# Electron Microscope Characterization of Carbohydrate Residues on the Body Wall of Xiphinema index[1]

Y. SPIEGEL,[2] W. M. ROBERTSON[3], S. HIMMELHOCH[4] and B. M. ZUCKERMAN[5]

*Abstract:* The location of carbohydrate moieties on the outer cuticle of *Xiphinema index* was examined by electron microscopy using several different reagents: a) The periodic acid-thiosemicarbazide-silver proteinate reaction was used as a general stain for carbohydrates. In sectioned material it stained the canal system and deeper layers of the cuticle as well as the outer surface. b) Cationized ferritin at pH 2.5, which identifies carboxyl and sulfate groups, was used to identify sialic acid residues and also labelled parts of the canal system. c) Ferritin-goat anti rabbit IgG coupled to a DNP ligand was used to label either sialyl or galactosyl/N-acetyl-D-galactosaminyl residues. d) Ferritin hydrazide, a new reagent, was used for the ultrastructural localization of glyco-conjugates. Reagents c) (with appropriate antisera) and d) were applied only to the outer surfaces of the cuticle; they showed that sialic acid residues were concentrated mainly on the outer body wall of the head, the lips, oral opening, amphid apertures, and outer surface of protruded odontostyles. Ferritin distribution was not altered by pretreatment with neuraminidase. Galactose oxidase treatments revealed galactose/N-acetyl-D-galactosamine residues along the entire body wall. These results confirmed earlier findings obtained by fluorescence microscopy. *Key words:* outer cuticle surface, cuticular canal system, sialic acid, galactose, electron microscopy, cryoultramicrotomy, cationized ferritin, ferritin hydrazide.

Journal of Nematology 15(4):528-534. 1983.

During recent years, a considerable body of evidence has accumulated which indicates that sugar moieties on the cell surface play a decisive role in intercellular communication (2) and in host parasite recognition (5). In a recent study, two fluorescent labelling techniques specific for sialic acid, galactose, and N-acetyl-D-galactosamine were used to locate these carbohydrate moieties on the outer body wall of *Xiphinema index* and several other plant parasitic nematodes (9).

The object of the current study was to characterize and map glycoconjugates and other sugar moieties on the surface of the body wall and in the upper layers of the cuticle in the plant parasitic nematode *X. index,* using four different reagents: a) periodic acid-thiosemicarbazide-silver proteinate, b) cationized-ferritin at pH 2.5, c) EM modification to the aforementioned fluorescence techniques, and d) ferritin-hydrazide.

## MATERIALS AND METHODS

*Abbreviations:* 2,4 dinitrophenyl (DNP); [4]N-dinitrophenyl-L-2,4, diaminobutyric acid hydrazide (DNP-DABH); ferritin hydrazide (FHZ); periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP); phosphate buffered saline, pH 7.4 (PBS); sodium cacodylate buffer, pH 7.2 (SCB).

*Nematodes:* Males, females, and larvae of *X. index* were extracted from soil around fig trees, washed three times with PBS, and

Received for publication 27 September 1982.

[1]Contribution from the Agricultural Research Organization (ARO), No. 568-E, 1982 Series.

[2]Division of Nematology, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50-250, Israel.

[3]Scottish Crop Research Institute, Dundee, DD2 5DA, Scotland.

[4]Section of Biological Ultrastructure, The Weizmann Institute of Science, Rehovot 76100, Israel.

[5]Department of Plant Pathology, CFNR, University of Massachusetts, Amherst, MA 01003.

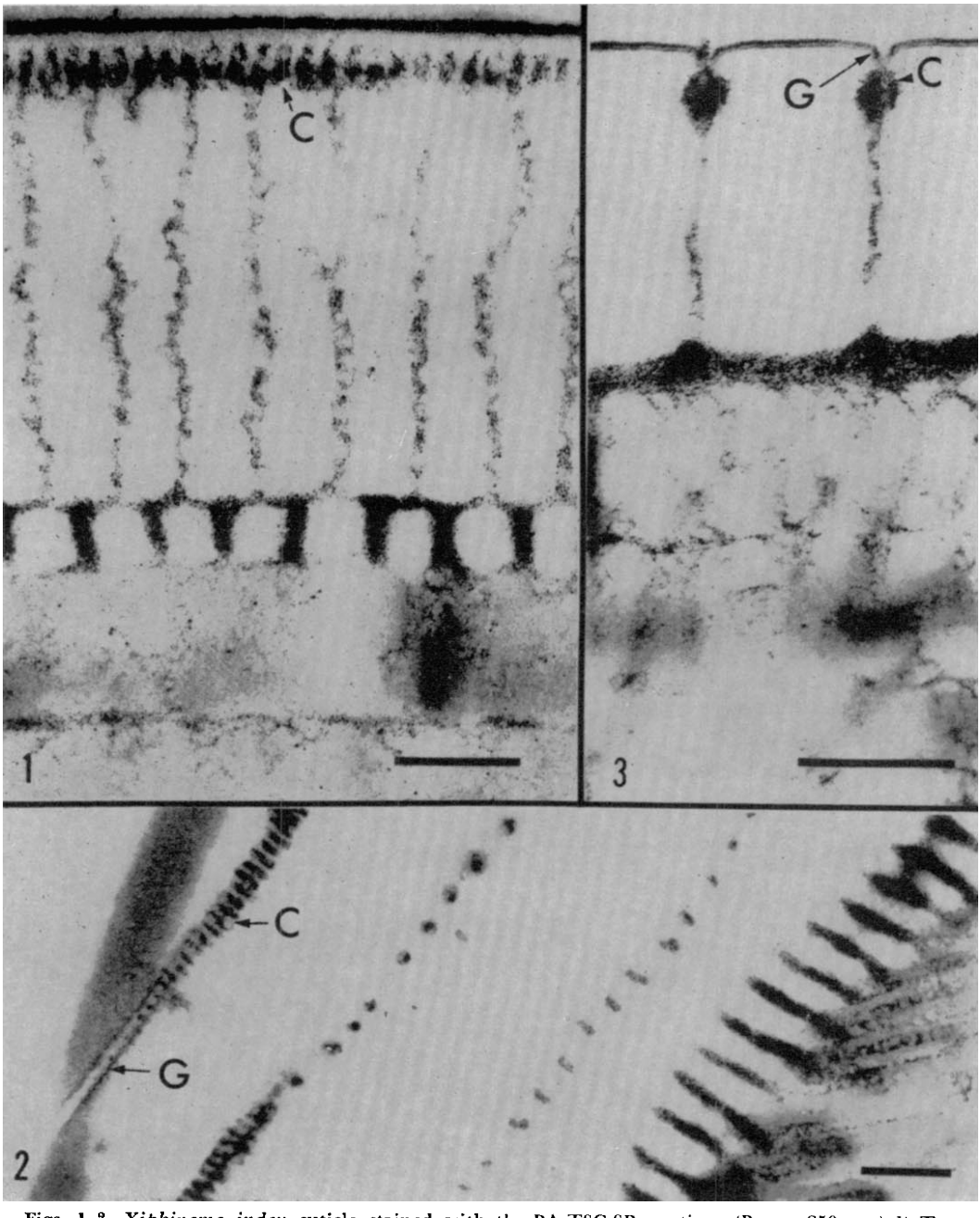
The authors thank Drs. C. E. Taylor and D. L. Trudgill of the S.C.R.I., Dundee, Scotland, and Dr. E. Cohn of the ARO, The Volcani Center, Bet Dagan, Israel, for reviewing the manuscript and for constructive advice. They also thank Mrs. C. Henry for assistance in the electron microscopy and Dr. Sarah Spiegel for her special help.

used immediately or kept at 5 C for no longer than 3 days.

*Chemicals:* Cationized ferritin (11.5 mg/ml) was obtained from Miles Laboratories, Rehovot, Israel.

DNP-DABH and purified rabbit-anti DNP were provided by M. Wilchek of the Weizmann Institute of Science, Rehovot, Israel.

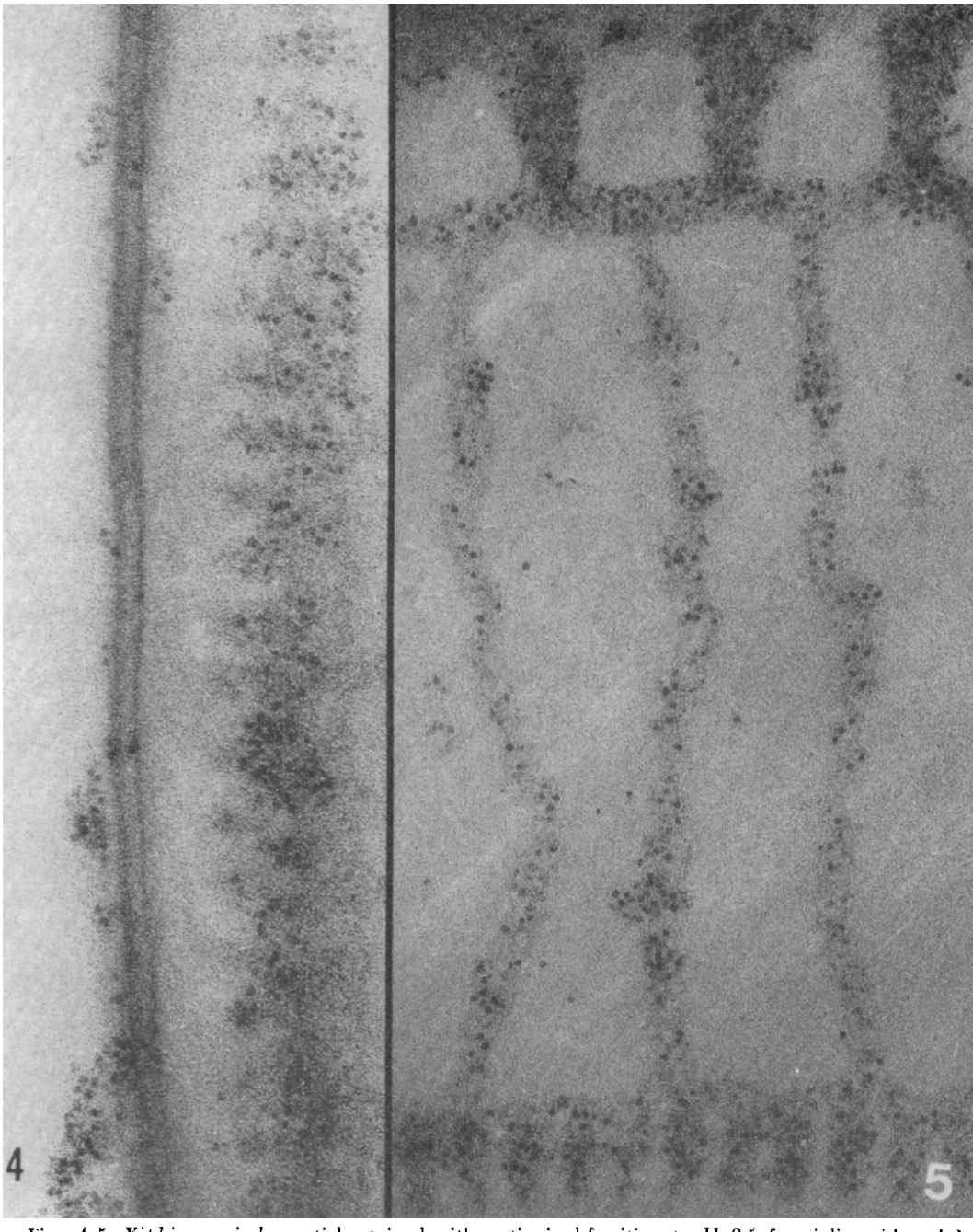
Emix resin was supplied by Emscope Laboratories, Ashford, Kent, U.K. Ferritin hydrazide was provided by E. Roffmann of the Weizmann Institute of Science. Ferritin-labelled goat anti rabbit IgG (1 mg/ml) was purchased from Miles Laboratories. Galactose oxidase (150 units) was



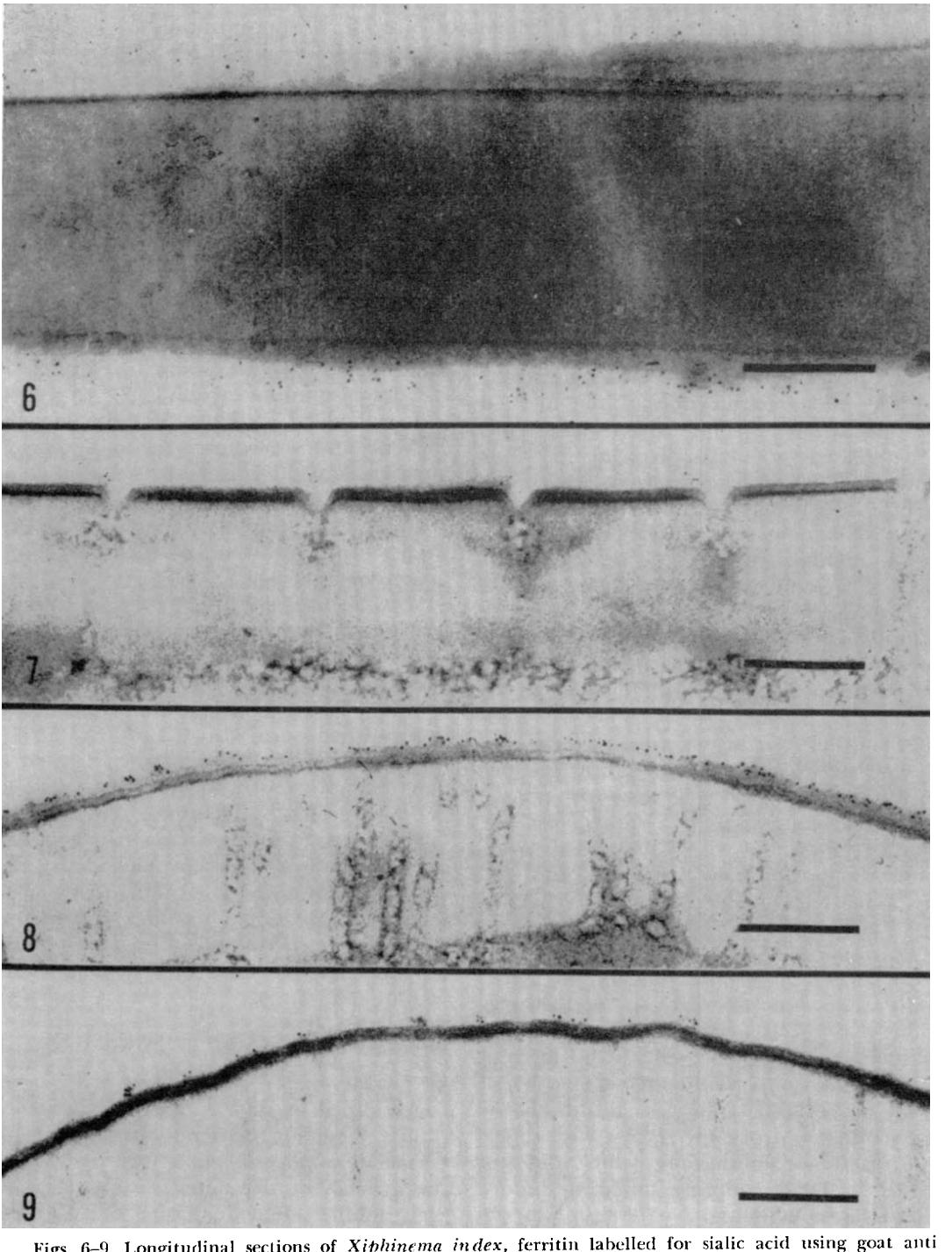

Figs. 1–3. *Xiphinema index* cuticle stained with the PA-TSC-SP reaction. (Bar = 250 nm.) 1) Transverse section showing canal system (C) which opens into the grooves in the cuticle. 2) Oblique section. Note canal system (C) at base of grooves (G). 3) Longitodinal section of juvenile cuticle showing the canal (C) and double outer membrane between grooves (G).

purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Glutaraldehyde, SCB, osmium tetroxide, and the embedding medium, Poly/Bed 812, were obtained from Poly Sciences. Neuraminidase (500 units/ml) was obtained from Boehringwerke, Marburg, W. Germany. Other PA-TSC-SP reagents were supplied by BDH Chemicals, Ltd., Poole, Dorset, U.K.

*General sugar characterization: Xiphinema index* were fixed in 3% paraformaldehyde in 0.1 *M* PBS for 1 hour, rinsed three times in PBS, embedded in 1% agar, and dehydrated in a graded ethanol series.




Figs. 4–5. *Xiphinema index* cuticle stained with cationized-ferritin at pH 2.5 for sialic acid and labelling (× 150,000). 4) Cuticle on side of the head. 5) Transverse section showing canal system (C) which opens into the grooves in the cuticle. Staining was carried out on ultrathin frozen sections.




Figs. 6–9. Longitudinal sections of *Xiphinema index*, ferritin labelled for sialic acid using goat anti rabbit serum (6,7,8) and ferritin hydrazide (9). 6) Odontostyle. 7) Cuticle on side of head. 8, 9) Anterior head region.

Specimens were transferred through two changes of propylene oxide and embedded in Emix (medium) resin. Thin sections were mounted on plastic-coated gold grids and stained using PA-TSC-SP reaction following the procedure of Lewis and Knight (6). Sections were viewed in a Hitachi HS-8 electron microscope operated at 50 KV using a 50-$\mu$m objective aperture.

*Sialic acid labelling:* i) *X. index* to be treated with cationized ferritin were fixed in a mixture of 2% glutaraldehyde and 1% paraformaldehyde, washed in PBS, infiltrated with 0.6 *M* sucrose in PBS, frozen in liquid nitrogen, and freeze-sectioned according to a modification of the method used by Tokuyashi and Singer (11). Grids were floated on 0.1 *M* glycerine in water and labelled with cationized ferritin at pH 2.5 according to Thiery and Ovtracht (10). Nematode sections were stained with 1% uranyl acetate (pH 7.0), rinsed quickly with water, and restained with 2% uranyl acetate. Sections were examined in either a Phillips 300 or JEM 7 electron microscope operated at 100 KV. This labelling identifies both carboxyl and sulfate groups. To verify the specificity of the observed binding, parallel control experiments were conducted in which frozen ultrathin sections of human red blood cells were first treated with neuraminidase for 2 hours at 37 C, washed four times with PBS, and then exposed to cationized ferritin at pH 2.5. ii) Nematodes to be reacted with ferritin goat anti rabbit IgG were suspended in PBS containing DNP-DABH, hydrogenated with potassium borohydride, and incubated with rabbit anti DNP IgG as described previously (9). The nematode suspension was washed twice, incubated with ferritin-labelled goat anti rabbit IgG (1 mg/ml) at 0 C for 30 min, and washed thoroughly with PBS. Control experiments with different reaction combinations which did not result in DNP coupling failed to induce this ferritin labelling. No ferritin was observed in the presence of unrelated normal rabbit IgG instead of rabbit anti DNP IgG. iii) Nematodes to be labelled with FHZ were first suspended in PBS at 0 C and 0.5 ml of 3 m*M* sodium periodate. After 20 min at 0 C, the nematodes were washed twice with PBS and once with 0.05 *M* SCB of PBS. The FHZ solution (0.5 ml) (7) was added to the suspended nematodes to give final concentration of 0.5 mg/ml FHZ, and the reaction was allowed to proceed for 30 min at room temperature.

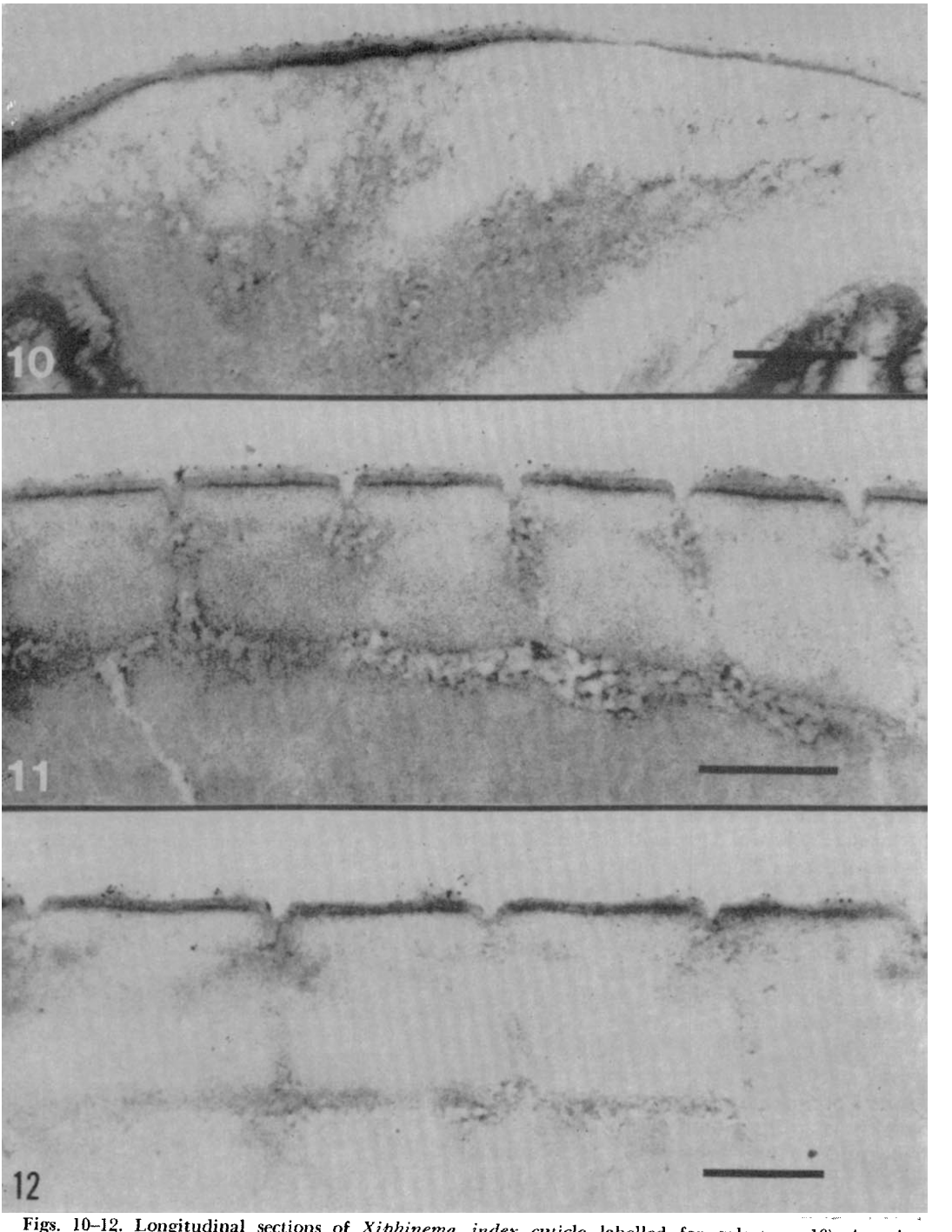
To obtain sections through nematodes treated with ferritin-labelled goat anti rabbit IgG or FHZ, specimens were washed twice with PBS and once with 0.05 *M* SCB and resuspended in 0.5 ml of SCB. Glutaraldehyde in SCB was added to give a final concentration of 0.8%. After 30 min at room temperature, the nematodes were washed twice with SCB and post fixed for 1 hour with 1% osmium tetraoxide in SCB. The nematodes were dehydrated in ethanolic solutions (from 50% to 100%) and embedded in Poly/Bed medium by conventional procedures. Sections approximately 500Å thick were cut, mounted on copper grids and examined in a Hitachi HS-8 electron microscope at 50 KV. Appropriate regions were recorded at magnifications of × 23,000.

*D-Galactose and N-acetylgalactosamine labellings:* Nematodes suspended in PBS (free of $Ca^{++}$ and $Mg^{++}$) were treated with galactose oxidase (5 units/ml) for 30 min at 37 C. After incubation, the nematodes were washed once with 0.2 *M* D-galactose, three times with PBS, and then treated as described previously for the sialic acid labellings, either by technique (ii) or (iii). Control experiments in which galactose oxidase was excluded were run in parallel.

*Neuraminidase pretreatment:* Prior to sialic acid labelling, 50 units/ ml of neuraminidase in special salt medium, pH 6.5 (8), were applied to washed nematodes for 60 min at 37 C.

## RESULTS AND DISCUSSION

Larvae, males, and females of *X. index* were stained by the PA-TSC-SP reaction. A positive reaction occurred throughout the cuticular canal system, which opens into the grooves of the outer cuticle (Figs. 1, 2): a double outer membrane was observed between the grooves (Fig. 3). Some sections were also treated with sodium borohydride before and after treatment with periodic acid, but no differences in the staining was observed. Sodium borohydride applied after the periodic acid treatment blocked all staining. Although this staining procedure is specific for carbohydrates (6), it still re-




Figs. 10–12. Longitudinal sections of *Xiphinema index* cuticle labelled for galactose. 10) Anterior head region. 11) Side of head just posterior to amphidial opening. 12) Main body cuticle.

acts with a wide group of polysaccharides, mucopolysaccharides, and glycoproteins. Using cationized ferritin at pH 2.5 enabled us to restrict the labelling only to carbohydrates carrying carboxyl and sulfate groups.

Frozen sections from the head area of *X. index* treated with cationized ferritin at pH 2.5 revealed the same labelling pattern obtained with the PA-TSC-SP reagents (Figs. 4, 5), suggesting that the components being stained were mainly mucopolysaccha-

ride residues (10). Pretreatment with neuraminidase did not change the pattern of staining. Control experiments with human red blood cells, treated with cationized ferritin and not treated with neuraminidase, were found to be labelled. Pretreatment with neuraminidase gave no labelling.

Alcian blue (pH 0.5—1.0) was used to differentiate between the carboxyl and sulphate groups (10). No reduction of labelling was noticed. We can, therefore, assume that we are dealing with carboxyl groups.

Ferritin hydrazide and ferritin goat anti rabbit IgG, after periodate treatment, reacted positively with larvae, males, and females of *X. index* (Figs. 6–9). These reagents (specific for sialic acid) revealed the presence of this sugar all over the body wall of the head; ferritin was concentrated mainly on the lips, oral opening, amphid apertures, and odontostyle. Farther back the nematode outer body wall did not show any ferritin labelling, thus confirming previous results obtained with fluorescent labelled antibody (9). Control experiments, in which sodium periodate treatment or the FHZ reagent was exluded, were run in parallel and gave no labelling. The distribution of ferritin was apparently not altered by prior treatment of the nematodes with neuraminidase. This is consistent with the reports of Himmelhoch and Zuckerman (3) and Himmelhoch et al. (4) who failed to reduce the labelling density of cationized ferritin with neuraminidase on *Caenorhabditis briggsae* and *Meloidogyne javanica,* and also agrees with earlier work on *X. index* using fluorescent microscopy (9). The inability of the neuraminidase to reduce the density of the ferritin labelling could be explained if the major constituent of reactive sialic acid residues occurs not on glycoproteins but on glycolipids (e.g., gangliosides), which are inaccessible to neuraminidase. Neuraminidase from *Vibrio cholerae* has failed to hydrolyze ganglioside sialic acids on the cell outer surface, although it acts on isolated gangliosides which contain terminated sialic acid. It is thought that cell surface sialolipids are localized within the plasma membrane in such a way that they are rendered sterically inaccessible to *V. cholerae* neuraminidase (1).

Sections from different regions of the outer body wall of *X. index,* after treating the nematodes with galactose oxidase followed by the double antibody technique or the FHZ, exhibited galactose and/or N-acetylgalactosamine residues along the nematode outer body wall (Figs. 10–12). Again, these results are in accordance with earlier finds obtained with fluorescence microscopy (9). Control experiments gave no labellings.

Ferritin staining in the grooves of the cuticular canal system indicates that the carbohydrates found on the body wall surface may have been secreted from inner layers, perhaps as a reaction to the chemical reagents.

## LITERATURE CITED

1. Barton, V. W., and A. Rosenberg. 1973. Action of *Vibrio cholerae* neuraminidase (sialidase) upon the surface of intact cells and their isolated sialolipid component. J. Biol. Chem. 248:7353-7358.

2. Cook, G. M. W., and R. W. Stoddart. 1973. Surface carbohydrates of the eukaryotic cell. London and New York: Academic Press.

3. Himmelhoch, S., and B. M. Zuckerman. 1978. *Caenorhabditis briggsae:* aging and the structural turnover of the outer cuticle surface and the intestine. Exp. Parasitol. 45:208-214.

4. Himmelhoch, S., D. Orion, and B. M. Zuckerman. 1979. Partial characterization of the cuticle surface of *Meloidogyne javanica* females. J. Nematol. 11:358-362.

5. Hugh, R. C. 1976. Membrane glycoproteins—a review of structure and function. London and Boston: Butterworths.

6. Lewis, P. R., and D. P. Knight. 1977. Staining methods for sectioned material. *In* A. M. Glauert, ed. Practical methods in electron microscopy. Amsterdam, New York, and Oxford: North-Holland Publishing Company.

7. Roffman, E., Y. Spiegel, and M. Wilchek. 1980. Ferritin hydrazide, a novel covalent electron dense reagent for the ultrastructural localization of glycoconjugates. Biochem. Biophys. Res. Commun. 97:1192-1198.

8. Skutelsky, E., D. Danon, M. Wilchek, and E. A. Bayer. 1977. Localization of sialyl residues on cell surfaces by affinity cytochemistry. J. Ultrastruc. Res. 61: 325-335.

9. Spiegel, Y., E. Cohn and S. Spiegel. 1982. Characterization of sialyl and galactosyl residues on the body wall of different plant parasitic nematodes. J. Nematol. 14:33-39.

10. Thiery, J. P., and J. Ovtracht. 1979. Differential characterization of carboxyl and sulfate groups in thin sections for electron microscopy. Biol. Cellul. 36:281-288.

11. Tokuyashi, K. T., and S. J. Singer. 1976. Improved procedures for immunoferritin labelling of ultrathin frozen sections. J. Cell Biol. 71:894-906 (with slight technical modifications).