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Ultrastructural Cytochemistry of Secretory Granules of Esophageal Glands of *Meloidogyne incognita*[1]

C. A. SUNDERMANN AND R. S. HUSSEY[2]

Abstract: Ultrastructural cytochemical tests for several enzymes, proteins, carbohydrates, and nucleic acids were conducted on secretory granules of dorsal and subventral esophageal glands of preparasitic second-stage juveniles and the dorsal gland of adult females of *Meloidogyne incognita.* Secretory granules in the subventral glands of juveniles stained positive for acid phosphatase. Peroxidase, DNase, RNase, cellulase, and nucleic acids were not detected in these granules. Secretory granules in the dorsal gland of adult females stained positive for peroxidase (pH 7.6) in < 50% of the tests. Acid phosphatase, β-glucuronidase, DNase, RNase, polyphenoloxidase, cellulase, and carbohydrates were not detected in dorsal gland granules in adult females. Positive staining with cobalt thiocyanate, a stain for amino groups of basic proteins, occurred in secretory granules in the dorsal gland, ribosomes, and chromatin in adult females. Ribosomes, nuclei, and secretory granules of the dorsal gland of adult females intensely stained when incubated in three reagents specific for nucleic acid.

Key words: cytochemistry, *Meloidogyne incognita,* secretory granule, esophageal gland, enzyme, root-knot nematode, electron microscopy.

Meloidogyne species are sedentary endoparasites that have evolved complex and intimate relationships with their host plants. Secretions from these nematodes transform recipient root cells in susceptible plants into elaborate nutritive feeding sites called giant cells. These multinucleate cells are initiated by infective second-stage juveniles (J2) and subsequently maintained by adult female nematodes as the source of nutrients needed for their growth and reproduction. Second-stage juveniles have three large and complex esophageal glands, one dorsal and two subventral. These glands produce membrane-bound secretory granules that are presumably involved in the initiation and maintenance of the giant cells. The contents of the secretory granules and their specific function in the life cycle of the nematode are unknown (15).

Secretory granules accumulate in the subventral glands and their cytoplasmic extensions in *M. javanica* J2 just before egg hatching (2). These large granules have a distinct membrane and contain protein but not nucleic acid (4). Granules that form in the subventral glands following the onset of parasitism change morphologically and chemically (3). Although nucleic acid is still not detected microspectrophotometrically in subventral gland extensions in parasitic juveniles, the protein absorption pattern differs from that recorded for preparasitic juveniles. Granules forming in the subventral glands during parasitism also stain positive for carbohydrate. The dorsal gland in preparasitic juveniles of *M. javanica* contains fewer granules than the subventral glands (5). Soon after juveniles penetrate roots, however, granules accumulate in the cytoplasmic extension and ampulla of the dorsal gland cell.

Elucidating the nature of secretions of esophageal glands of different stages of *Meloidogyne* species is necessary for understanding the molecular basis of their pathogenesis. Before any secretory component from *Meloidogyne* species can be implicated in inducing a feeding site, the fact that it is synthesized in an esophageal gland should be established. Ultrastructural cytochemical analyses of secretory granules in the esophageal glands of these nematodes might provide evidence on the nature of the contents of the granules. Our objective was to conduct in situ ultrastructural cytochemical analyses of secretory granules

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[2] Postdoctoral Research Associate and Professor, Department of Plant Pathology, University of Georgia, Athens, GA 30602. Present address of first author: Department of Zoology, 101 Cary Hall, Auburn University, Auburn, AL 36849.

TABLE 1. Ultrastructural cytochemical tests conducted on secretory granules of dorsal (D) and subventral (SV) esophageal glands of second-stage juveniles and adult females of *Meloidogyne incognita*.

Cytochemical test	Second-stage juvenile			Adult female	
	No.†	D	SV	No.†	D
Enzyme					
Cellulase	1	−	−	2	−
Acid phosphatase	3	−	+	5	−
Peroxidase					
pH 7.6	3	−	−	12	±
pH 4.5	1	−	−	1	−
pH 9.4	−	NT	NT	2	−
Polyphenoloxidase	−	NT	NT	1	−
β-glucuronidase	−	NT	NT	3	−
DNase	1	−	−	2	−
RNase	1	−	−	1	−
Protein (basic)	1	−	−	3	+
Carbohydrate	−	NT	NT	1	−
Nucleic acid					
Sodium tungstate	2	−	−	2	+
Indium trichloride	2	−	−	2	+
Feulgen-silver methenamine	2	−	−	2	+

− = negative reaction. + = positive reaction. ± = inconsistent reaction.
NT = not tested.
† Number of times test was conducted.

of dorsal and subventral esophageal glands of *M. incognita* J2 and adult females. A preliminary report of this research has been published (16).

MATERIALS AND METHODS

Culture and fixation

M. incognita (Kofoid & White) Chitwood was propagated on greenhouse-grown tomato (*Lycoperiscon esculentum* Mill. cv. Rutgers). All cytochemical tests were performed on 30-day-old adult female nematodes, whereas selected tests were conducted on preparasitic J2 (Table 1).

Adult females were placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4 C immediately after they had been carefully dissected from galled root tissue. The specimens were fixed 15 minutes after which their posterior ends were punctured with a pin. After an additional 30 minutes in fixative, the anterior one-third of the nematode was severed under a stereomicroscope with a double edged razor blade. Primary fixation was complete after the anterior portions had remained in fixative for 2.5–3.0 hours at 4 C. All samples were washed in at least three changes of buffer before proceeding.

Second-stage juveniles were hatched from eggs as previously described (24). Juveniles were transferred to 2.5% buffered glutaraldehyde and, after 15 minutes, their tails were severed. Juveniles were fixed an additional 2 hours at 4 C and washed three times in buffer.

Cytochemical tests

Acid phosphatase: A modification of the Gomori (13,20) procedure was used as follows: Fixed nematodes were incubated in Gomori medium (pH = 5.0; substrate = Na glycerophosphate) for 1 hour at 37 C, rinsed in buffer, postfixed in 2% OsO_4 for 1 hour, dehydrated in an ascending series of ethanols (70–100%) followed by propylene oxide, and then infiltrated with a 1:1 mixture of propylene oxide and epon-araldite for at least 1 hour. Specimens were flat embedded in Mollenhauer's (21) mixture of epon and araldite which was then cured in a 60 C oven for 3 days. Thin (75–80 nm) sections were cut with a Sorvall Porter-Blum MT-2 ultramicrotome and viewed with a Zeiss 10 A transmission electron microscope operating at 60 kV. Samples were not poststained. Sectioned material was photographed with Kodak electron image film, and all negatives were printed on Kodabrome II RC paper with F4 hardness with similar exposure settings.

Peroxidase: These tests were conducted at three pHs. 1) pH 7.6. Adult females and juveniles were fixed in glutaraldehyde and washed as before. Samples were incubated in a solution containing 10 ml 50 mM Tris-HCl, 5 mg DAB (3,3'-diaminobenzidine tetrahydrochloride) and 0.1 ml 1% H_2O_2 for 1 hour (14). Control experiments omitted either DAB or H_2O_2. All samples were postfixed, dehydrated, infiltrated, and embedded as described above. 2) pH 9.4. After primary fixation, adult females were incubated for 1 hour at 37 C in a medium consisting of 0.05 M 2-amino-2 methyl-1,3-

propanediol buffer, 0.2 ml 1% H_2O_2, and 20 mg DAB (22). Control preparations lacked H_2O_2. Samples were postfixed, dehydrated, infiltrated, and embedded as before. 3) pH 4.5. After primary fixation and rinsing, nematodes were placed in a solution containing 20 mg DAB, 8.9 ml 0.05 M sodium acetate buffer (pH 4.0), 1.0 ml 0.05 M manganous chloride, and 8.9 ml 0.1% H_2O_2 (22) for 1 hour at 25 C. H_2O_2 was omitted from control samples. After rinsing, samples were postfixed, dehydrated, infiltrated, and embedded as described.

Polyphenoloxidase: Primary fixation of adult female nematodes was accomplished as described. After the buffer rinse, control samples were heat inactivated (100 C, 5 minutes). All samples were then incubated in 0.05 g D-DOPA in 12 ml 0.1 M cacodylate buffer for 2 hours at 25 C (12). Control and experimental material was rinsed, postfixed, and embedded as described. All sections were viewed unstained.

Cellulase: Nematodes were fixed in Karnovsky's (19) fixative and washed extensively in phosphate buffer. Tissue was then incubated in 0.02% carboxymethylcellulose (omitted for control samples) in Na phosphate buffer (pH 6.0) for 30 minutes, placed in Benedict's solution (100 C) for 15 minutes, and washed in distilled water (1). Specimens were postfixed, embedded, and sectioned as described. Sections were viewed without further staining.

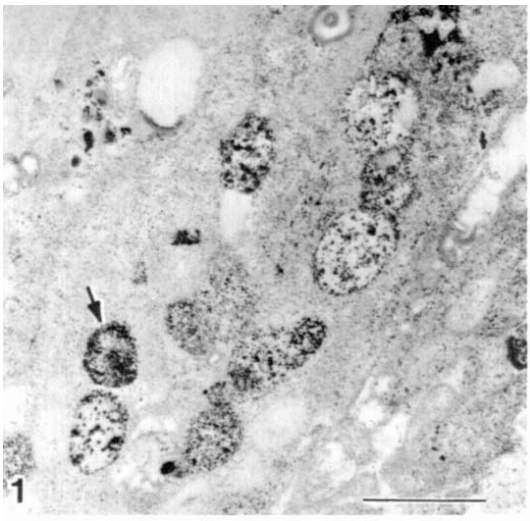
DNase: After primary fixation and rinsing, nematodes were placed in 0.2 M sodium acetate buffers with descending pH (7.0, 6.5, 6.0) for 30 minutes each at 30 C. Samples were then incubated at 37 C in a medium containing 2 mg DNA (from calf thymus Type I; Sigma Chemical Co., St. Louis, MO), 5 mg acid phosphatase, 12.5 ml 0.2 M acetate buffer, 0.25 ml 0.4 M lead nitrate, and 36 ml water (9,28). Specimens were rinsed in 0.2 M acetate buffers (pH 6.0, 6.5, 7.0) for 30 minutes each, rinsed in distilled water, and postfixed, dehydrated, and embedded as described.

RNase: The procedure used for the detection of RNase was identical to that used for DNase, except that 10 mg RNA (from calf liver, Type IV, Sigma) was substituted for 10 mg DNA.

β-glucuronidase: After primary fixation, nematodes were incubated for 3 hours at 37 C in a solution containing 6-bromo-2-naphthyl-β-D-glucopyranoside in 2-methoxyethanol (1 mg/ml) and 0.1 M phosphate buffer (pH 5.4, final substrate concentration of 0.1 mg/ml) (7). After a phosphate buffer rinse, tissue was placed in a coupling medium, 0.14% hexazonium pararosaniline in 0.1 M phosphate buffer (pH 7.0) at 0 C for 15 minutes. After a brief rinse, samples were postfixed, dehydrated, and embedded as described.

Carbohydrate: After primary fixation in glutaraldehyde, adult females were rinsed, dehydrated, infiltrated, and embedded as described. Postfixation in OsO_4 was omitted. Thin sections of the embedded material were mounted on nickel grids and immersed in 1% periodic acid for 20 minutes at 25 C; this step was omitted for control grids. Grids were washed twice with distilled water and placed in the dark in a solution containing silver nitrate and methenamine in sodium tetraborate buffer for 40 minutes at 60 C (11,27). Grids were transferred to water for 2 minutes and then washed for 5 minutes in 5% sodium thiosulfate. After a final water rinse, sections were dried and viewed.

Nucleic acid: 1) Sodium tungstate. After primary fixation in glutaraldehyde, nematodes were rinsed, dehydrated, infiltrated, and embedded as stated above. Fixation in OsO_4 was omitted. Thin sections on grids were incubated for 1 hour in 10% sodium tungstate (26), rinsed with distilled water, and viewed without further heavy metal staining. 2) Indium trichloride. Primary fixation and rinsing were accomplished as described. Samples were dehydrated in an ascending series of acetone to absolute acetone, rinsed in pyridine for 30 minutes at 4 C, and reduced in pyridine saturated with lithium borohydride for 2 hours (29,30). After a 30-minute wash in pyridine, samples were left overnight in pyridine and acetic anhydride saturated with sodium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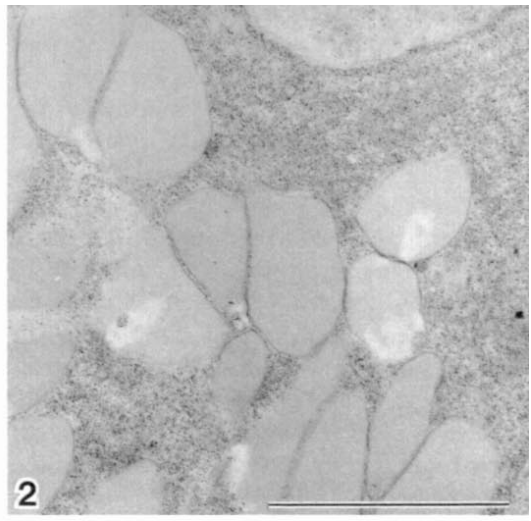
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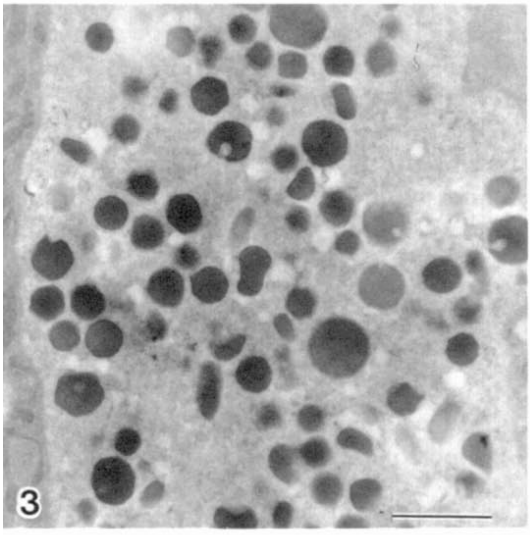
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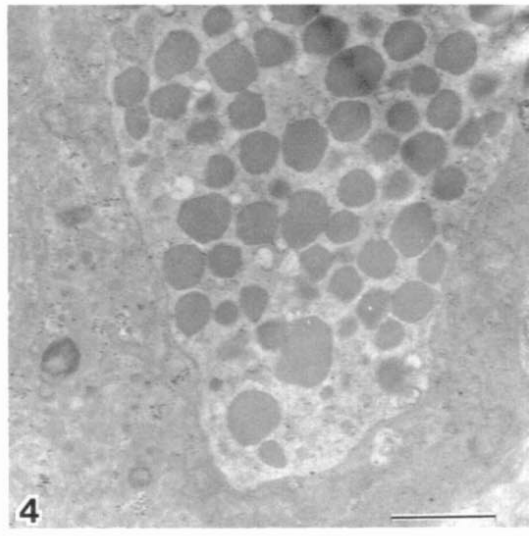
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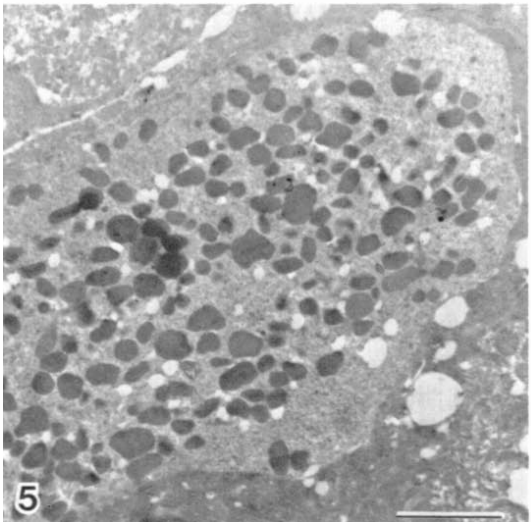
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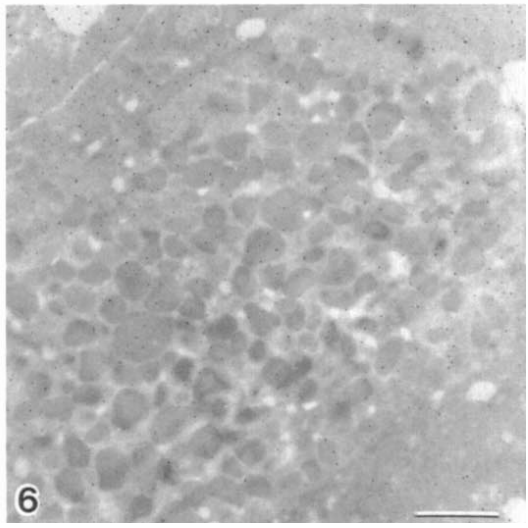
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acetate (6:4) at 25 C. Tissue was then washed in acetone, stained with 2.5% indium trichloride (in acetone) for 2 hours, washed in acetone followed by propylene oxide, and embedded as described. Sections were viewed without heavy metal staining.

DNA: After primary fixation and rinsing, nematodes were dehydrated, infiltrated, and embedded as stated above. Thin sections of the embedded tissue were placed on gold grids and hydrolyzed with 5 N HCl for 30 minutes; control sections were not hydrolyzed. After a distilled water wash, sections were stained with a silver nitrate-methenamine solution for 1 hour at 60 C and rinsed (23). All material was viewed without further staining.

Some control sections for the nucleic acid tests were subjected to digestion by DNase. Two different procedures were employed. 1) After primary fixation, nematodes were dehydrated in 70% ethanol and then incubated for up to 24 hours in DNase (0.1 ml DNase, Type I, Sigma, in 0.15 M NaCl and 1.0 ml 0.05 M Tris); samples were then rinsed, dehydrated, and embedded. 2) Grids containing thin sections of nematodes were immersed in the DNase solution for 24 hours, rinsed, dried, and then incubated in nucleic acid stains.

Protein: After primary fixation, samples were dehydrated and embedded as stated above. Fixation in OsO_4 was omitted. Thin sections mounted on grids were stained with a solution containing 15 g $Co(NO_3)_2$, 70 g NH_2SCN, and 100 ml water (diluted 1:4 with water before use) for 3 minutes at 25 C (10). Experimental grids were rinsed with acetic acid, and control grids were rinsed with distilled water. All sections were viewed without further staining.

Results

Cytochemical tests for the presence of cellulase, peroxidase (pH 4.5), peroxidase (pH 9.4), polyphenoloxidase, β-glucuronidase, DNase, RNase, and carbohydrate in secretory granules were negative in adult females and, when tested, J2 (Table 1). Secretory granules in the dorsal gland in adult females and J2 were negative for acid phosphatase, but granules in the J2 subventral glands stained positive for this enzyme (Fig. 1) as evidenced by the pattern of deposition of lead phosphate. No such dense precipitate was observed in control sections (Fig. 2). In cytochemical tests for peroxidase activity at pH 7.6, granules in the dorsal gland of adult females frequently appeared extremely osmiophilic when compared to granules in sections of control nematodes (Figs. 3, 4). In some repetitions of the procedure, however, secretory granules of experimental and control nematodes appeared similar. Granules in the J2 dorsal and subventral glands were always negative for peroxidase.

The presence of basic protein in secretory granules of adult females was indicated by the cobalt thiocyanate stain when grids containing experimental or control material were compared (Figs. 5, 6). Granules in the J2 dorsal and subventral glands gave negative results when the same basic protein stain was used.

Cytochemical tests using three reagents for nucleic acids in secretory granules in the adult female dorsal gland were positive (Table 1). The three reagents used (sodium

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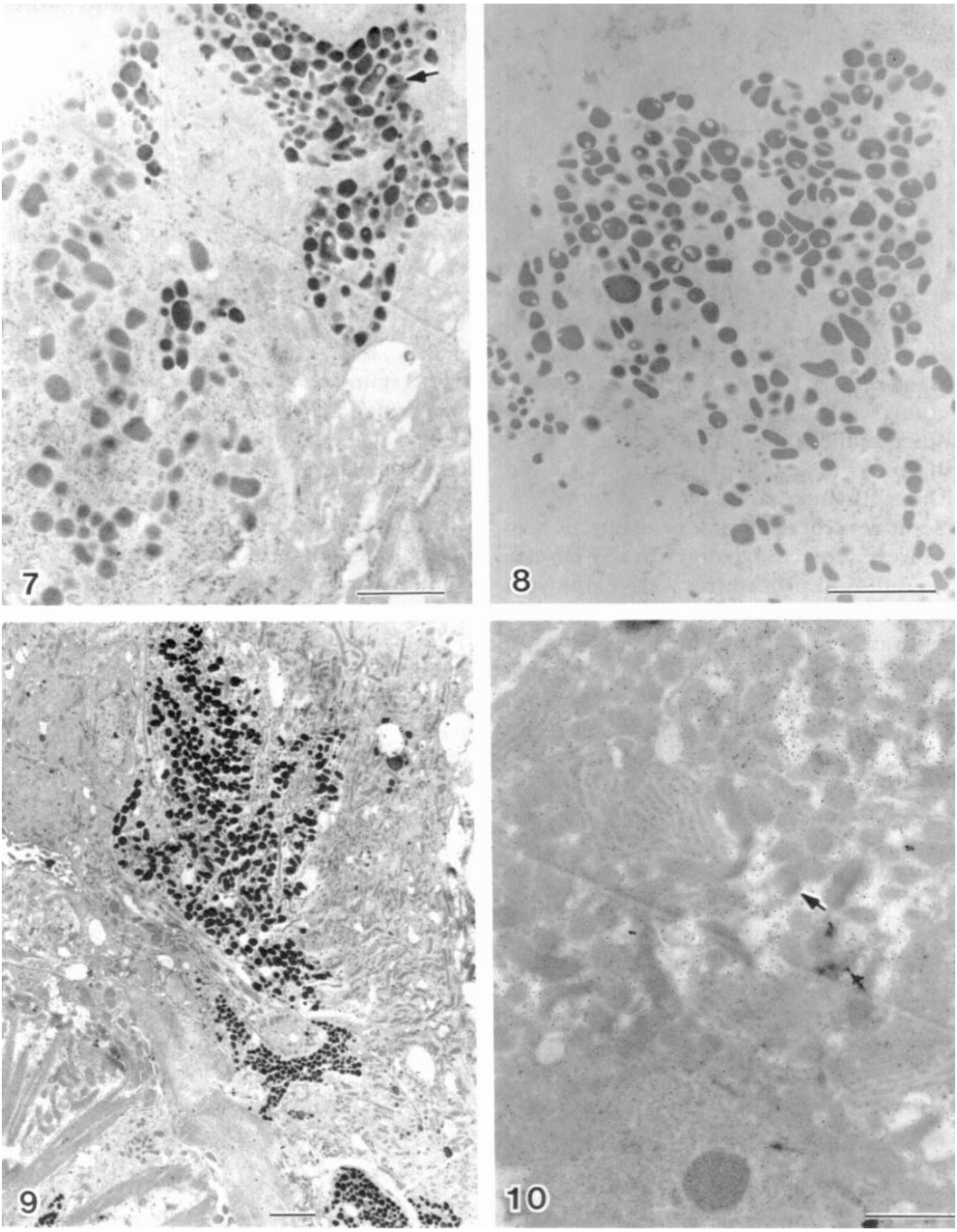
Figs. 1–6. Transmission electron micrographs of secretory granules in esophageal glands in *Meloidogyne incognita*. 1, 2. Granules tested for acid phosphatase in a subventral gland in a preparasitic second-stage juvenile. 1) Lead phosphate deposits on granules (arrow) indicate the presence of acid phosphatase. Bar = 1 μm. 2) Control for acid phosphatase when substrate was omitted from incubation mixture. Bar = 1 μm. 3, 4. Dorsal gland granules of adult female tested for peroxidase at pH 7.6. 3) Experimental material. Bar = 1 μm. 4) Control sample in which hydrogen peroxide was omitted. Bar = 1 μm. 5, 6. Dorsal gland granules of adult female stained for basic protein with cobalt thiocyanate. 5) Section from an experimental grid which received an acetic acid wash after staining. Bar = 2 μm. 6) Control section washed with water after staining. Bar = 1 μm.



FIGS. 7–10. Transmission electron micrographs of secretory granules tested for nucleic acid in the dorsal gland of *Meloidogyne incognita* adult female. 7) Section stained with sodium tungstate. Electron-dense spot is evident in some granules (arrow). Bar = 2 μm. 8) Section stained with indium trichloride. Bar = 2 μm. 9) Section stained by the Feulgen-silver methenamine method. Bar = 2 μm. 10) Control for Feulgen-silver methenamine staining. This grid received no treatment with periodic acid before staining. A faint background of light precipitate is present, but heavy deposition on granules (arrow) is lacking. Bar = 1 μm.

tungstate, indium trichloride, and Feulgen-silver methenamine) indicated the presence of a nucleic acid associated with the secretory granules (Figs. 7–10). Chromatin also stained in treated sections. The fact that most secretory granules in control sections were not electron dense indicated that no stain was deposited; one exception was the DNase-treated sections of nematodes. After 24 hours incubation in DNase, secretory granules and chromatin still gave a positive reaction. The digestion was apparently inadequate, because structures known to contain DNA remained positive; therefore, the DNase treated samples were not valid controls. Secretory granules in the J2 esophageal glands were negative in all tests for nucleic acids; experimental and control sections appeared similar.

Discussion

Secretions of plant-parasitic nematodes involved in pathogenesis are presumably synthesized in esophageal glands. Ultrastructural cytochemistry provides a means for detecting certain compounds directly in the matrix of granules formed in esophageal glands of different life stages of these nematodes. Although acid phosphatase was detected in granules in the subventral glands of *M. incognita* J2, its functional significance is unknown.

Secretory granules in the dorsal esophageal gland of adult female nematodes stained positive for peroxidase, although the reaction occurred only at pH 7.6 and was variable. Poor diffusion of the DAB substrate (19) could account for the inconsistent results. Peroxidase activity has been previously detected in stylet secretions from *M. incognita* adult females (17), but other studies (18,25) indicated that this enzyme activity might be from plant peroxidase ingested by adult females and regurgitated during the incubation procedure. Although our findings indicate that peroxidase might be present in secretory granules, it needs to be confirmed using other methods.

Root cell modifications during parasitism are regulated by *Meloidogyne* species. Secretion of nucleic acid by these nematodes was investigated, even though current evidence provides little support for this type of induction or regulatory mechanism (15). In our study two methods for nucleic acids and one (Feulgen-silver methenamine) for DNA gave positive reactions in secretory granules in the dorsal esophageal gland of adult females and not J2 of *M. incognita*. Of the three methods used to detect nucleic acids, the Feulgen-silver methenamine procedure was the most specific. Hydrolysis with HCl produces aldehyde groups that are extremely reactive, and material that has not been hydrolyzed serves as a control (20). Although granules and nuclear material were not successfully digested by DNase in the present study, electron density was absent in control material in which the other stains, indium trichloride or sodium tungstate, were omitted. Before any conclusions can be made about the presence of nucleic acid in these secretory granules, these results need to be confirmed using other techniques. Ultrastructural studies of esophageal glands indicate that they are sites of synthesis of large amounts of proteins for export and provide no evidence that they are sites where nucleic acids are sequestered for export (15). Nucleic acid was not detected in esophageal gland granules of *M. javanica* adult females (3,5). On the other hand, Cardin and Dalmasso (8) showed in light microscopy studies that granules observed in the lumen of the esophagus of parasitic juveniles and adult females of *Meloidogyne* species stained positive for DNA. The origin of these granules was not established, however.

Secretory granules in the dorsal gland of adult females in both experimental and control specimens were stained by periodic acid-silver methenamine (PAS), indicating nonspecific staining had occurred. Therefore this method was not specific enough to indicate the presence of glycoproteins in the granules, even though earlier studies (5) with adult females showed that these granules were PAS positive.

Granules in the dorsal and subventral

glands of J2 and adult females stained differently, possibly indicating different functions for the two types of glands during the life cycle of the nematode. Whether only secretions from the dorsal gland or both types of glands are involved in pathogenesis has not been determined (15).

Additional ultrastructural cytochemical studies might help elucidate the nature of the contents of secretory granules. However, the usefulness of this technique is limited by the relatively few enzymes that can be localized cytochemically, and negative results are not necessarily proof of the absence of an enzyme. Most cytochemical tests require the substrate to diffuse through tissue to the enzyme site. The relative impermeable cuticle probably restricts substrate diffusion to occur near the cut ends of nematodes. Cellulase was not detected in secretory granules, even though it was previously demonstrated to be exuded from *M. javanica* J2 (6). Nonetheless, the knowledge already obtained from cytochemical studies indicates that the contents of esophageal glands from J2 and adult females of *Meloidogyne* species are distinctively different.

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