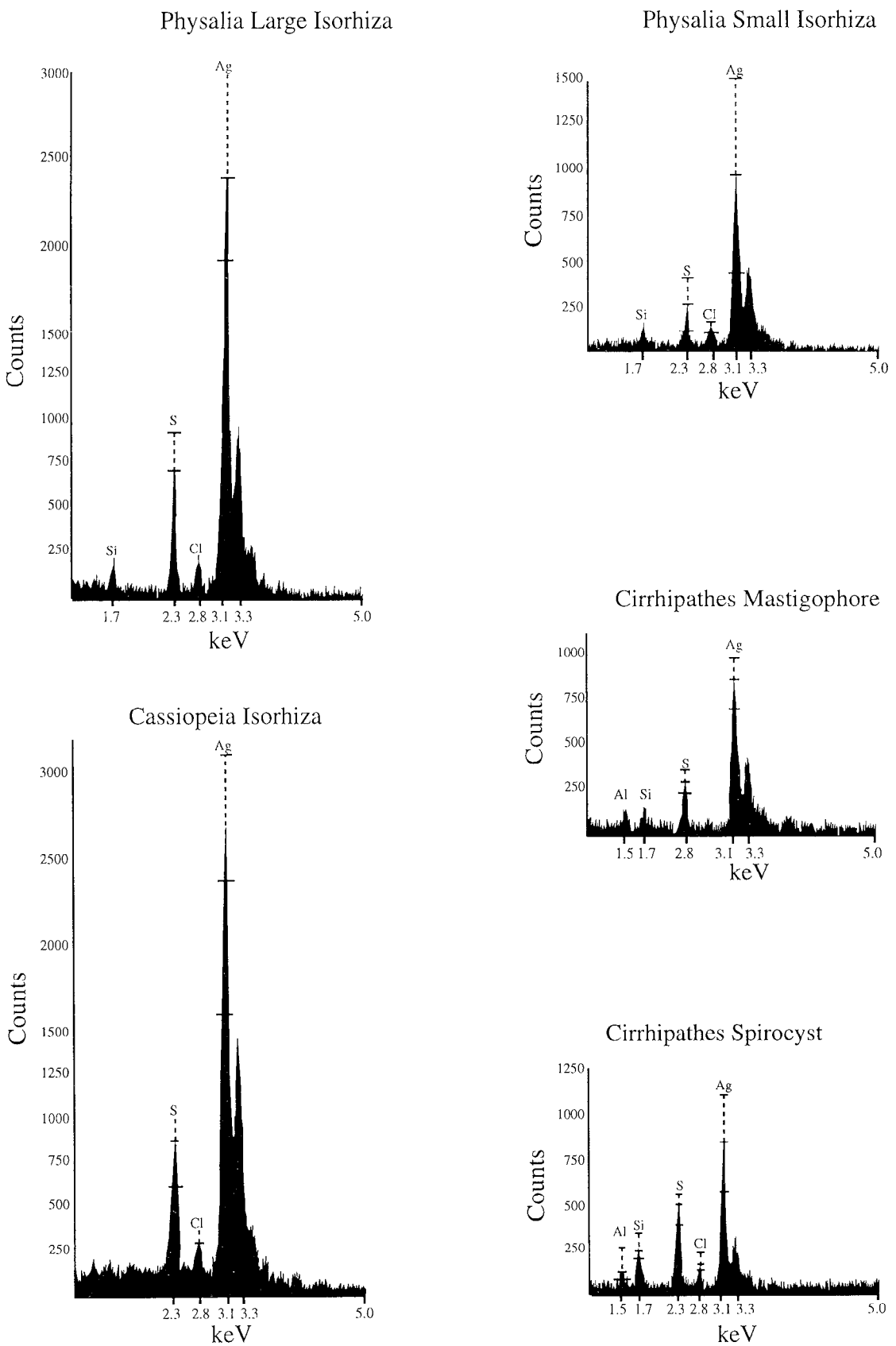


Figure 21. Composite TEM X-ray spectra of cnidae capsules. Methenamine-silver preparations show relative amounts of silver compared to natural constituents of the capsule. Mean counts for each element

Table I

Glycine, proline/hydroxyproline and S-amino acid content of cnidae as mole.% (pmoles amino acid as a percent of total pmoles ± std dev of 3 samples)

Amino acid	*Physalia physalis*	*Cassiopeia xamachana*	*Cirrhipathes luetkeni*
Gly	20.56 ± 0.15%	18.4 ± 3.9%	19.49 ± 1.97%
Pro	23.94 ± 0.06%	12.79 ± 3.55%	11.28 ± 2.6%
Hypro	10.72 ± 0.04%	1.81 ± 0.81%	1.70 ± 0.45%
Cys*	4.36 ± 0.17%	4.72 ± 1.13%	4.14 ± 0.61%
Met**	0.25 ± 0.01%	2.12 ± 0.29%	0.44 ± 0.06%

* Cys is the combined result of cysteic acid and small amounts of residual cystine, unconverted by performic acid.

** Met is methionine converted by performic acid to metsulfone.

0.48 mole.%), whereas metsulfone never constituted more than 0.49 mole.%. However, in contrast to the other types of cnidae examined, in *C. luetkeni* cnidae a substantial amount of cystine (1.14% ± 0.23% of the amino acids or 27.5% of the S-amino acids) was left unhydrolyzed by performic acid.

Discussion

Alkaline silver solutions have long been used to demonstrate neurofibrils, reticulum, Golgi, and other components of tissues and cells. The nature of the reaction is complex, and the degree of specificity often depends upon the type of fixation employed, the counter ion of the silver (nitrate, hydroxide, carbonate, methenamine, etc.) and the post-silver treatment of the tissue (*e.g.*, Humason, 1979). Specificity is further complicated by the action of silver as both an oxidizing stain and a redox reagent (Hayat, 1993). Methenamine-silver, introduced by Gomori (1946), was initially adapted for electron microscopy as a periodic acid–Schiff reagent. Despite nonselective staining of nuclear chromatin, melanin granules and ribosomes, neutral carbohydrate could be specifically demonstrated by periodic acid oxidation along with aldehyde blockade reactions (see reviews by Kiernan, 1990; Hayat, 1993). Methenamine-silver has also been employed to demonstrate disulfides in keratin and in other cystine-containing tissues. The mechanism probably involves alkaline hydrolysis of the disulfide and subsequent reduction of an undescribed methenamine-silver complex to metallic silver (Swift, 1968, 1973; Thompson and Colvin, 1970). The specificity of the reagent is dependent upon site-specific silver reduction in tissues that have been aldehyde-blocked and extracted with thiosulfate to eliminate unreduced silver. In addition, disulfide reduction and subsequent alkylation must prevent deposition of reduced silver at those previously reactive sites. Finally, the method should be employed in conjunction with parallel chemical techniques to confirm the cytochemical results (Thompson and Colvin, 1970). The silver cytochemistry of cnidae capsules is consistent with the results of post-reduction alkylation controls, X-ray microanalysis, and amino acid analysis. All of these data suggest that the argentophilia in the cnidae we have studied is due to the presence of capsular disulfides.

We know of only one other study in which electron cytochemistry has been used to detect disulfides in cnidae. Watson and Mariscal (1984) used performic acid oxidation coupled with alcian blue staining to study nematocyst development in the anemone *Haliplanella luciae.* They found that nematocyst maturation in that species is characterized by a 50% reduction in the thickness of the capsule wall, coincident with the formation of disulfide linkages. Both of these conclusions are in accordance with our observations of *Cassiopeia xamachana.* Capsule thinning also occurs with maturity in *Physalia physalis,* judging from the differences between immature and mature isorhizas. We did not attempt to study maturational changes in *Cirrhipathes luetkeni* cnidae.

If the alcian blue technique has an advantage over methenamine-silver, it is in the capacity to distinguish sulfhydryl from disulfide groups. We were not able to directly determine the contribution of either group specifically because both are argentophilic. The application of iodoacetic acid as a blockade prevented pre-existing sulfhydryl groups from reacting with silver, but increased the background in all but *P. physalis.* No differences were noted in this species between mature cnidae treated with iodoacetic acid and those left unblocked, suggesting that their argentophilia may be due entirely to disulfide groups. In *C. xamachana* and *C. luetkeni,* on the other hand, there was a small decrease in capsular argentophilia with iodoacetate controls, suggesting that some reactivity was contributed by sulfhydryl groups, although significantly less than that contributed by disulfides. In either case, the weak reactivity of developing cnidae in our study strongly suggests that neither sulfhydryl nor disulfide groups are present until very late in the process of capsular maturation.

The amino acid composition of coelenterate cnidae has been examined by a number of authors (Phillips,

Figure 21. (*Continued*) are represented by bar atop solid peaks; dashed lines indicate highest counts; lowermost bar represents lowest counts. Peaks without bars or dashes represent elements that were not consistently present.

1956; Lenhoff *et al.*, 1957; Lenhoff and Kline, 1958; Lane and Dodge, 1958; Fishman and Levy, 1967; Lane, 1968, 1974; Phelan and Blanquet, 1985; Blanquet, 1988) or the patterns of their constituent proteins (Kurz *et al.*, 1991; Brand *et al.*, 1993; Holstein *et al.*, 1994); but the universe of species examined is surprisingly small, representing only four genera with nematocysts (*Hydra* spp., *Physalia physalis, Aiptasia pallida, Metridium* spp.) and *Pachycerianthus torreyi,* a species with ptychocysts. Elevated levels of glycine, proline, and hydroxyproline are characteristic of collagen, and all cnidae analyzed thus far, including those in the present study, appear to contain these structural proteins. Glycine in nematocyst protein varies from 31.2–19.2 mole percent (Fishman and Levy, 1967; Stone *et al.*, 1970; Lane, 1974; Phelan and Blanquet, 1985). Glycine in our study is within this range, albeit at the lower limits. In *P. physalis* the proline content we reported is consistent with levels given by Lane (1968; 1974). Proline levels in *C. xamachana* and *C. luetkeni* cnidae were not unlike those of *P. physalis.* Hydroxyproline in *P. physalis,* at 10.7 mole percent, is slightly higher than the range of 6.9–9.2 mole percent reported by Fishman and Levy (1967) and Phelan and Blanquet (1985). However, hydroxyproline levels have also been reported as 20%, 8.5%, and 5.4% of the nematocyst protein (Kline, 1961; Blanquet and Lenhoff, 1966; Stone *et al.*, 1970, respectively). Conversion of our data to this form yields 12.5% hydroxyproline for *P. physalis,* but only 1.9% for *C. luetkeni* and 1.0% for *C. xamachana.* Thus a considerable range of this imino acid appears to occur in the proteins of the capsule and the tubule. However, these figures have not taken into account the ratio of collagen to the total nematocyst protein, and we cannot state with certainty that all of the cnidae pooled in our analyses (*e.g.,* spirocysts in *C. luetkeni*) contain collagen.

The amount of cystine in cnidocysts is only occasionally reported, and the range is considerable. Phelan and Blanquet (1985) found 7.24 and 2.39 mole percent cysteine in *Aiptasia pallida* and *Pachycerianthus torreyi* respectively, and Fishman and Levy (1967) reported 25.7 mole percent in *Metridium marginatum.* The most frequent and readily obtainable observation of cnidocyst chemistry is by X-ray microanalysis. In every case in which such analyses of capsule composition have been performed, sulfur has been the dominant element (Mariscal, 1980; Lubbock *et al.*, 1981, 1988; Gupta and Hall, 1984; Tardent *et al.*, 1990; Zierold *et al.*, 1991; this study). In addition, each author has suggested that disulfide linkage of collagen is the most likely role of that element. Several studies have shown that certain nematocysts dissolve in thiol reagents (*e.g.,* Yanagita, 1959; Blanquet and Lenhoff, 1966; Fishman and Levy, 1967; Mariscal, 1980; Phelan and Blanquet, 1985; Brand *et al.*, 1993). Yet it is not clear from this database whether the presence of sulfur alone in nematocysts, or even the presence of cystine, means that the capsule will be dissolved by disulfide reducing agents. This uncertainty was first indicated by Mariscal and Lenhoff (1969) who tested the solubility in dithiothreitol of 16 nematocysts from squash preparations of 10 coelenterate species. Five of the nematocysts from four species failed to depolymerize in this reagent, as did spirocyst preparations from two additional species. Phelan and Blanquet (1985) found that ptychocysts from the cerianthid *P. torreyi* were similarly resistant to the effects of disulfide reducing agents, although cysteic acid residues were present in their hydrolysates.

In light of these results, reasonable questions have been raised as to whether this insolubility might be due to (a) the absence of disulfide bonds in some cnidae, (b) the interference of reducing agent activity by mucus in the fresh preparations used by Mariscal and Lenhoff (1969), or (c) the inaccessibility of disulfides to reducing agent activity resulting from the tertiary structure of the capsule proteins (Blanquet, 1988). As in Phelan and Blanquet's observations of ptychocysts, our study has shown that cnidae from *C. luetkeni* contain cysteic acid residues. In addition, electron cytochemistry and X-ray microanalysis strongly suggest that in spite of their resistance to DTT and thioglycolate, nematocysts and spirocysts of this species both possess disulfide linkages. Our use of clean cnidae preparations rules out chemical interference with disulfide reduction in this study. With respect to the last suggestion (c) above, disulfide linkages located deep within hydrophobic regions of the protein could account for the relatively large amount of cystine remaining after performic acid hydrolysis in this species. However, since disulfide reducing agents fail to cause capsular depolymerization in *C. luetkeni,* it is possible that disulfides occur within rather than between peptide chains, or that disulfides are secondary to other types of covalent, intermolecular linkages as they are in bivalve byssus (*e.g.,* Benedict and Waite, 1986; Van Ness *et al.*, 1988) and dogfish egg capsule (*e.g.,* Rusaouën *et al.*, 1976). A lesser degree of dependence on disulfides as a means of capsule protein stabilization may also account for the partial depolymerization responses noted in the small isorhizas of *P. physalis* and *C. xamachana* (this study) and in nematocysts of the sea nettle (Goldner *et al.*, 1969; Stone *et al.*, 1970).

Spirocysts have long been considered a separate class of cnidae (*e.g.,* Hyman, 1940), a perspective reinforced by their distinctive tubule structure (*e.g.,* Mariscal *et al.*, 1977; Rifkin, 1991; Goldberg and Taylor, 1996). The chemistry of the spirocyst capsule is essentially unknown, except that, like some nematocysts, those of two actiniarians are resistant to disulfide reducing agents

(Mariscal and Lenhoff, 1969). Despite this property, Mariscal (1984) found that sulfur was the most strongly represented element in the spirocyst capsule of the anemone *Haliplanella luciae,* as it was in the nematocyst capsules of that species. Our study of *C. luetkeni* not only shows that sulfur is the principal element of the capsule (as well as the tubule wall and pleats), but electron cytochemistry strongly suggests that capsular sulfur occurs primarily in the form of disulfide linkages, as it does in nematocysts.

Surprisingly, the presence of disulfide linkages in spirocyst capsules is not entirely new information. Hamon (1955) observed that spirocysts from *Anemonia sulcata* reacted more strongly to nitroprusside after disulfide reduction than did the basitrichs of the same species. Unfortunately, the effects of disulfide reducing agents on unfixed cnidae were not investigated. Further study will be necessary to determine the distribution of resistance to disulfide reduction among coelenterate cnidae generally, and spirocysts specifically. Additional work should also focus on the characterization of spirocyst capsule proteins and the alternative forms of capsular stabilization reflected in their resistance to thiol reagents.

Acknowledgments

We thank P. Blackwelder of E.M. Analytical, Inc., for assistance with and use of X-ray analytical equipment, and C. Bigger and B. Fry for assistance in collecting specimens. This work benefited from discussions with K. Downum and D. Kuhn. We thank R. N. Mariscal (Florida State University) for helpful critique of the manuscript. Support from Florida International University is gratefully acknowledged.

Literature Cited

Benedict, C. V., and J. H. Waite. 1986. Location and analysis of byssal structural proteins of *Mytilus edulis. J. Morphol.* **189:** 171–181.

Bigelow, R. P. 1990. The anatomy and development of *Cassiopeia xamachana. Mem. Boston Soc. Nat. Hist.* **5:** 191–236.

Blanquet, R. S. 1988. The chemistry of cnidae. Pp. 407–425 *The Biology of Nematocysts,* D. A. Hessinger and H. M. Lenhoff, eds. Academic Press, New York.

Blanquet, R. S., and H. M. Lenhoff. 1966. A disulfide-linked collagenous protein of nematocyst capsules. *Science* **154:** 152–153.

Brand, D. D., R. S. Blanquet, and M. A. Phelan. 1993. Collagenous, thiol-containing proteins of cnidarian nematocysts: a comparison of the chemistry and protein distribution patterns in two types of cnidae. *Comp. Biochem. Physiol.* **106B:** 115–124.

Brown, C. H. 1950. Keratins in invertebrates. *Nature* **166:** 439.

Cormier, S. M., and D. A. Hessinger. 1980. Cellular basis for tentacle adherence in the Portuguese Man-of-War (*Physalia physalis*). *Tissue Cell* **12:** 713–721.

Fishman, L., and M. Levy. 1967. Studies on the nematocyst capsule protein from the sea anemone *Metridium marginatum. Biol. Bull.* **133:** 464–465.

Goldberg, W. M., and G. T. Taylor. 1989. Cellular structure and organization of the black coral *Antipathes aperta:* 1. Organization of the tentacular epidermis and nervous system. *J. Morphol.* **202:** 239–253.

Goldberg, W. M., and G. T. Taylor. 1996. The structure of the spirocyst tubule in black corals (Anthozoa: Antipatharia) and its taxonomic significance. *Mar. Biol.* **125:** 655–662.

Goldner, R., J. W. Burnett, J. S. Stone, and M. S. Dilaimy. 1969. The chemical composition of sea nettle nematocysts. *Proc. Soc. Exp. Biol. Med.* **131:** 1386–1388.

Gomori, G. 1946. The study of enzymes in tissue sections. *Am. J. Clin. Pathol.* **16:** 347–352.

Gupta, B. L., and T. A. Hall. 1984. Role of high concentrations of Ca, Cu, and Zn in the maturation and discharge in situ of sea anemone nematocysts as shown by X-ray microanalysis of cryosections. Pp. 77–95 in *Toxins, Drugs, and Pollutants in Marine Animals.* L. Boils *et al.*, eds. Springer-Verlag, Berlin.

Hamon, M. 1955. Cytochemical research on coelenterate nematocysts. *Nature* **176:** 357.

Hayat, M. A. 1993. *Stains and Cytochemical Methods.* Plenum Press, New York. 455 pp.

Heinrickson, R. L., and S. C. Meredith. 1984. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivitization with phenylisothiocyanate. *Anal. Biochem.* **136:** 65–74.

Hessinger, D. A., and M. T. Ford. 1988. Ultrastructure of the small cnidocyte of the Portuguese Man-O-War (*Physalia physalis*) tentacle. Pp. 75–94 in *The Biology of Nematocysts,* D. A. Hessinger and H. M. Lenhoff, eds. Academic Press, New York.

Holstein, T. W., and P. Tardent. 1984. An ultrahigh-speed analytical exocytosis: nematocyst discharge. *Science* **223:** 830–833.

Holstein, T. W., M. Benoit, G. v. Herder, G. Wanner, C. N. David, and H. E. Gaub. 1994. Fibrous mini-collagens in *Hydra* nematocysts. *Science* **265:** 402–404.

Hulet, W. H., J. L. Belleme, G. Musil, and C. E. Lane. 1974. Ultrastructure of *Physalia* nematocysts. Pp. 99–114 in *Bioactive Compounds from the Sea,* H. J. Humm and C. E. Lane, eds. Marcel Dekker, New York.

Humason, G. L. 1979. Pp. 162–207 in *Animal Tissue Techniques.* W. H. Freeman, San Francisco,

Hyman, L. H. 1940. Pp. 365–661 in *The Invertebrates: Protozoa through Ctenophora.* McGraw-Hill, New York.

Kiernan, J. A. 1990. *Histological and Histochemical Methods: Theory and Practice.* Pergammon Press, New York. 433 pp.

Kline, E. S. 1961. Chemistry of nematocyst capsule and toxin of *Hydra littoralis.* Pp. 153–168 in *The Biology of* Hydra *and of Some Other Coelenterates,* H. M. Lenhoff and W. F. Loomis, eds. University of Miami Press, Coral Gables, FL.

Kurz, E. M., T. W. Holstein, B. M. Petri, J. Engel, and C. N. David. 1991. Mini-collagens in *Hydra* nematocysts. *J. Cell Biol.* **115:** 1159–1169.

Lane, C. E. 1960. The Portuguese Man-of-War. *Sci. Am.* **13:** 371–393.

Lane, C. E. 1968. Coelenterata: chemical aspects of ecology: pharmacology and toxicology. Pp. 263–284 in *Chemical Zoology* v. 2, M. Florkin and B. T. Scheer, eds. Academic Press, New York.

Lane, C. E. 1974. Nematocyst toxins of coelenterates. Pp. 123–137 in *Bioactive Compounds from the Sea,* H. J. Humm and C. E. Lane, eds. Marcel Dekker, New York.

Lane, C. E., and E. Dodge. 1958. The toxicity of *Physalia* nematocysts. *Biol. Bull.* **115:** 219–226.

Lenhoff, H. M., E. S. Kline, and R. Hurley. 1957. A hydroxyproline-rich, intracellular, collagen-like protein of *Hydra* nematocysts. *Biochim. Biophys. Acta* **26:** 204–205.

Lenhoff, H. M., and E. S. Kline. 1958. High imino acid content of the capsule from *Hydra* nematocysts. *Anat. Rec.* **130:** 425.

Locke, M., and N. Krishnan. 1971. The distribution of phenoloxidases and polyphenols during cuticle formation. *Tissue Cell* **3:** 103–126.

Lubbock, R., and W. B. Amos. 1981. Removal of bound calcium from nematocyst causes discharge. *Nature* **290:** 500–501.

Lubbock, R., B. L. Gupta, and T. A. Hall. 1981. Novel role of calcium in exocytosis: mechanism of nematocyst discharge as shown by X-ray microanalysis. *Proc. Natl. Acad. Sci. USA* **78:** 3624–3628.

Mariscal, R. N. 1971. The effect of a disulfide reducing agent on the nematocyst capsules from some coelenterates with an illustrated key to nematocyst classification. Pp. 157–168 in *Experimental Coelenterate Biology*, H. M. Lenhoff, L. Muscatine, and V. Davis, eds. Univ. Hawaii Press, Honolulu.

Mariscal, R. N. 1974. Nematocysts. Pp. 129–178 in *Coelenterate Biology: Reviews and New Perspectives*, L. Muscatine and H. M. Lenhoff, eds. Academic Press, New York.

Mariscal, R. N. 1980. The elemental composition of nematocysts as determined by X-ray microanalysis. Pp. 337–342 in *Developmental and Cellular Biology of Coelenterates*, P. Tardent and R. Tardent, eds. Elsevier/North-Holand, Amsterdam.

Mariscal, R. N. 1984. Cnidaria. Pp. 57–67 in *Biology of the Integument* vol. 1 Invertebrates, J. Bereiter-Hahn, A. G. Matolsky, and K. S. Richards, eds. Springer-Verlag, Berlin.

Mariscal, R. N. 1988. X-ray microanalysis and perspectives on the role of calcium and other elements in cnidae. Pp. 95–114 in *The Biology of Nematocysts*, D. A. Hessinger and H. M. Lenhoff, eds. Academic Press, New York.

Mariscal, R. N., and H. M. Lenhoff. 1969. Effect of a disulfide reducing agent on coelenterate nematocyst capsules. *Experientia* **25:** 330–331.

Mariscal, R. N., and C. H. Bigger. 1976. A comparison of putative sensory receptors associated with nematocysts in an anthozoan and a scyphozoan. Pp. 559–567 in *Coelenterate Ecology and Behavior*, G. O. Mackie, ed. Plenum, New York.

Mariscal, R. N., and R. B. McLean. 1976. The form and function of cnidarian spirocysts 2. Ultrastructure of the capsule tip and wall and mechanism of discharge. *Cell Tissue Res.* **169:** 313–321.

Mariscal, R. N., R. B. McLean, and C. Hand. 1977. The form and function of cnidarian spirocysts 3. Ultrastructure of the thread and function of spirocysts. *Cell Tissue Res.* **178:** 427–432.

Phelan, M. A., and R. S. Blanquet. 1985. Characterization of nematocyst proteins from the sea anemones *Aiptasia pallida* and *Pachycerianthus torreyi* (Cnidaria: Anthozoa). *Comp. Biochem. Physiol.* **81B:** 661–666.

Phillips, J. H. 1956. Isolation of active nematocysts of *Metridium senile* and their chemical composition. *Nature* **178:** 932.

Rambourg, A. 1967. An improved silver-methenamine technique for the detection of periodic acid reactive complex carbohydrates with the electron microscope. *J. Histochem. Cytochem.* **15:** 409–412.

Rusaouën, M., J-P. Pujol, L. Bocquet, A. Veillard, and J-P. Borel. 1976. Evidence of collagen in the egg capsule of the dogfish, *Scyliorhinus canicula*. *Comp. Biochem. Physiol.* **53B:** 539–543.

Rifkin, J. F. 1991. A study of the spirocytes from the Ceriantharia and Actiniaria (Cnidaria: Anthozoa). *Cell Tissue Res.* **266:** 365–393.

Smith, H. G. 1937. Contribution to the anatomy and physiology of *Cassiopeia frondosa*. Carnegie Inst. Wash. Pub. #475, *Pap. Dry Tortugas Lab.* **31:** 17–52.

Stone, J. H., J. W. Burnett, and R. Goldner. 1970. The amino acid content of sea nettle (*Chrysaora quinquecirra*) nematocysts. *Comp. Biochem. Physiol.* **33:** 707–170.

Swift, J. A. 1968. The electron histochemistry of cystine-containing proteins in thin transverse sections of human hair. *J. R. Microsc. Soc.* **88:** 449–460.

Swift, J. A. 1973. The electron cytochemical demonstration of cystine disulfide bonds using the silver-methenamine reagent. *Histochimie* **35:** 307–310.

Tardent, P. 1988. History and current state of knowledge concerning discharge of cnidae. Pp. 309–332 in *The Biology of Nematocysts*, D. A. Hessinger and H. M. Lenhoff, eds. Academic Press, New York.

Tardent, P., K. Zierold, M. Klug, and J. Weber. 1990. X-ray microanalysis of elements present in the matrix of cnidarian nematocysts. *Tissue Cell* **22:** 629–643.

Thompson, E., and J. R. Colvin. 1970. Electron cytochemical localization of cystine in plant cell walls. *J. Electron Microsc.* **91:** 87–98.

Van Ness, K. P., T. J. Koob, and D. R. Eyre. 1988. Collagen cross-linking: distribution of hydroxypyridinium cross-links among invertebrate phyla and tissues. *Comp. Biochem. Physiol.* **91B:** 531–534.

Watson, G. M., and R. N. Mariscal. 1984. Ultrastructure and sulfur cytochemistry of nematocyst development in catch tentacles of the sea anemone *Haliplanella luciae* (Cnidaria: Anthozoa). *J. Ultrastruct. Res.* **87:** 159–171.

Yanagita, T. M. 1959. Physiological mechanism of nematocyst responses in sea-anemone 1. Effects of trypsin and thioglycolate upon the isolated nematocysts. *Jpn. J. Zool.* **12:** 361–375.

Yanagita, T. M., and T. Wada. 1954. Effects of trypsin and thioglycolate upon the nematocysts of the sea anemone. *Nature* **173:** 171.

Zierold, K., P. Tardent, and S. V. Buravakov. 1991. Elemental mapping of cryosections from cnidarian nematocytes. *Scanning Microsc.* **5:** 439–444.