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THE USE OF SILVER NITRATE STAINING AND BACKSCATTERED ELECTRON IMAGING TO VISUALIZE NEMATODE SENSORY STRUCTURES.

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Abstract

Parasitic nematodes of the species Cosmocercoides variabilis were stained with silver nitrate and examined with backscattered electron imaging (BEI). Sensory papillae were selectively highlighted in backscatter images. Silver stain deposited on papillae was located on the papillary surface as well as on the underlying dendritic process. Portions of the body cuticle were also stained. Some cuticular staining was attributed to non-specific deposition of silver but, consistent patterns of cuticular staining were noted in the anterior and posterior regions. This observation suggests that some staining of the cuticle was specific. Results of this preliminary work suggest that BEI is a technique useful to the study of nematode form.

KEY WORDS: Silver Staining, Backscattered Electron Imaging, Transmission Electron Microscopy, Parasitic Nematodes, Nematode Sensory Structures, Form and Function.

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Introduction

Staining procedures involving the reduction of silver have long been used by light microscopists to visualize specific tissues such as nerve fibers (see Ungewitter 1951) and blood vessel endothelia (see Gottlob and Hoff 1968). Silver staining for the light microscope has also been employed on protozoological material especially to reveal the cortical patterns of ciliates (Corliss 1953; Foissner 1977; Lee et al. 1985). More recently, silver staining has become prominent in studies utilizing the scanning electron microscope. Structures within a specimen, which have been stained with a heavy metal (such as silver), can be imaged using backscattered electrons (Becker and Sogard 1979). Investigators have used backscattered electron imaging (BEI) of silver to visualize cell nuclei, connective tissue fibers, basement membranes, and mucopolysaccharides, as well as nerve fibers (see Becker and Sogard 1979; Piché et al. 1983; Horiguchi et al. 1984; Taylor et al. 1984; Ushiki and Fujita 1986). In addition, BEI is now being used to examine ciliates which have been stained with silver proteinate (Small et al. 1980; Tellez et al. 1982; Paulin 1986).

Surface papillae of nematodes are sensory in nature (Chitwood and Chitwood 1950) and should, therefore, be stainable with silver and detectable with BEI. In the present study, a common nematode parasite, Cosmocercoides variabilis, of toads (see Vanderburgh and Anderson 1987) was stained with a silver nitrate staining method not previously used for nematodes and examined using BEI in order to determine whether or not papillae are selectively stained.

Cosmocercoides variabilis was chosen for this study because it possesses (i) well developed papillae on lips surrounding the oral opening and (ii) numerous simple papillae arranged in rows along the body. Further, male specimens only have many prominent papillae such as (i) a double row of rosettes in the caudal region, each rosette consisting of a central papilla surrounded by cuticular tubercles, (ii) a prominent preanal papilla, and (iii) simple and complex papillae on the tail. All of these papillae, some of which may be sensory, are easily identified with secondary electron imaging (SEI).

Materials and Methods

More than a dozen male specimens of Cosmocercoides variabilis (Harwood, 1930) Travassos, 1931 (see Harwood 1930; Travassos, 1931 for descriptions) were removed from the rectum of toads (Bufo americanus) and placed in 0.7% NaCl at 4° C for at least 12 h to remove any debris adhering to the cuticle. C. variabilis readily survives these conditions so that nematodes were alive at the time of fixation. Worms were fixed for 5 minutes with 1% osmium tetroxide in 0.1 M HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.2) prior to fixation for 12 h with 2.5% glutaraldehyde in 0.1 M HEPES (pH 7.2) containing 1% sodium chloride. Following 3 washes with 1% sodium chloride, nematodes were immersed in 3% silver nitrate for 1 h, washed thoroughly in distilled water, and exposed to ultraviolet light for 1 h.

Specimens were then dehydrated in an ascending ethanol series. Some were critical point dried using liquid CO_2 and others were infiltrated, embedded, and polymerized in LR White resin. Critical point dried specimens were mounted on stubs, sputter coated with enough gold palladium to eliminate charging, and viewed and photographed using a JEOL JSM 35C scanning electron microscope equipped with a BEI detector. Secondary electron images (SEI) as well as BEI were taken for comparison. Ultrathin sections were obtained from LR White embedded material, mounted on grids and viewed unstained in a JEOL 100CX transmission electron microscope. Controls consisted of specimens prepared as described above without silver nitrate and with or without sodium chloride.

Results

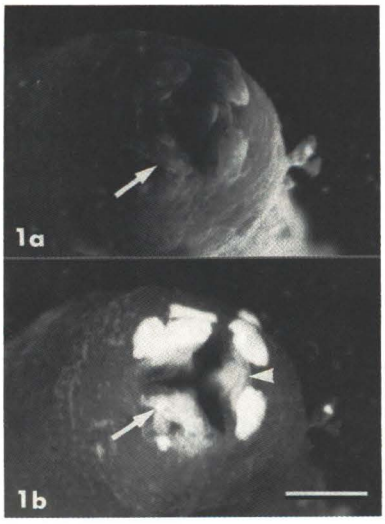
The large cephalic papillae surrounding the mouth of C. variabilis (Fig. 1a, arrow) were highlighted in backscattered electron images of worms treated with silver nitrate and sodium chloride (Fig. 1b, arrow), indicating that silver was deposited in these papillae. Stain was also taken up by oesophageal tissue protruding into the oral opening (Fig. 1b, arrowhead). Silver was deposited on all simple body (sp), rosette (rp), and tail (tp) papillae (Fig. 2) as well as the preanal papilla (Fig. 3). The central papilla of rosettes was always stained while surrounding tubercles were typically unstained (Fig. 4), although staining of tubercles was noted in some specimens (Fig. 2). No staining occurred in control specimens prepared without silver nitrate and with or without sodium chloride (Figs. 5,6). Transmission electron micrographs of stained rosette and body papillae revealed accumulations of silver grains on the papillary dendritic process (Fig. 7, dp) and on the papillary surface (Figs. 8,9).

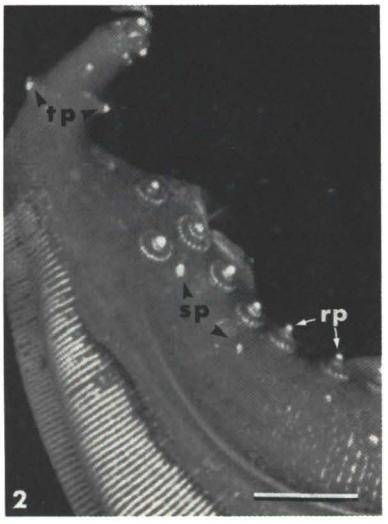
Silver was deposited around striae of the cuticle in various regions of the nematode body (Figs. 10,11, s). Cuticle in the anterior two-thirds of the body tended to be stained dorsally, laterally and ventrally but considerable variation in staining was noted among specimens except for the anterior extremity which was always unstained (Fig. 11, an). Posteriorly, a patch of ventral cuticle distal to the anus was prominently stained (Fig. 12, p) in all specimens as was the cuticle around the anus (Fig. 12, a) and the ventral surface between rows of papillae (Fig. 12, vs). Dorsal cuticle in the caudal region was also stained but lateral and latero-ventral regions were always unstained (Figs. 2, 12).

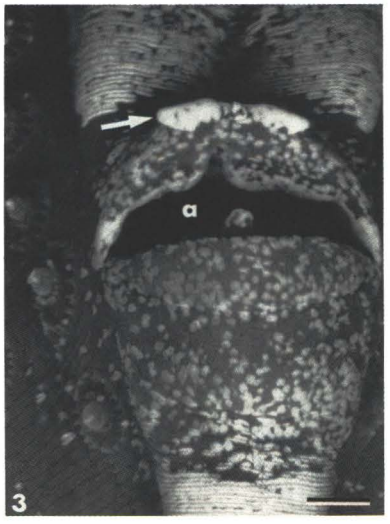
Discussion

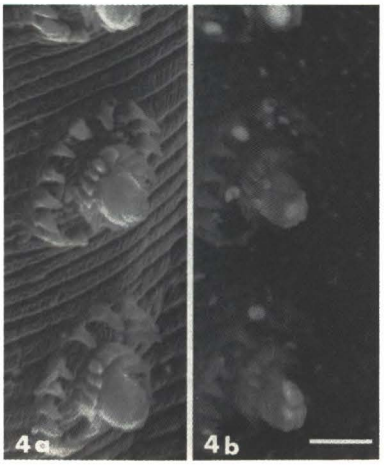
Silver stains are specific to invertebrate nerves (Rowell 1963). Silver nitrate has been used to visualize the nematode nervous system in light microscopical studies (e.g., Sanwal 1957). The finding, in the present study, of silver deposits on papillary dendritic processes suggests that staining of papillae was selective and was based upon an affinity of stain for the underlying nervous tissue. Nevertheless, staining of the papillary surface also appeared to be selective and the mechanism by which this was achieved can only be speculated upon. Davey (1964) noted the presence of neurosecretory cells in the cephalic papillae of the nematode Ascaris lumbricoides and suggested that such cells may become active in situations stressful to the worm. Possibly, during the 12 h saline wash or fixation of C. variabilis, neurosecretory products from papillary dendritic processes are released through the cuticle and are stained at the surface by silver nitrate.

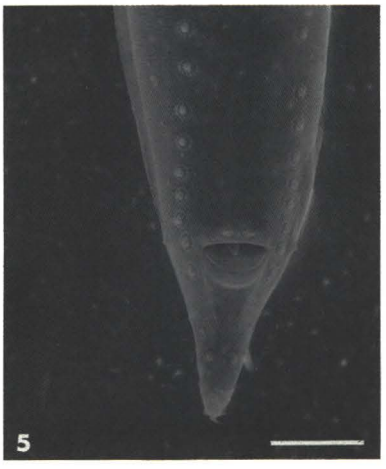
Variable staining of tubercles in rosettes suggests that, in addition to specific deposition of silver on sensory papillae, some non-specific deposition of silver occurred. Tubercles of rosettes are not thought to be innervated although no detailed TEM studies have been done to address this question. Non-specific deposition of silver probably accounts for variation noted in staining of body cuticle. Tissue specificity of silver stain is dependent on the initial formation of small silver "nuclei" in that tissue (Peters 1955; Gottlob and Hoff 1968). Under the action of suitable reducing agents, an autocatalytic process occurs in which additional silver dissociates from complexes dispersed in surrounding tissues and is reduced (deposited) around existing "nuclei". Results of experiments by Gottlob and Hoff (1968) suggested that tissue complexes may be in the form of silver chloride and this is supported, in the present study, by the finding that worms processed without sodium chloride were not stained. If the developing agent is too strong, silver which would otherwise have dissociated from (or remained complexed in) surrounding tissues may become reduced, resulting in non-specific deposition. Free aldehyde groups created during fixation may also provide non-specific sites on which silver can readily be reduced (see Lhotka 1956). In the present study, the reducing agent was ultraviolet light and it is possible that its reducing ability was too strong. The use of a weaker, chemical "developer" might, therefore, eliminate the problem of non-specific silver deposition on the cuticle.

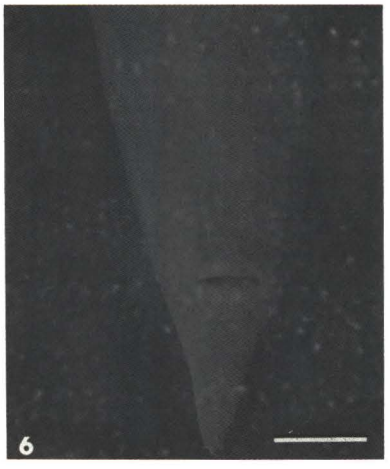












All figures are of male specimens of Cosmocercoides variabilis.

Fig. 1. SEI (a) and corresponding BEI (b) of cephalic end. Note large papillae (one shown by arrow at left) surrounding the oral opening, which are selectively highlighted with BEI. Some oesophageal tissue (arrowhead) is also highlighted. Bar = 10 um.

Fig. 2. BEI of caudal region (lateral view, dorsal aspect to the left) of another specimen showing staining of simple body papillae (sp), rosette papillae (rp), and tail papillae (tp). Tubercles surrounding the central papilla of rosettes are stained in this specimen Dorsal cuticle is stained but lateral/latero-ventral cuticle is not. Bar = 50 um.

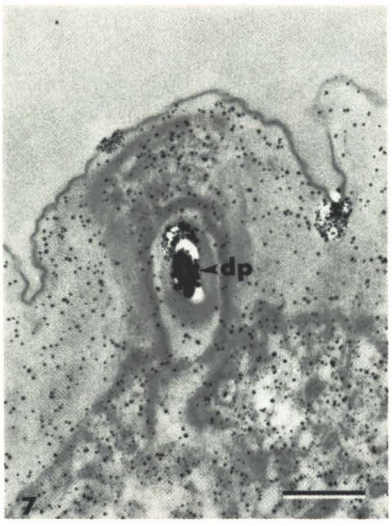
Fig. 3. BEI close-up of anal region showing staining of preanal papilla (arrow). a, anus. Bar = 10 um.

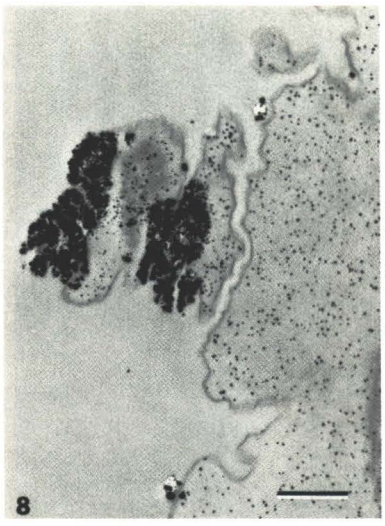
Fig. 4. SEI (a) and corresponding BEI (b) of rosette papillae of specimen in Fig. 3. Note tips of central papillae are stained but surrounding tubercles generally are not. Bar = 5 um.

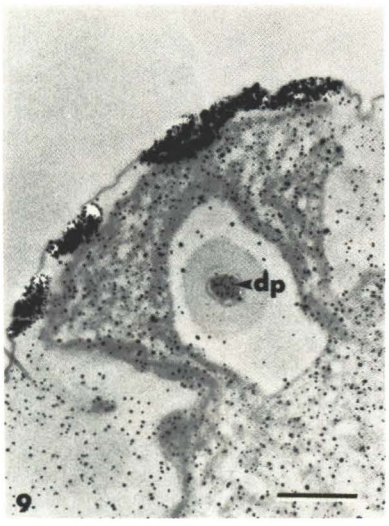
Fig. 5. SEI of control specimen fixed without sodium chloride. Bar = 25 um.

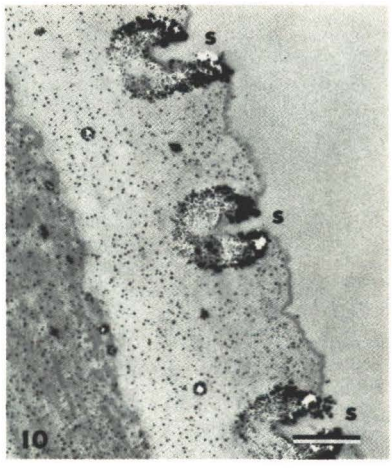
Fig. 6. BEI of specimen in Fig. 5. No staining is detected. Bar = 25 um.

Consistent patterns of cuticular staining were evident in the anterior and posterior regions of C. variabilis and could not readily be explained by non-specific deposition of silver. It is not known if these patterns are indicative of underlying nervous structure or whether cuticle in stained regions is somehow different from cuticle in unstained regions. Croll and Maggenti (1968), using a silver nitrate stain, argued for the existence of a peripheral nervous network in nematodes and Hirumi et al. (1971) provided ultrastructural evidence. The presence of neurosecretory nerve endings in the cuticle









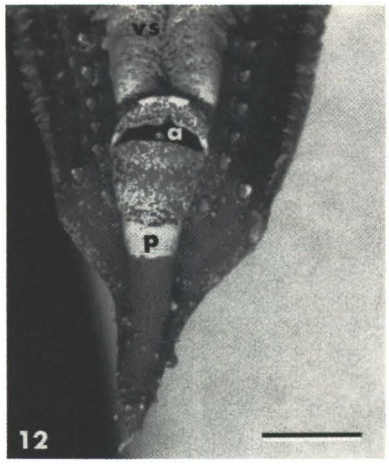


Fig. 7. Transmission electron micrograph (longitudinal section) of central papilla of a stained rosette showing accumulation of silver grains on the papillary dendritic process (dp). Bar = 1 um.

Fig. 8. Transmission electron micrograph (longitudinal section) of central papilla of a stained rosette showing accumulation of silver grains on the papillary surface. Bar = 1 um.

Fig. 9. Transmission electron micrograph (longitudinal section) showing accumulation of silver on the papillary surface. At this level, the dendritic process (dp) is not stained as it is in Fig. 7. Bar = 1 um.

Fig. 10. Transmission electron micrograph (longitudinal section) of portion of stained body cuticle showing accumulation of silver grains around striae (s). Bar = 1 um.

Fig. 11. BEI of lateral view of the anterior extremity (an), showing region of unstained cuticle. General cuticular staining can be seen to the rear. Bar = 25 um.

Fig. 12. BEI of caudal extremity of the ventral surface (vs), showing patch (p) of stained cuticle posterior to the anus (a) and staining of cuticle around anus and between rows of rosette papillae. Note that lateral/latero-ventral cuticle remains unstained. Bar = 50 um.

has been noted in Haemonchus contortus and Meloidogyne javanica (Rogers 1968; Bird 1971). Bird (1971) suggested that cuticular nerve endings may be more extensive in nematodes than previously thought.

This preliminary work is the first involving silver staining and BEI of nematodes. The apparent affinity of stain to sensory structures and its convenient visualization by BEI suggests that the technique is of potential use in the study of nematode form and function.

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Discussion with Reviewers

H.E. Buhse, Jr.: What relationship, if any, can be made between the staining pattern observed for nematodes, presumably sensory structures, and the silver impregnation pattern observed by BEI or light microscopy of silver impregnated ciliates ? Do the authors think there is (are) any common structure(s) that stain by silver ?

Authors: There are no obvious anatomical similarities in any sensory structures, although some nematode sensory structures do have modified cilia (see Bird, 1971) and some macromolecules, such as glycoproteins, may reside in/on both nematode and ciliate cell membranes. Native sulfhydryl groups and free aldehyde groups produced by this fixation and staining method may be a common feature.

W.G. Henk: Were specimens examined light microscopically to assess stain specificity at that level ?

Authors: For routine anatomical examination under the light microscope, nematodes require clearing in hot alcohol-glycerine due to their thick cuticle. With the use of OsO_4 during our

staining regimen, the specimens were rendered completely opaque.

W.G. Henk: Why was gold palladium used instead of carbon to coat specimens ?
J.J. Paulin: Did you try coating specimens with carbon or aluminum to obtain a stronger backscatter electron signal ?
Authors: Extremely thin gold coating has been used to render specimens conductive for BEI that have been labelled with colloidal gold. We do not feel that the BEI signal was interfered with, especially when one considers the amount of silver deposited compared to the amounts of colloidal gold. Furthermore, carbon evaporation is time consuming in contrast to gold-palladium sputter coating.

W.G. Henk: Why do the authors feel that the dendritic process in Fig. 9 is specifically stained ?
Authors: Figs. 7 and 9 are sequential sections of the same dendritic process. Our supposition is that stain intensity varies along the terminal portions of the neuron.

J.J. Paulin: Considering the difficulty in penetrating the cuticle of nematodes, have the authors experimented with staining protocols longer than one hour ?
Authors: We have not experimented with longer time periods as our interest has primarily been in the cuticular sensory structures, some of which appear to react specifically in our procedure. Presumably, longer staining times would permit staining of structures deeper in the organism.

V.C. Barber: Why do the authors feel the BEI/silver method has advantages over secondary electron imaging ?
Authors: As you point out in your comments, a selective stain for nervous tissue would be useful. If our silver procedure selectively stains nerve processes, then the BEI/silver method allows discrimination of innervated papillae. This would not be possible using SEI alone.

R.P. Becker: Have your tried a heat reduction method (such as used by Becker and Sogard, 1979, in text references) ?
Authors: We have not tried a heat reduction method. Perhaps this approach would reduce the background staining.