Cuticle Formation in *Hemicycliophora arenaria*, *Aphelenchus avenae* and *Hirschmanniella gracilis*[1]

P. W. JOHNSON, S. D. VAN GUNDY AND W. W. THOMSON[2]

Abstract: The onset of molting in all stages of *Hemicycliophora arenaria* was preceded by the appearance of numerous, discrete globular structures which were termed "molting bodies" because they were present in the hypodermis only during the production of the new cuticle. In all parasitic stages the molt commenced with the separation of the cuticle from the hypodermis from which the new sheath and cuticle were differentiated. Following completion of the new sheath and cuticle most of the old outer covering was apparently absorbed before ecdysis. Electronmicrographs of body wall cross sections in molting L4 male specimens revealed the final molt to be a double molt in which an additional sixth cuticle was produced. Since both a new sheath and cuticle were produced during the molt of each stage, the sheath must be considered as an integral part of the cuticle and not as a residual cuticle or the result of an incomplete additional molt. Molting in *Aphelenchus avenae* and *Hirschmanniella gracilis* was less complex and "molting bodies" were not observed. After cuticle separation the hypodermis gave rise to a new trilaminate zone, the future cortex, and (later) the matrix and striated basal layers. *Key Words:* Cuticle, Molting, Ultrastructure, *Hemicycliophora arenaria, Aphelenchus avenae, Hirschmanniella gracilis.*

The generalized life cycle of free-living or parasitic nematodes consists of six stages, egg, four larval stages and adult. Development beyond the first larval stage depends upon successful molting; a process about which very little is known, particularly at the ultrastructure level. The electron microscope studies by Bird and Rogers (1), Lee (9) Samoiloff and Pasternak (13) and Watson (18) are the only ones dealing with nematode molting.

The molting nematode may be at one of the weakest points in its life cycle for withstanding periods of stress (17). The main purpose of the present ultrastructural studies of molting was to gain a better understanding of both the molting process and cuticle structure. Considerable controversy exists concerning the origin of the sheath in *Hemicycliophora arenaria* in particular and *Hemicycliophora* spp. and *Hemicriconemoides* spp. in general. A second purpose of this study was to clarify this situation. Comparative ultrastructural studies of cuticle formation in *H. arenaria, Aphelenchus avenae* and *Hirschmaniella gracilis* were undertaken with these objectives in mind.

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[2] Department of Nematology and Department of Life Sciences, University of California, Riverside, California 92502. Present address of senior author: Canada Department of Agriculture, Research Station, Harrow, Ontario, Canada.

MATERIALS AND METHODS

All nematodes were maintained and extracted as previously reported (7). The molting sequence in *H. arenaria* was studied in active, handpicked, fourth-stage (L4) male and female larvae held in tap water in a moist chamber at 26.5 C. When nematodes became quiescent and relaxed, it was assumed the molting process had been initiated and this was arbitrarily called "time zero." Molting specimens were selected after various time intervals, depending on the developmental stage of the nematode, from time zero to exsheathment. Second and third-stage (L2, L3) molting larvae were visually selected in various stages of the molt, without regard to time, directly from the washings from the 325-mesh screen. Molting specimens of *A. avenae* and *H. gracilis* were selected directly from the washings without regard to elapsed time in the molt.

All histological techniques were the same as those previously described (7).

RESULTS

HEMICYCLIOPHORA ARENARIA: The onset of molting in all stages was preceded by the appearance of numerous, peculiar, globular structures, termed "molting bodies" (Fig. 1-A, D): intimately associated with the production of the new cuticular outer covering appearing just prior to molting and disappearing shortly after its completion. They varied considerably in size (0.2–1.2 μ in diameter) and were confined to the hypodermal chords (Fig. 1-A). Internally the molting bodies exhibited three distinctive types of substructure: crystalline areas, discoid structures and amorphous areas (Fig. 1-B). In some sections they appeared to be releasing material into the hypodermis (Fig. 1-C, arrow). In later stages of the molt, molting bodies were frequently observed to be deteriorating (Fig. 1-D). Just prior to and during the molt, the hypodermis stained more intensely than during intermolt periods (Fig. 1-C).

In L4 female specimens (at 26.5 C the molting and exsheathing processes required approximately 72 hr) the first ultrastructural sign of molting was an increase in thickness of the interchordal hypodermis (Fig. 2-B) concurrent with its separation from the old cuticle (Fig. 2-A, B, arrows). An electron dense (osmiophilic), multilayered region then formed at the outer edge of the hypodermis as the fibrillar matrix and striated basal layer began to differentiate (Fig. 3-A, B, C). At this time many folds began to form in the new cuticle (Fig. 3-B, C, arrows, Fig. 4-D) and the fibrillar structure of the matrix was easily distinguished (Fig. 4-A, B). The matrix also increased in thickness as the striated basal layer became further differentiated (Fig. 4-A, C).

Approximately 50 hr after initiation of the molt, the trilaminate cortex of the new cuticle became visible (Fig. 5-A, arrow) in the trailing edge of the multilayered region, resulting in a decrease in its total thickness (Fig. 5-A). Continued differentiation of electron-dense layers (Fig. 5-B, arrows) occurred in the multilayered region as the new sheath developed (Fig. 5-B, C.S5). The outer osmiophilic layers of the sheath were probably derived from what remained of the multilayered region after the other osmiophilic layers of the cuticle and sheath had been differentiated from it. The new cuticle and sheath were essentially complete and the old

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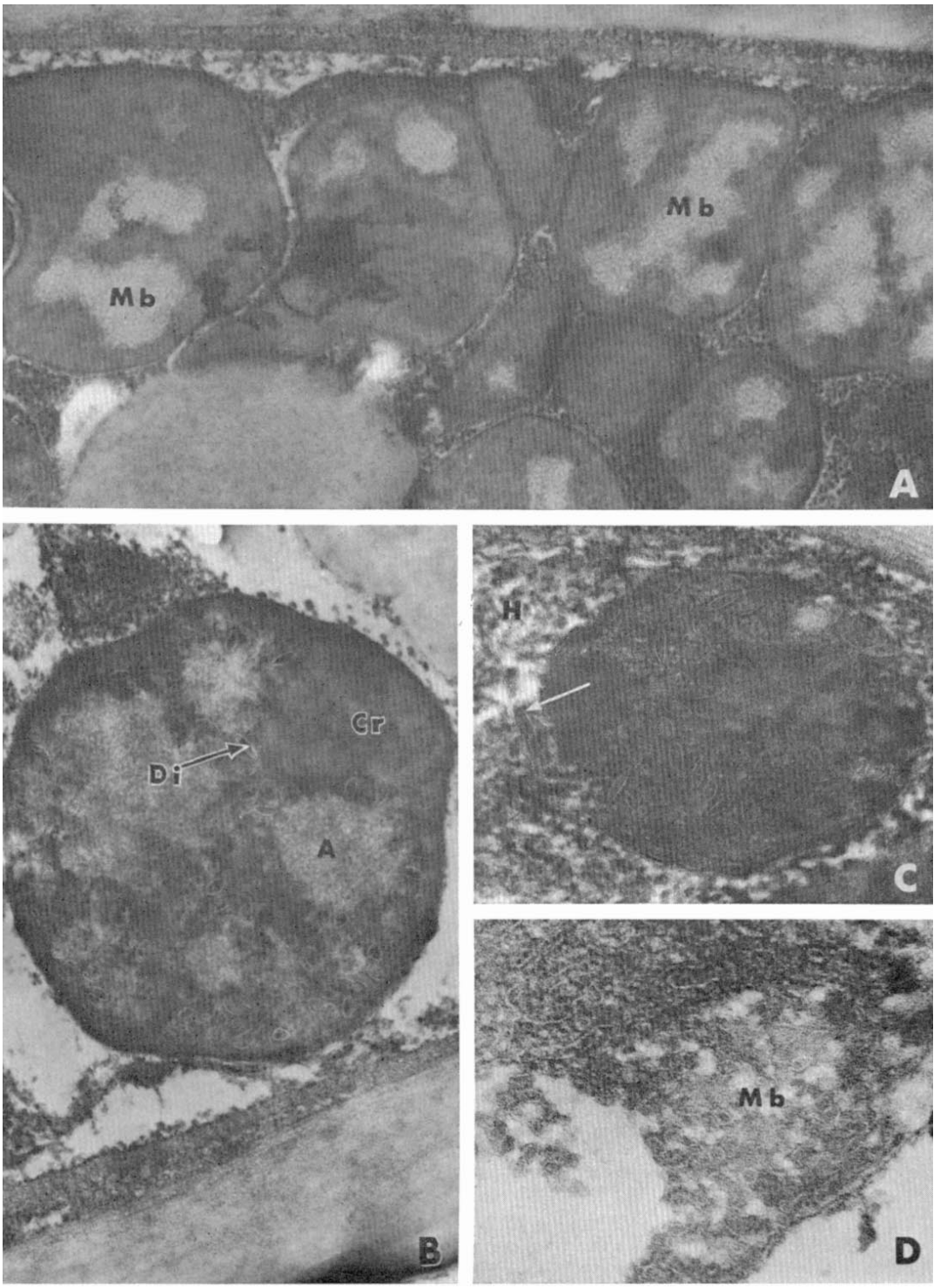
FIG. 1. Electron micrographs of molting bodies in *H. arenaria*. **A**. Longitudinal section just prior to molt with numerous molting bodies in chordal area. × 53,000; **B**. Transverse section of large molting body showing three types of substructure. × 47,000; **C**. Transverse section showing molting body apparently releasing (arrow) material into hypodermis. × 78,000; **D**. Transverse section showing molting body in apparent state of breakdown in late stages of the molt. × 78,000.

KEY TO LETTERING OF FIGURES

A. amorphous area in molting body
bl. basal lamella
C. cortex
Cr. crystalline area in molting body
Cu. cuticle
Cu2, Cu3, etc. cuticle of stage designated by numeral
Cu6. sixth cuticle of male *H. arenaria*
Di. discoid shaped structure in molting body
F. fibrillar layer
F1, F2, etc. fibrillar layers signified in centripetal order
H. hypodermis
M. matrix
Mb. molting body
Ms. modified sheath of male *H. arenaria*
Mu. somatic musculature
R. multilayered region
S. sheath
S2, S3, etc. sheath of stage designated by numeral
St. striated layer
T. trilaminate zone


Mb
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striated layer was no longer visible (Fig. 5-C) after approximately 60 hr.

During the next 10–12 hr much of the old cuticle and sheath degenerated and was either reabsorbed or simply broken down. Observations of micrographs indicate material from the old cuticle and sheath may have been reabsorbed (Fig. 5-D, arrows) although conclusive proof has not been obtained. After approximately 72 hr, only remnants of the old cuticle and sheath remained (Fig. 6-A, B). The exsheathment process in water required as little as 15 min to occasionally more than 24 hr and resembled the molt of *Caenorhabditis briggsae* (6).

Molting of **L2** larvae (Fig. 7-A, B) and **L3** larvae (Fig. 7-C, D) were comparable to molting of the **L4** females; in each case a new sheath and cuticle were produced. There was no evidence of retention of the sheath and/or cuticle of the previous stage.

L4 male specimens, also required approximately 72 hr for molting but actually underwent a double molt; two new cuticles, only the second of which included a sheath, were produced. The first of these new cuticles was shed at ecdysis.

Initially, as in the other stages, the hypodermis thickened and separated from the old **L4** cuticle (Fig. 8-A, arrows); a trilaminate osmiophilic zone formed at the outer edge of the hypodermis (Fig. 8-B, arrows), a striated layer formed inside this (Fig. 8-B, C), and a narrow fibrillar matrix region appeared between the two (Fig. 9-A, B). Within 25–30 hr after the beginning of the molt a new cuticle consisting of a trilaminate cortex, a matrix and a striated basal layer was complete (Fig. 9-A) and the striated and matrix layers of the old **L4** cuticle appeared to be breaking down (Fig. 9-A, B). Some micrographs (Fig. 9-C, arrows) suggest that this breakdown material was probably reabsorbed but, as with the **L4** females, absolute proof of this was not obtained. The cuticle breakdown may or may not be completed before the next stage of the molt, 30–40 hr after molt initiation.

The hypodermis separated from the newly-completed cuticle (Fig. 10-A, Cu5) and a densely staining (osmiophilic) multilayered region, similar to that formed in the other stages, was formed at its outer edge (Fig. 10-A, arrows). A new striated layer formed inside this region (Fig. 10-B). The multilayered region and striated layer became further separated as the fibrillar matrix layer continued to increase in thickness (Fig. 10-C) and the trilaminate cortical zone and modified four-layered sheath became differentiated (Figs. 10-C, D; 11-A). In the region of the lateral field, the striated layer was forked (Fig. 10-D, arrows) and replaced by a layer of fibrillar material which appeared as two layers in mature male specimens. Dense-staining material accumulated at the base of the matrix adjacent to the striated layer (Fig. 10-C) and represented an apparent fluid-filled space in adult males (Fig. 11-B).

In all stages of *H. arenaria*, breakdown and reformation of the somatic musculature was observed concurrent with molting. In premolt larvae (Fig. 12-A) the somatic musculature was still intact; however, as the molt commenced deterioration started (Fig. 12-B) after 5–10 hr and proceeded through 40 hr (Fig. 12-C). Muscle reformation was essentially complete after 60–65 hr (Fig. 11-A).

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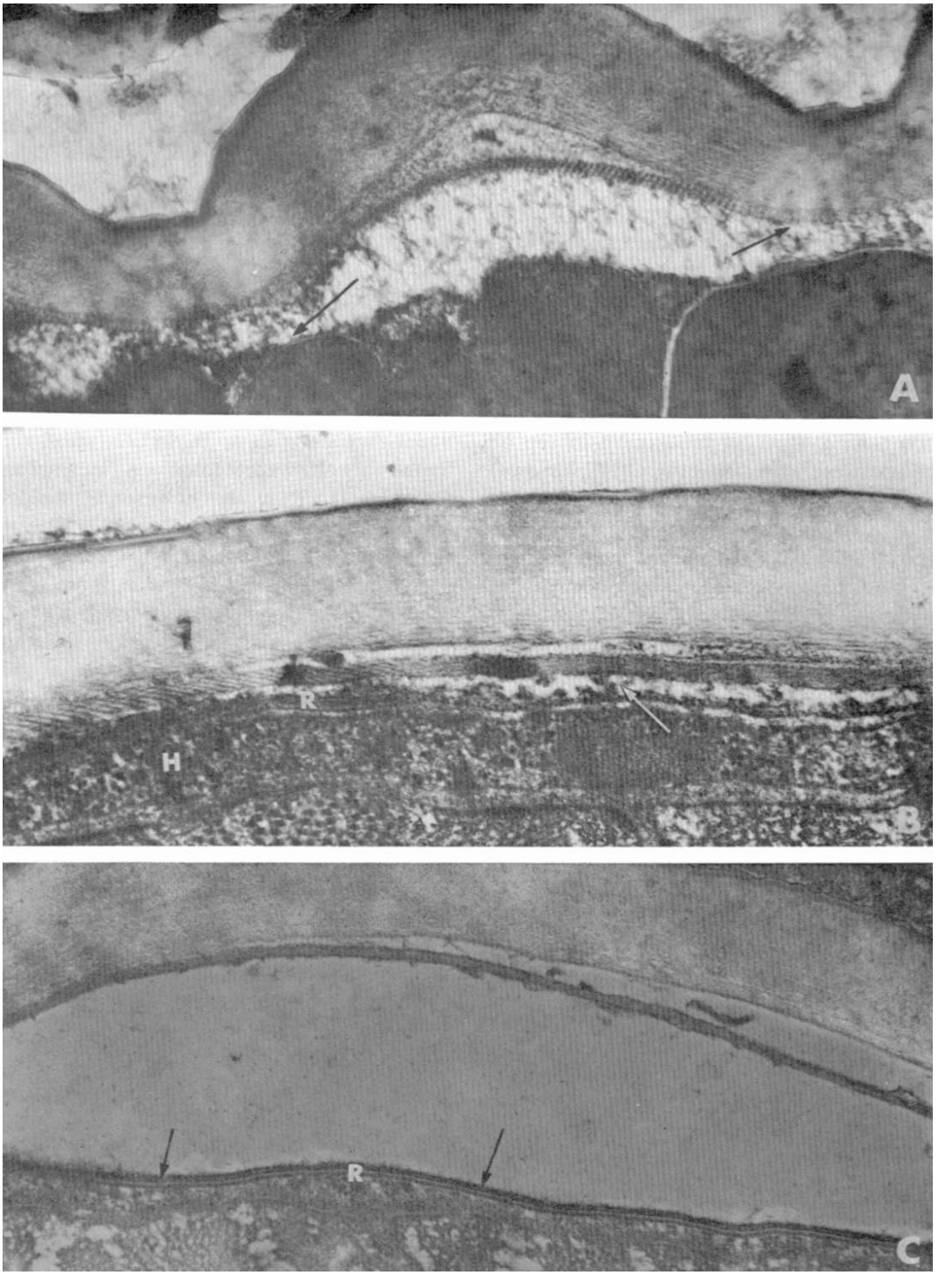
FIG. 2. Electron micrographs of molting *H. arenaria* **L4** females. **A.** Longitudinal section 0–10 hr in molt. Note separation of hypodermis from old cuticle (arrows). × 37,500; **B.** Transverse section showing thickened hypodermis and forming (osmiophilic) multilayered region. × 51,500; **C.** Transverse section showing differentiated multilayered region (arrows). × 37,500.


A
R
H
B
R
C

APHELENCHUS AVENAE: The molting process of *A. avenae* appeared to be a simpler process than in *H. arenaria*. No molting bodies were seen and although the hypodermis became densely stained, no great increase in thickness of the interchordal zone was noted.

The hypodermis separated from the old cuticle and a trilaminate zone formed at its outer edge (Fig. 13-A, arrow). Immediately interior of this trilaminate zone (cortex), the striated layer was formed (Fig. 13-B) and continued to develop as the fibrillar matrix layer appeared between the two (Fig. 13-B, C). As the new cuticle approached completion, signs of breakdown in the old cuticle were observed (Fig. 13-C, arrows). Some folding of the new cuticle was observed during the molt, particularly in the area of the lateral fields, but less than in *H. arenaria*.

HIRSCHMANNIELLA GRACILIS: Only a limited number of molting *H. gracilis* specimens were observed. Molting was similar to that described for *A. avenae* with the separation of the hypodermis from the old cuticle followed by the differentiation of the various layers of the new cuticle (Fig. 14-B). As molting neared completion, signs of breakdown in the old cuticle were also observed (Fig. 14-C, D).

In the region of the lateral field, the striated basal layer tapered off and was replaced by four fibrillar layers (Fig. 14-D). Densely-staining material accumulated at the base of the matrix layer adjacent to the striated layer (Fig. 14-B, C). At maturity this area represented an apparent fluid-filled space. Some folding of the new cuticle was observed during the molt, particularly in the area of the lateral field (Fig. 14-A), but not as much as in *H. arenaria*.

DISCUSSION

The molting process and sheath formation in *Hemicycliophora* and *Hemicriconemoides* has been a controversial subject. All stages of *Hemicycliophora*, except the male, have been reported to possess an extra cuticle or sheath. It has now been shown that a modified sheath is also present in the male (7). The extra cuticle (sheath) of the larval stages and the female has been described as the retention of the previous larval cuticle by Tarjan (14), Thorne (15), Colbran (2), Luc (11) and Raski (12). Van Gundy (16, 17) suggested the sheath on a female *Hemicycliophora arenaria* was not a larval cuticle, but an additional sixth cuticle representing an incomplete fifth molt. Fassuliotis (5) suggested that *Hemicriconemoides chitwoodi* underwent only three molts and that the female sheath represented an incomplete fourth molt. Recently, however, Dasgupta *et al.* (3) have shown that *H. chitwoodi* does undergo four molts and have suggested that, as in *H. arenaria*, the female sheath probably represents an incomplete fifth molt.

The results reported here show conclusively that the sheath in *H. arenaria*, and probably in all *Hemicycliophora* and *Hemicriconemoides* must be considered an integral part of the cuticle since both a new sheath and a new cuticle are produced jointly at each molt. The sheath of the dauer larvae of animal parasitic nematodes such as *Haemonchus* and *Trichostrongylus* is probably produced as the result of an incomplete molt (failure of exsheathment) and thus may be structurally unlike the sheath in *Hemicycliophora*.

Two additional nematode genera, *Criconema* and *Criconemoides*, closely related to *Hemicycliophora*, possess thick, coarsely annulated cuticles but the presence of a sheath has not been reported in either. Ultrastructural studies of the cuticle and its formation in these two genera might reveal interesting phylogenetic relationships, particularly if they are found to possess layers

resembling those in the sheath of *Hemicycliophora.*

The final molt of molting L4 males can be considered a double molt in which a sixth cuticle is produced. Initially, during this final molt, a thin, fifth cuticle containing the three basic layers, cortex, matrix and striated layers is produced. Subsequently, this cuticle is cast and a sixth cuticle, along with a modified sheath is produced. After ecdysis, the cuticle of the mature male is not a fifth cuticle, as in the case of the female, but represents a sixth cuticle. The significance or survival value of this fact is not known.

The origin of the "molting bodies" in *H. arenaria* and what stimulates their formation is not known. They appear just before the initiation of the molt, apparently release material into the hypodermis during the molt, and disappear during or shortly after its completion. It is suggested these structures either produce or transport material which is liberated into the hypodermis and used in the production of the new cuticle.

Davey (4) attributed a role in cuticle formation in *Phocanema decipiens* to secretions from the muscle cells. Later, Kan and Davey (8) suggested the muscle cells of *P. decipiens* were actively synthesizing protein at a time when the cuticle was being formed but that this was correlated with nutrition rather than with cuticle deposition. Observations on *H. arenaria* indicated the somatic musculature was broken down and then reformed before completion of the molt. The process was not observed in detail, but it seems logical that secretion in muscle cells at this time would probably be involved in the reformation of muscle filaments rather than new cuticle. Breakdown of the somatic musculature probably causes the characteristic relaxed quiescence of nematodes in the early stages of the molt. Resumption of movement in later stages is probably correlated with the regeneration of somatic musculature.

Molting in *A. avenae* and *H. gracilis*, even though not examined in as much detail as that of *H. arenaria*, appeared to be less complex. Initial stages in the molt of all three nematodes were similar to those described for *Meloidogyne javanica* (1), *Panagrellus silusiae* (13) and *Turbatrix aceti* (18). As cuticle separation occurred, a trilaminate osmiophilic zone was laid down at the outer edge of the hypodermis in *A. avenae* and *H. gracilis.* This zone, as in *M. javanica* (1), represented the future cortical layers. In *H. arenaria* a dense, multilayered, osmiophilic region was produced at the outer edge of the thickened hypodermis. During the molt, this region differentiated into the cortical layers of the cuticle and the electron dense (osmiophilic) layers of the sheath. It appears that all the osmiophilic material in the cuticle is laid down simultaneously even though these layers may later be interspaced with non-osmiophilic material.

The newly-forming cuticle of *H. arenaria* was convoluted in much the same manner as that of *M. javanica* (1) and *P. silusiae* females (13). This presumably allows for rapid growth of the nematode after ecdysis. The cuticles of *A. avenae* and *H. gracilis* exhibited only slight folding, particularly in the area of the lateral field, and may more closely resemble cuticle formation in *P. silusiae* male and larval stages (13) and *T. aceti* (18).

Cuticle reabsorption has been reported in molting *M. javanica* (1); a similar process appeared to occur in *H. arenaria.* In L3, L4 stages and females, reabsorption of the inner layers of the cuticle, as well as portions of the sheath, appeared to occur just prior to ecdysis. In males, reabsorption apparently occurred at an earlier stage just after the formation of the fifth cuticle but prior to the production of the sixth cuticle. Although it

was not observed, it is probable that reabsorption of the fifth cuticle occurred prior to ecdysis.

In *A. avenae* and *H. gracilis* signs of breakdown in the old cuticle were observed but signs of reabsorption were not. This may indicate the specimens observed were not close to ecdysis or that not as much of the old cuticle is reabsorbed as was suggested for *P. silusiae* (13) and *T. aceti* (18).

It seems likely that reabsorption of the inner layer of the cuticle, well documented in insects (10), also occurs in some molting nematodes as suggested by Bird and Rogers (1). In *H. arenaria* it appears that all but the osmiophilic components of the cuticle and sheath may be reabsorbed.

Much additional information is required on molting in nematodes to extend our knowledge of this vital process and to provide a more complete understanding of cuticle formation and structure.

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FIGURE LEGENDS

FIG. 3. Electron micrographs of molting *H. arenaria* **L4** females. **A.** Transverse section 20–30 hr in molt showing formation of the various regions. × 49,000; **B.** and **C.** Transverse sections 23–30 hr in molt. Note folding of new cuticle (arrows). (**B.** × 50,000 **C.** × 40,000).

FIG. 4. Electron micrographs of molting *H. arenaria* **L4** females. **A.** Transverse section 30+ hr in molt. × 40,000; **B.** Transverse section 30+ hr in molt showing fibrillar structure of matrix. × 40,000; **C.** Longitudinal section approx. 40 hr in molt. × 29,500; **D.** Longitudinal section approx. 40 hr in molt showing folding of the new cuticle. × 18,000.

FIG. 5. Electron micrographs of molting *H. arenaria* **L4** females. **A.** Transverse section approx. 50 hr in molt showing differentiation of the trilaminate cortical zone just behind the trailing edge of the now somewhat narrowed multilayered region. × 35,000; **B.** Transverse section showing development of the sheath. × 55,000; **C.** Transverse section approx. 60 hr in molt. The old striated layer is no longer visible. × 40,000; **D.** Late stage in molt showing breakdown of the old cuticle and its possible reabsorption (arrows). × 40,000.

FIG. 6. Electron micrographs of body wall of exsheathing *H. arenaria* female and cast exuvia. **A.** Transverse section of exsheathing female approx. 72 hr in molt. Note breakdown of old cuticle and sheath so only the trilaminate cortex and portions of the old sheath remain. × 50,000; **B.** Transverse section of cast exuvia. × 135,000.

FIG. 7. Electron micrographs of molting *H. arenaria* **L2** and **L3** larvae. **A.** Transverse section of **L2** larvae near mid-molt showing folding of the new cuticle. × 20,000; **B.** Transverse section of **L2** larvae near completion of molt showing signs of breakdown and possible reabsorption of the old cuticle (arrows). × 50,000; **C.** Transverse section of **L3** larvae near mid-molt. × 50,000; **D.** Transverse section of **L3** larvae in late molt, showing signs of breakdown and possible reabsorption of the old cuticle (arrows). × 59,000.

FIG. 8. Electron micrographs of molting *H. arenaria* **L4** males. **A.** Transverse section showing separation of old cuticle (arrows) from the hypodermis. × 59,000; **B.** Transverse section 10–15 hr in molt. Note widened interchordal hypodermis and formation of outer trilaminate zone (arrows). × 56,000; **C.** As in B. Note further development of striated layer (St.) × 56,000.

FIG. 9. Electron micrographs of molting *H. arenaria* **L4** males. **A.** Transverse section 25–30 hr in molt. Note signs of deterioration of old striated layer. × 51,500; **B.** Transverse section 25–30 hr in molt showing signs of deterioration in matrix of previous stage. × 51,500; **C.** Transverse section approx. 30 hr in molt showing apparent reabsorption (arrows) of old matrix material. × 70,500.

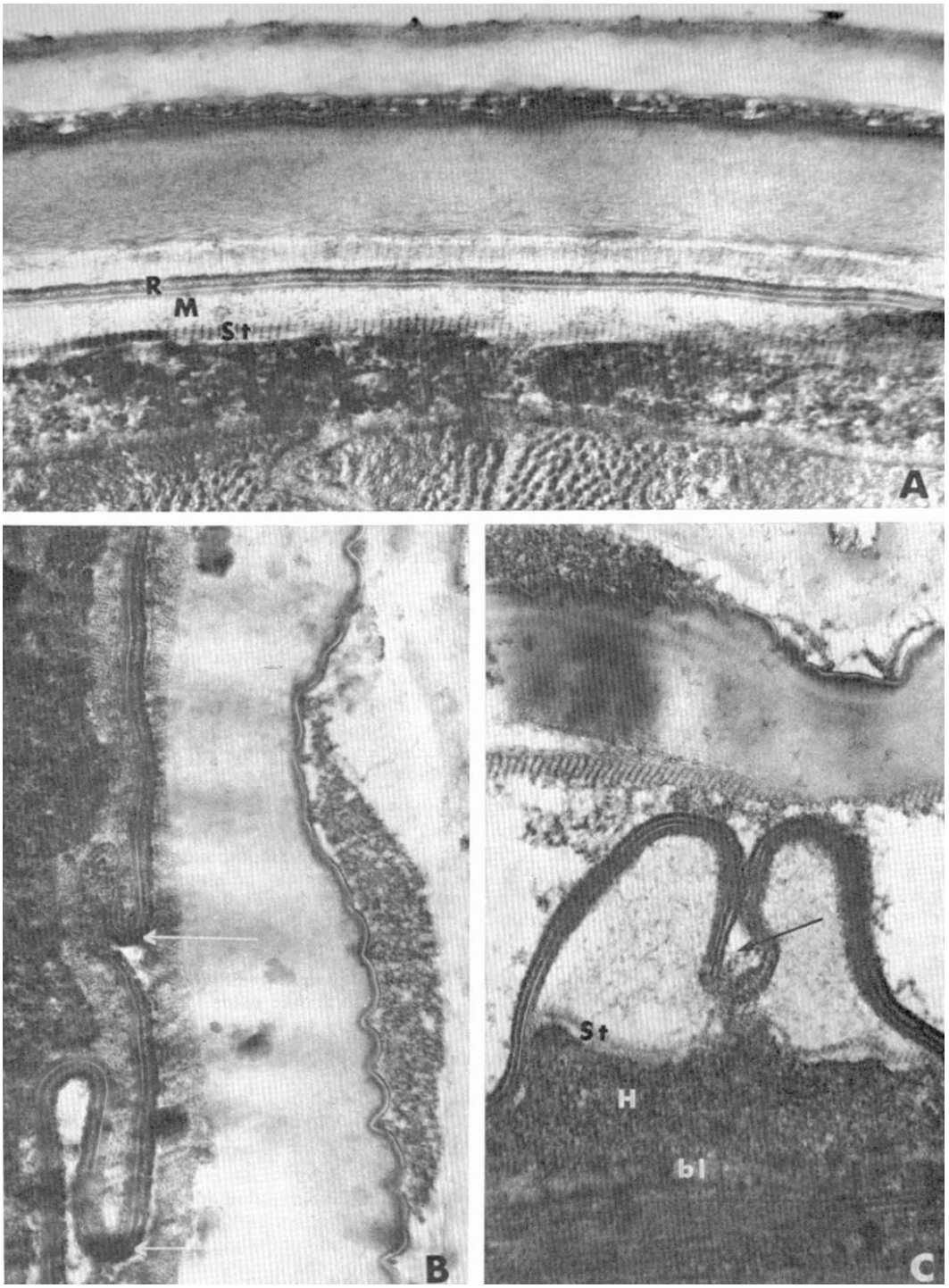
FIG. 10. Electron micrographs of molting *H. arenaria* **L4** males. **A.** Transverse section 30–40 hr in molt showing the separation from the new cuticle and the formation of a multilayered region (arrows) at the outer edge of the hypodermis. × 70,500; **B.** Transverse section showing an early stage in the formation of the sixth cuticle. × 70,500; **C.** Transverse section of later stage than above. Note dense staining material at base of the matrix adjacent to the striated layer. × 56,000; **D.** Transverse section through the developing lateral field showing forking in the striated layer (arrow) and its replacement by a layer of fibrillar material. × 56,000.

FIG. 11. Electron micrographs of molting **L4** and mature *H. arenaria* males. **A.** Transverse section near completion of molt. × 36,000; **B.** Transverse section of a mature male. × 56,000.

FIG. 12. Electron micrographs showing degeneration of somatic musculature in molting *H. arenaria*. **A.** Transverse section of a pre-molt larva. × 47,000; **B.** Transverse section 5–10 hr in molt. × 59,000; **C.** Transverse section approx. 40 hr in molt. × 65,000.

FIG. 13. Electron micrographs of molting *A. avenae*. **A.** Transverse section early in molt. × 81,000; **B.** Longitudinal section approx. mid-molt. × 59,000; **C.** Longitudinal section in late-molt showing signs of breakdown in old cuticle (arrows). × 65,000.

FIG. 14. Electron micrographs of molting *H. gracilis*. **A.** Transverse section approx. mid-molt. Note folding in region of developing lateral field. × 59,000; **B.** Longitudinal section of late stage in molt of an **L4** male. × 51,500; **C.** Transverse section of **L4** female near completion of molt. Note signs of breakdown in old cuticle and accumulation of dense staining material at base of matrix. × 51,500; **D.** Longitudinal section through the developing lateral field of an unknown stage showing presence of four fibrillar layers. × 22,000.



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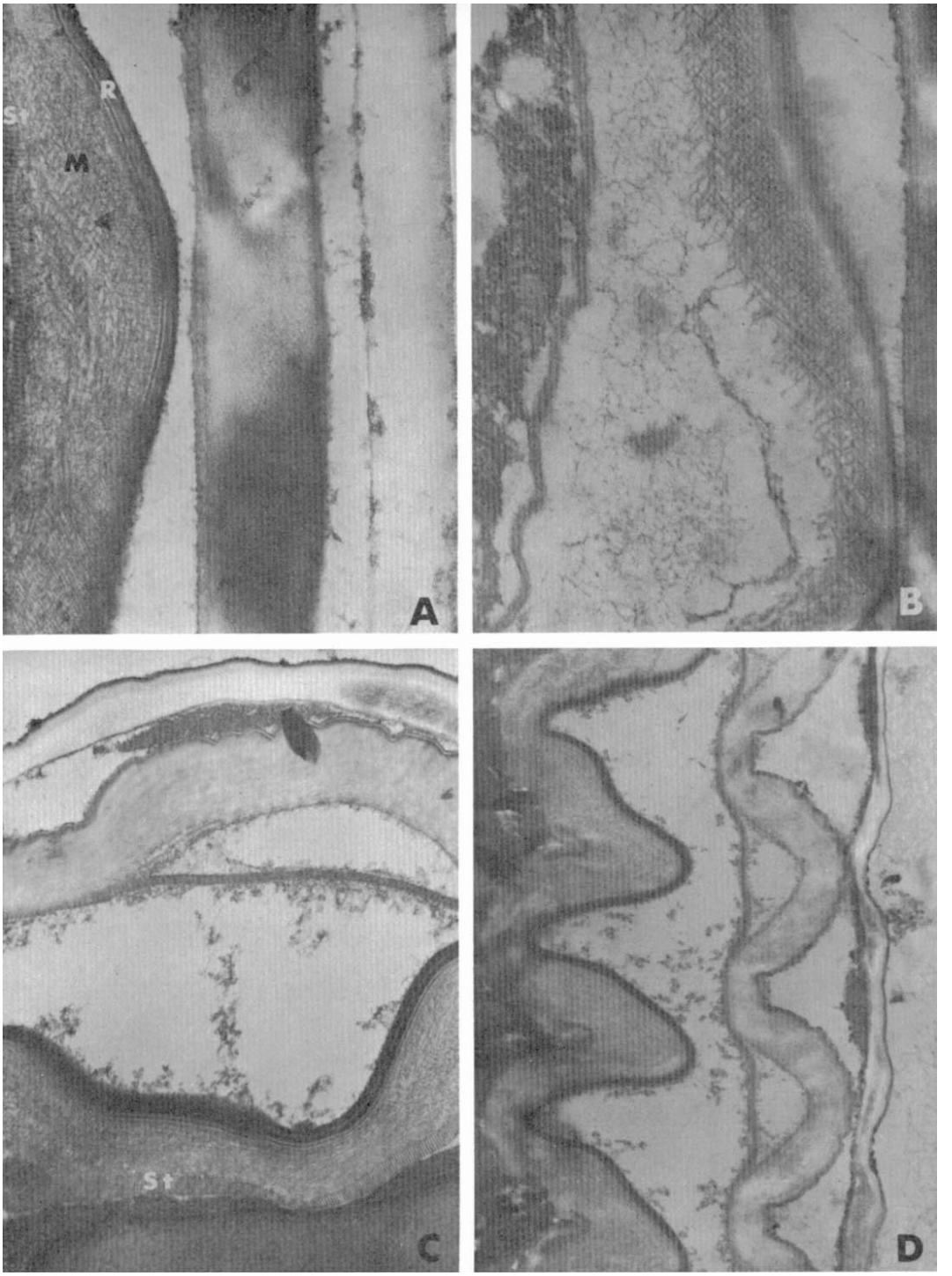

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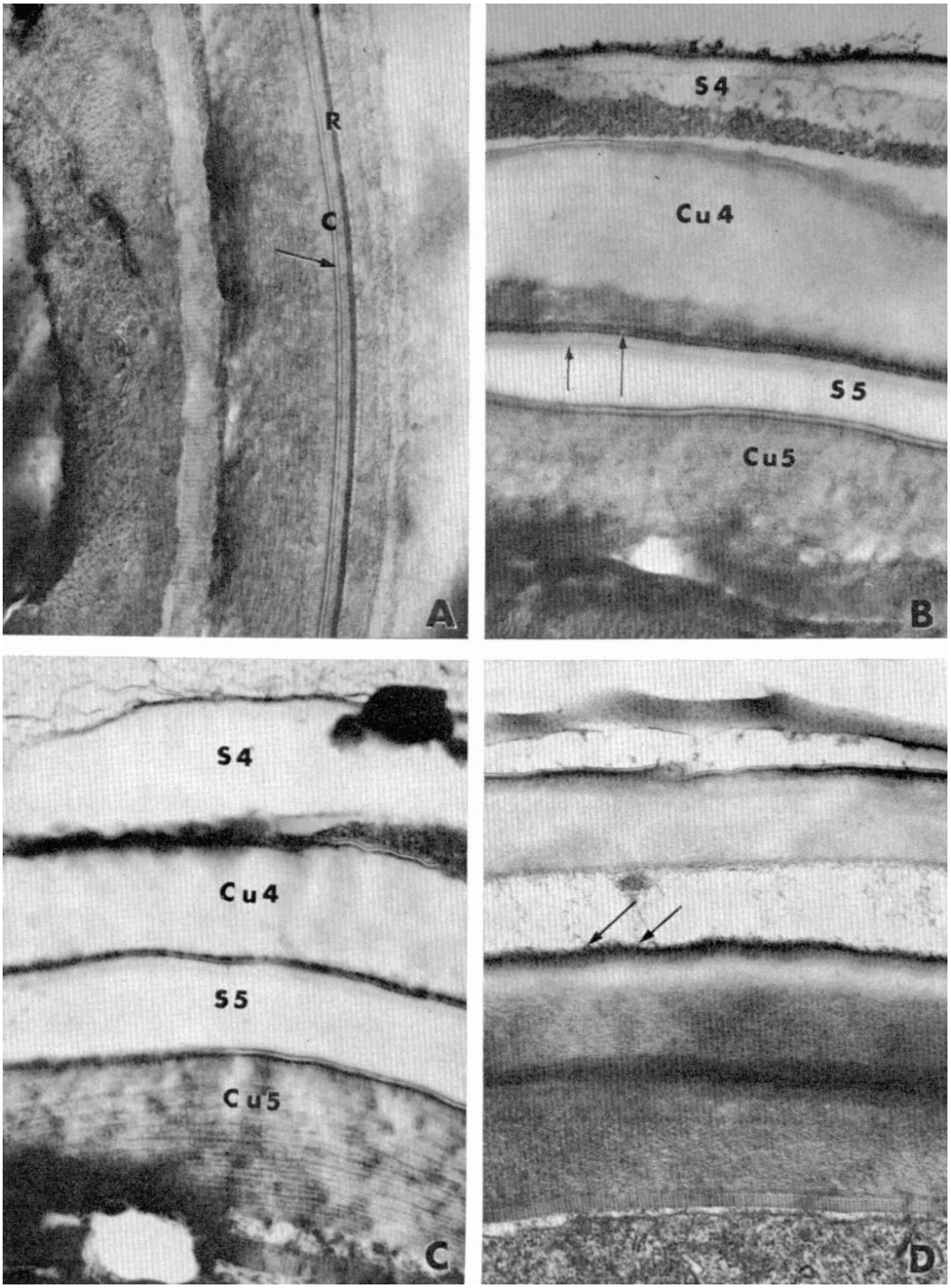


Figure 5.

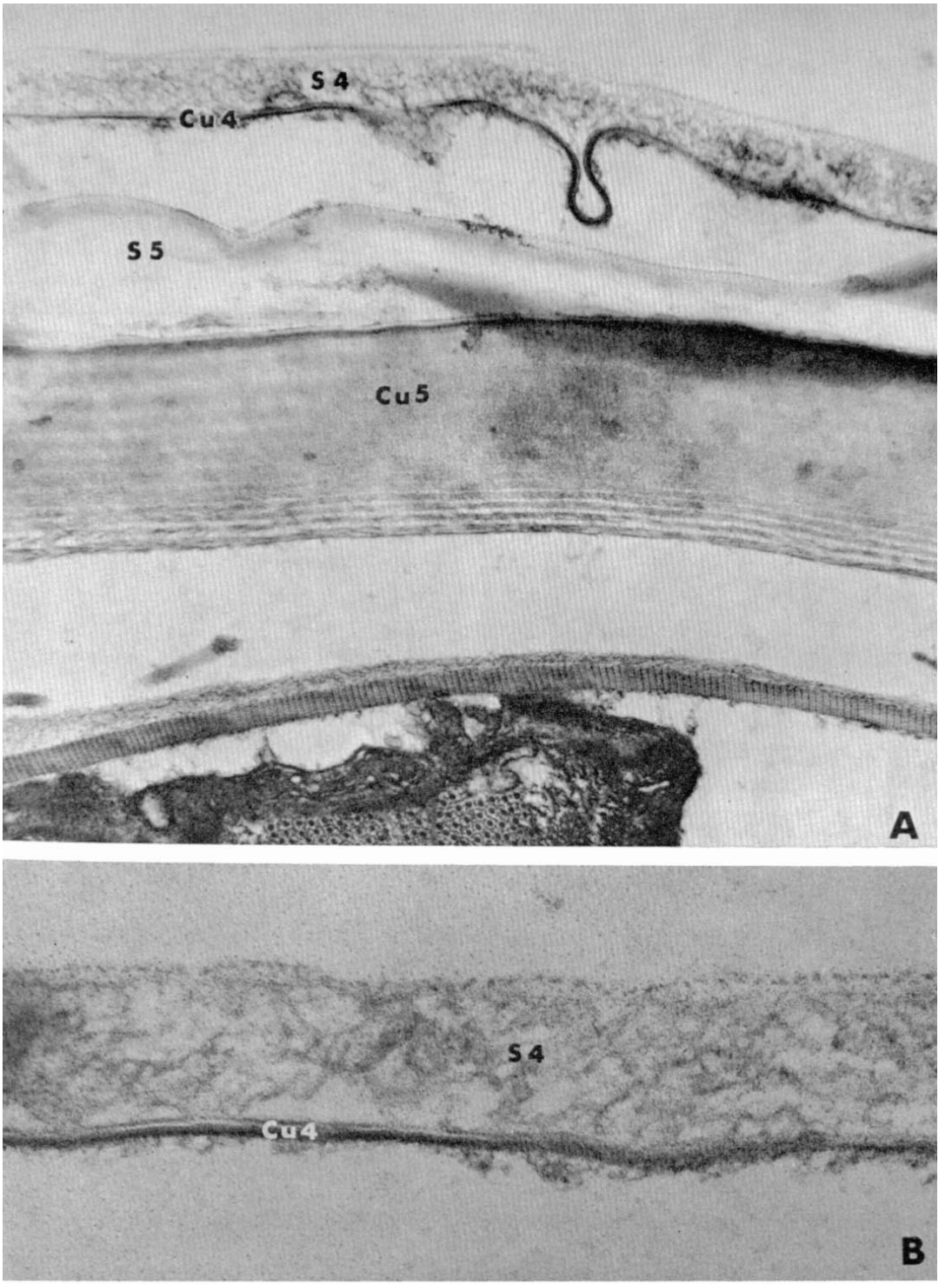
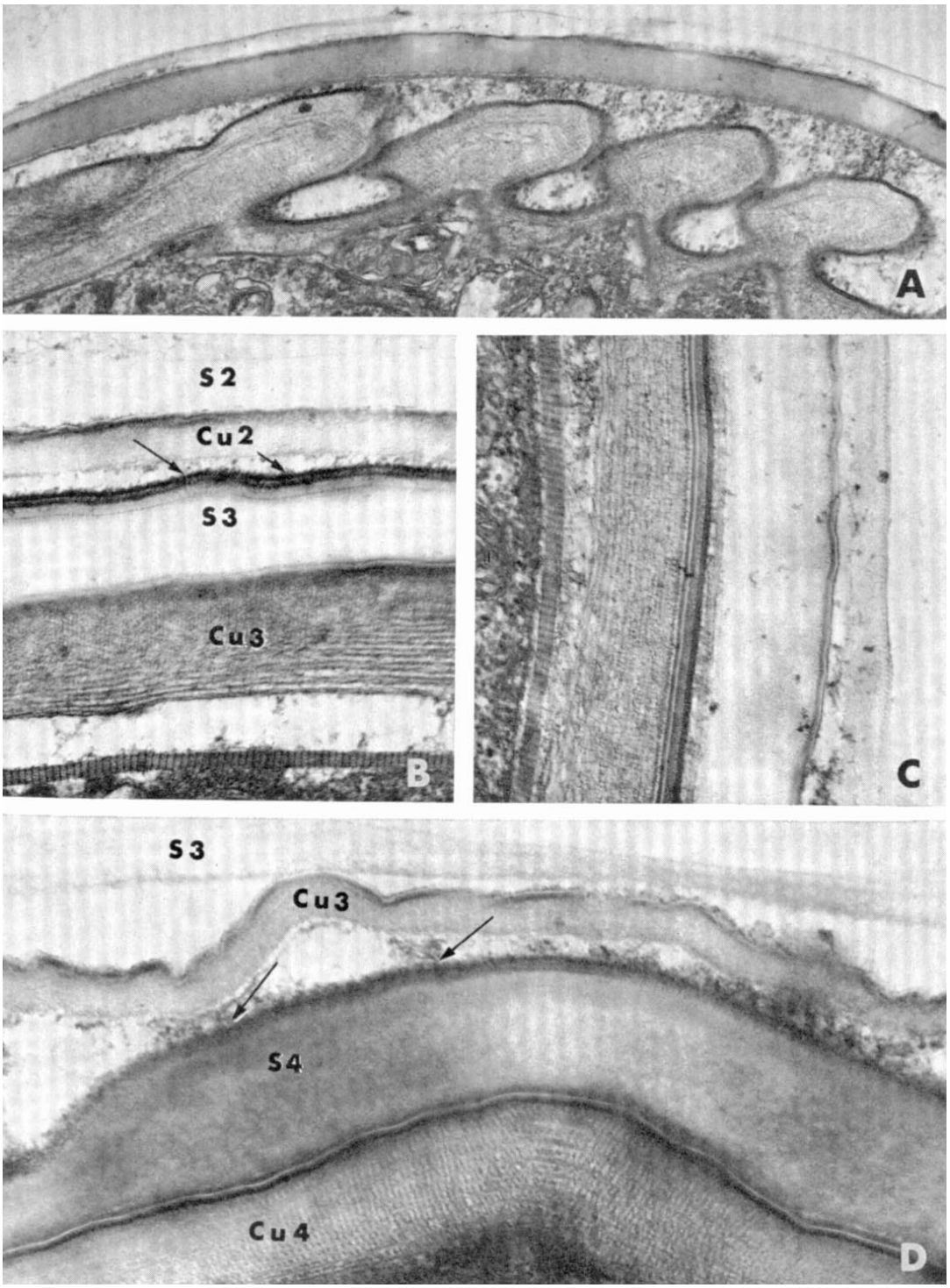


Figure 6.



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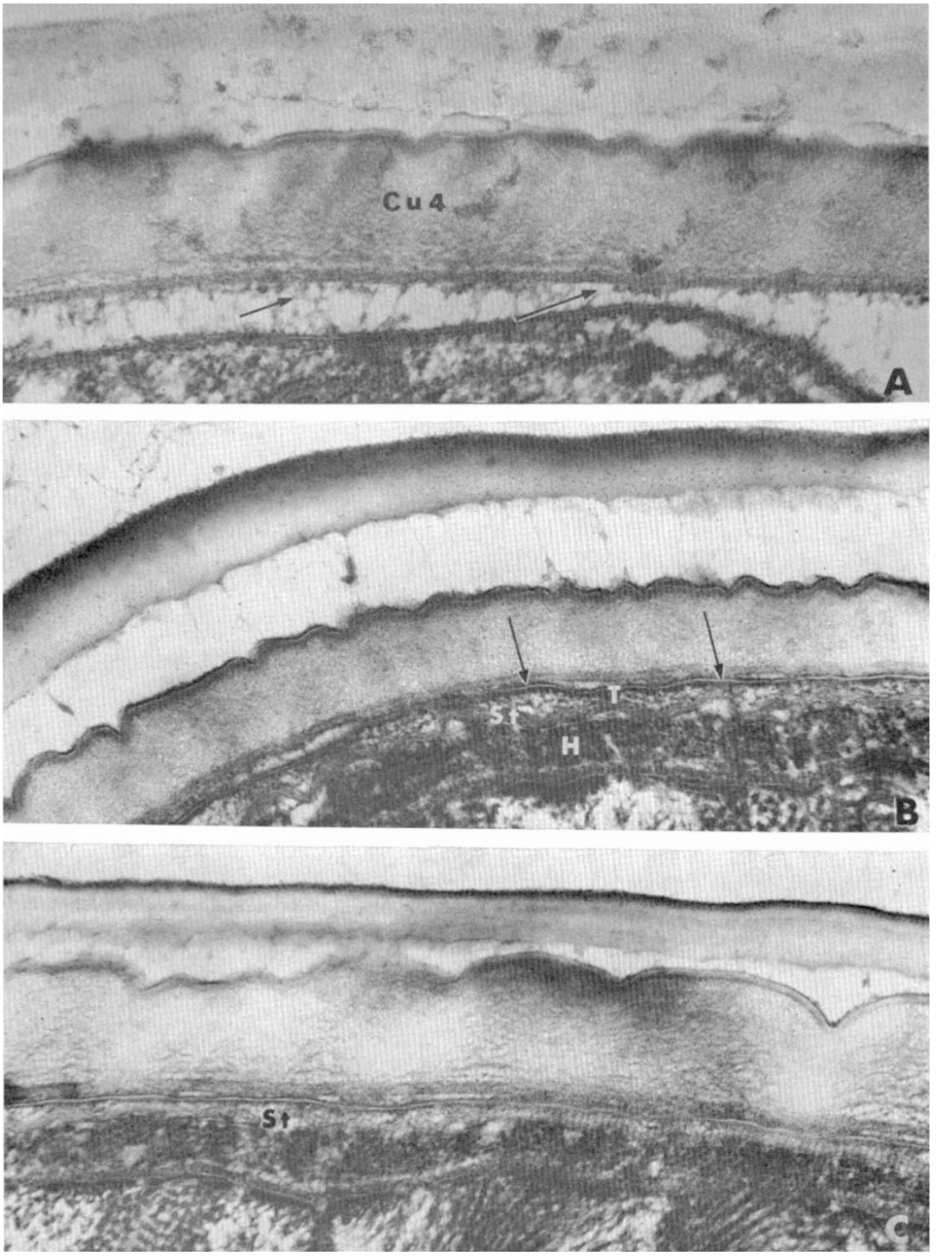

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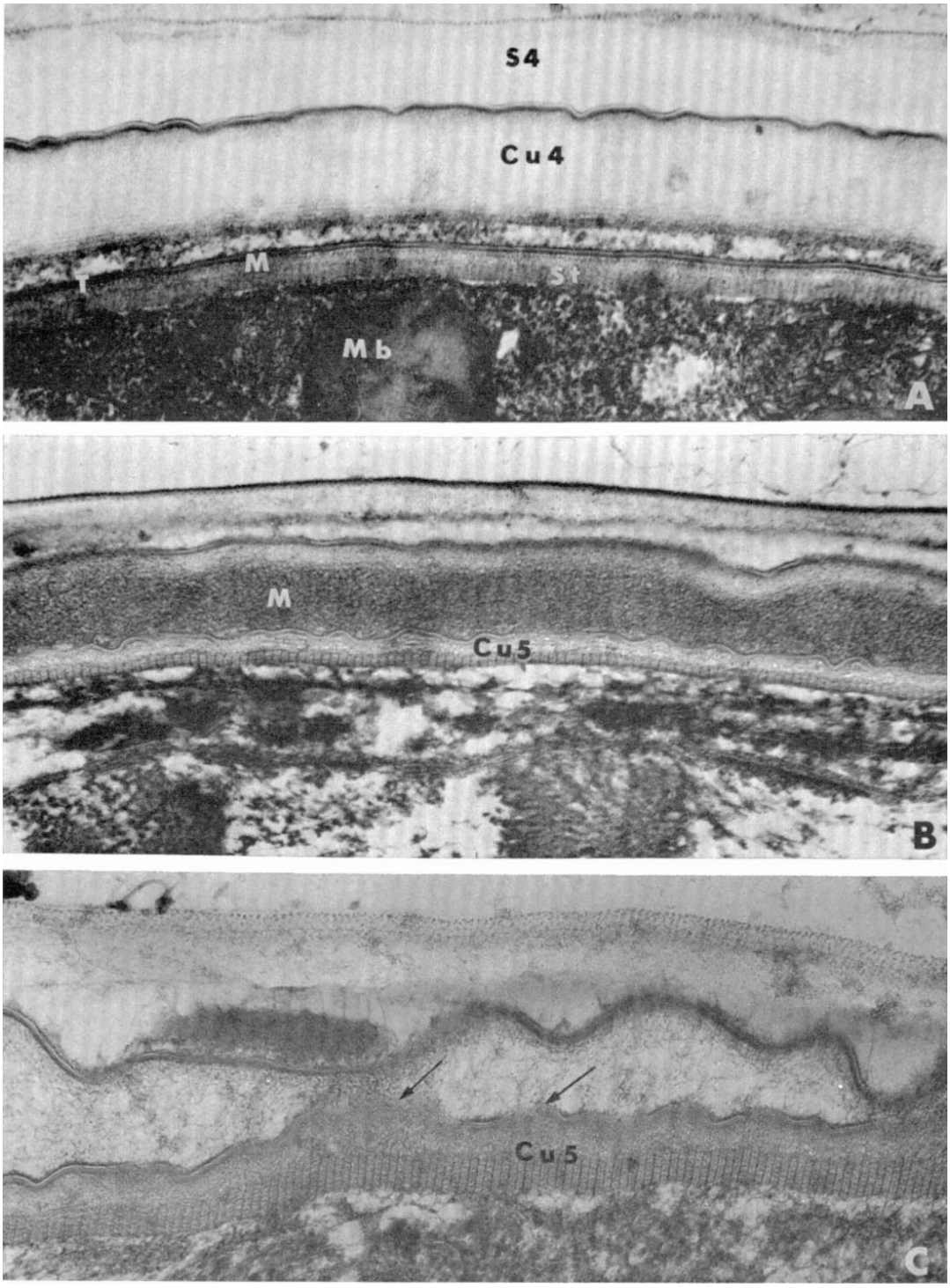

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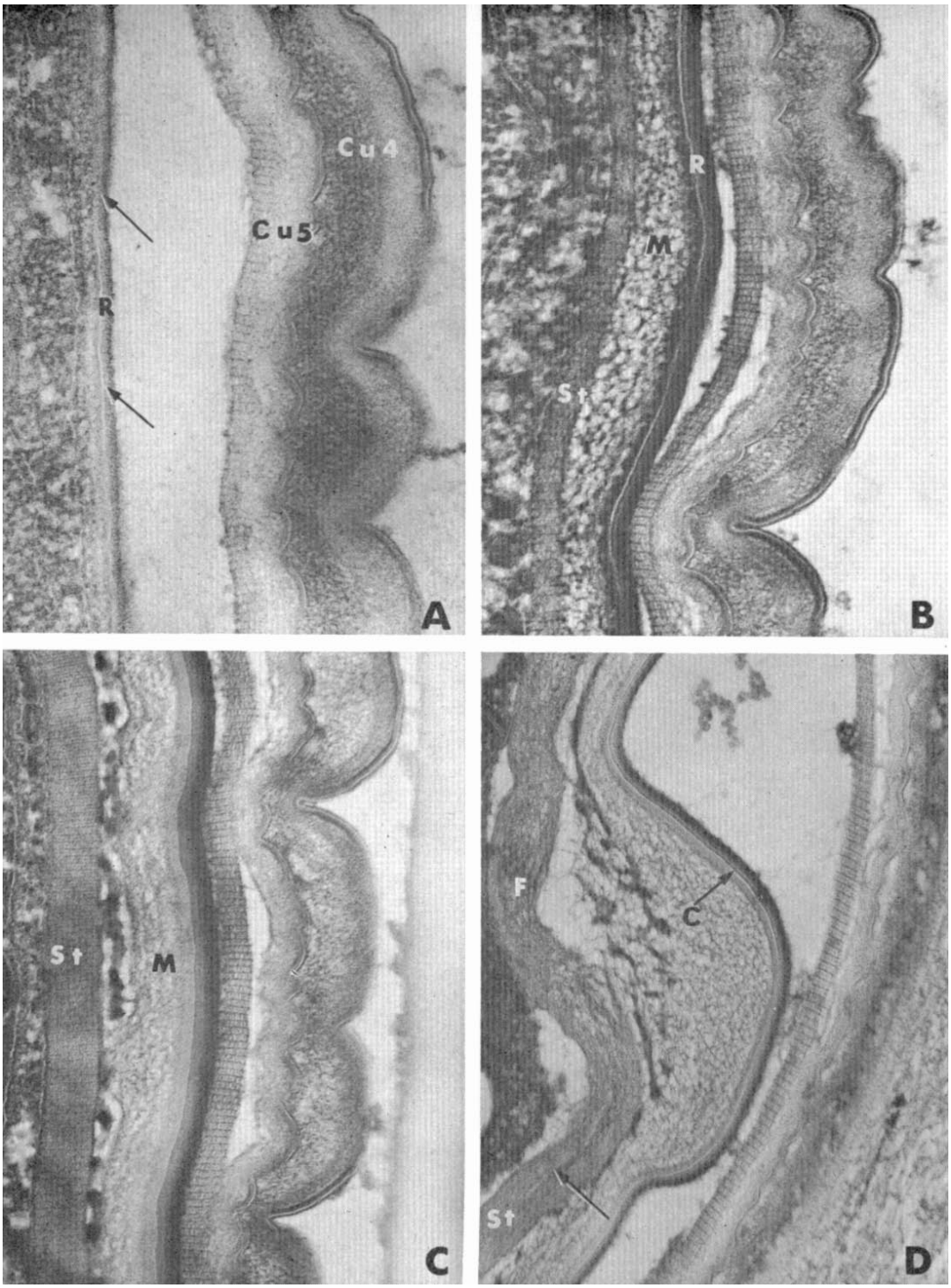

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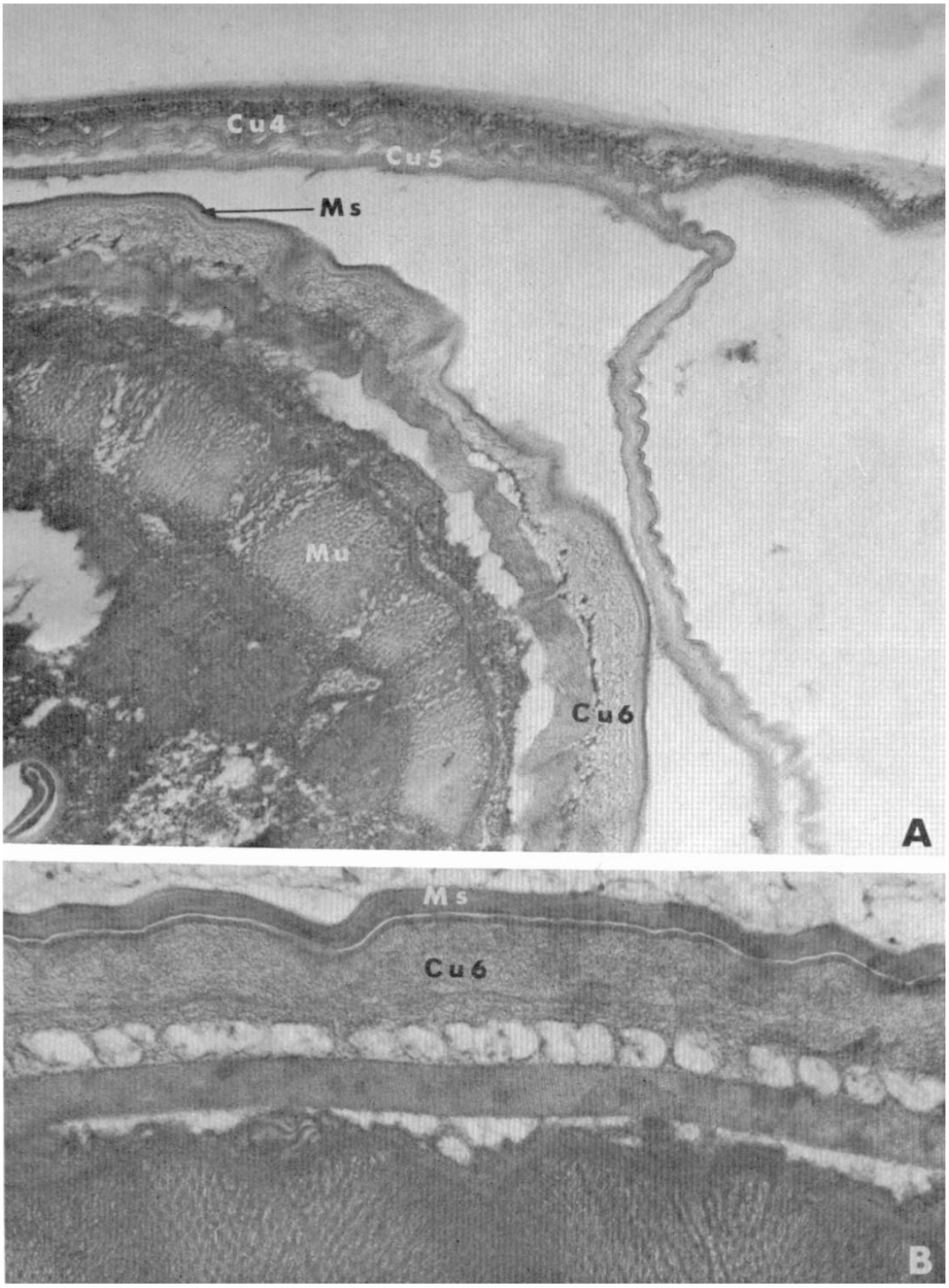

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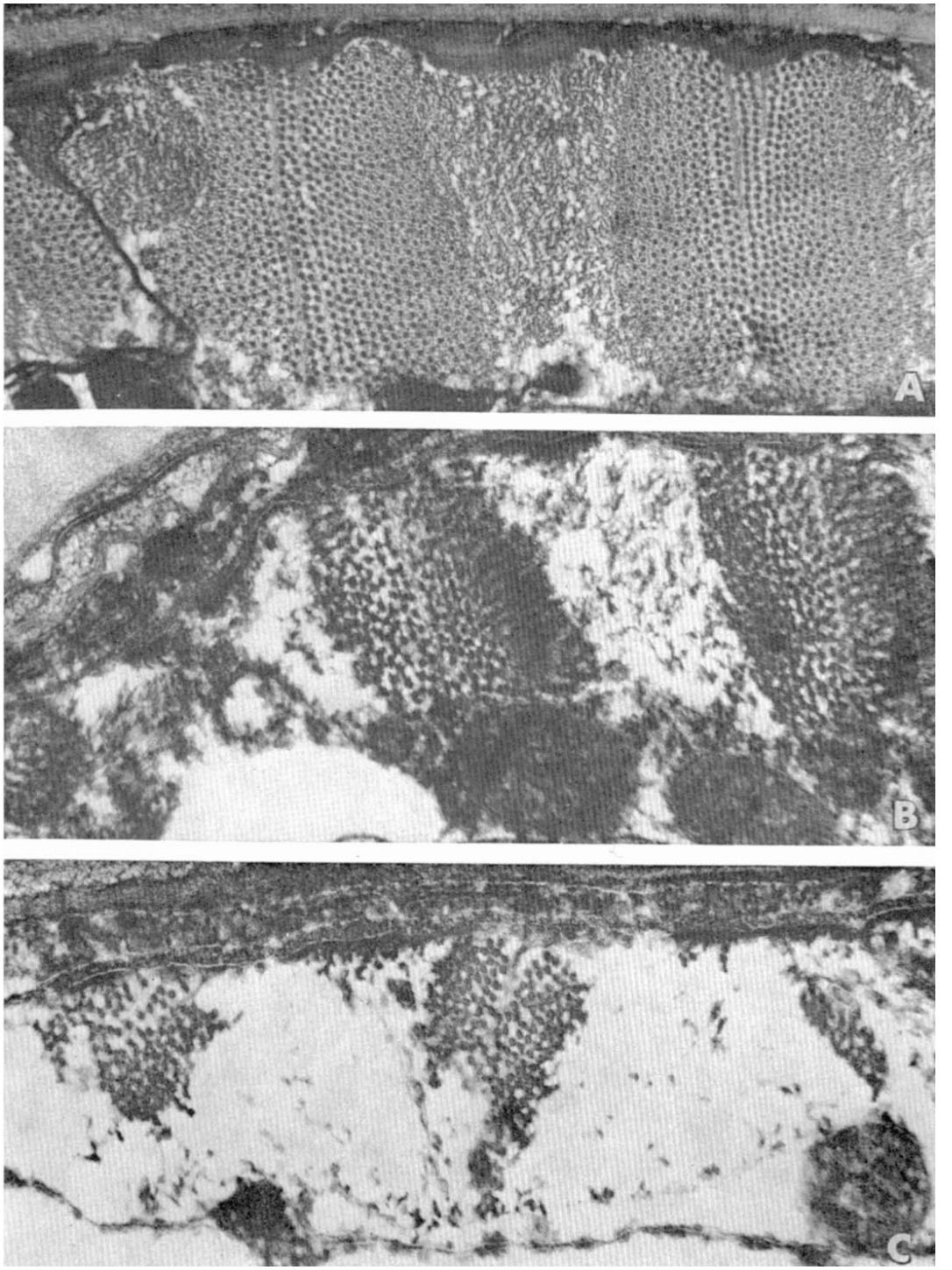

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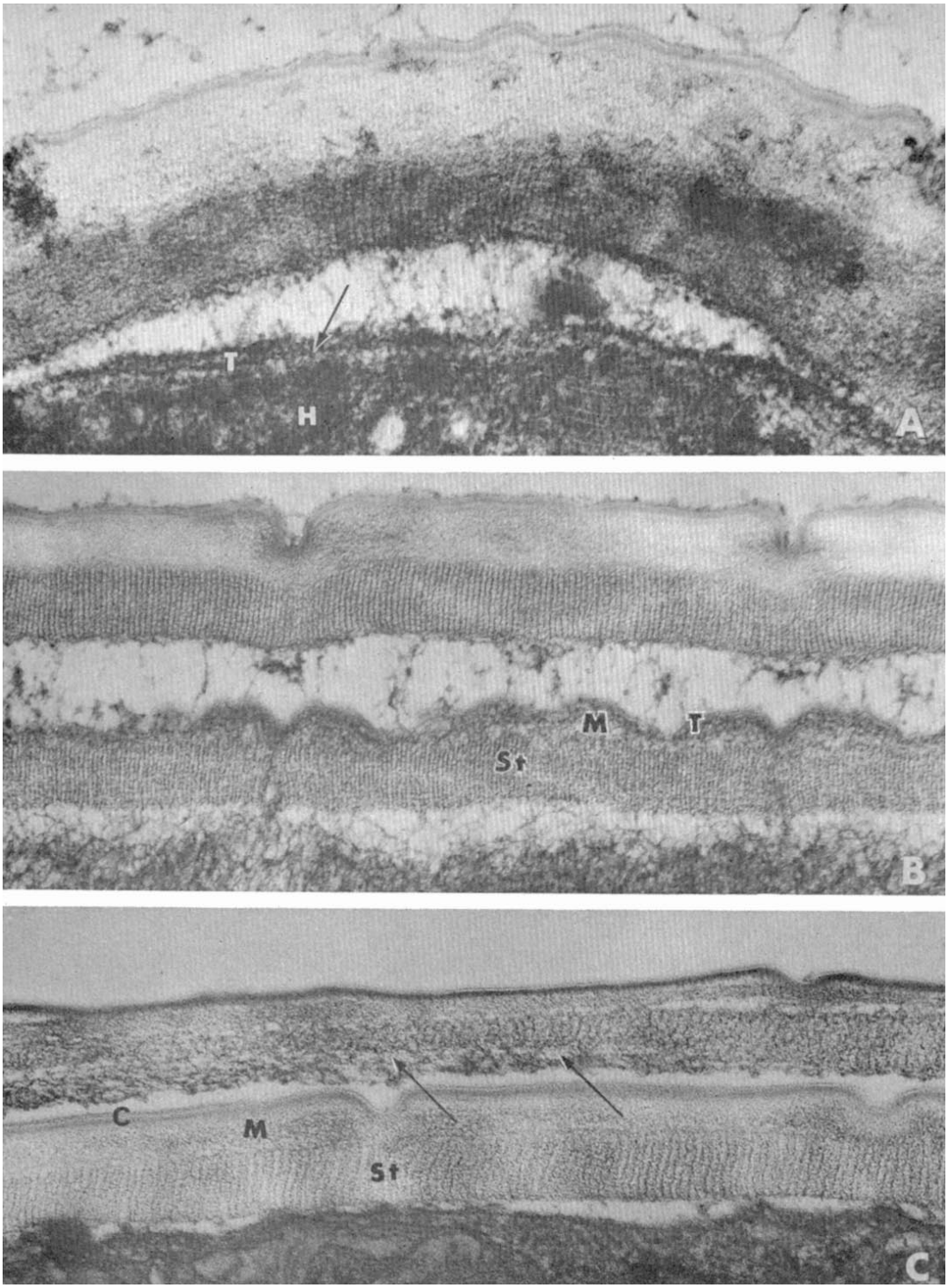

Figure 13.

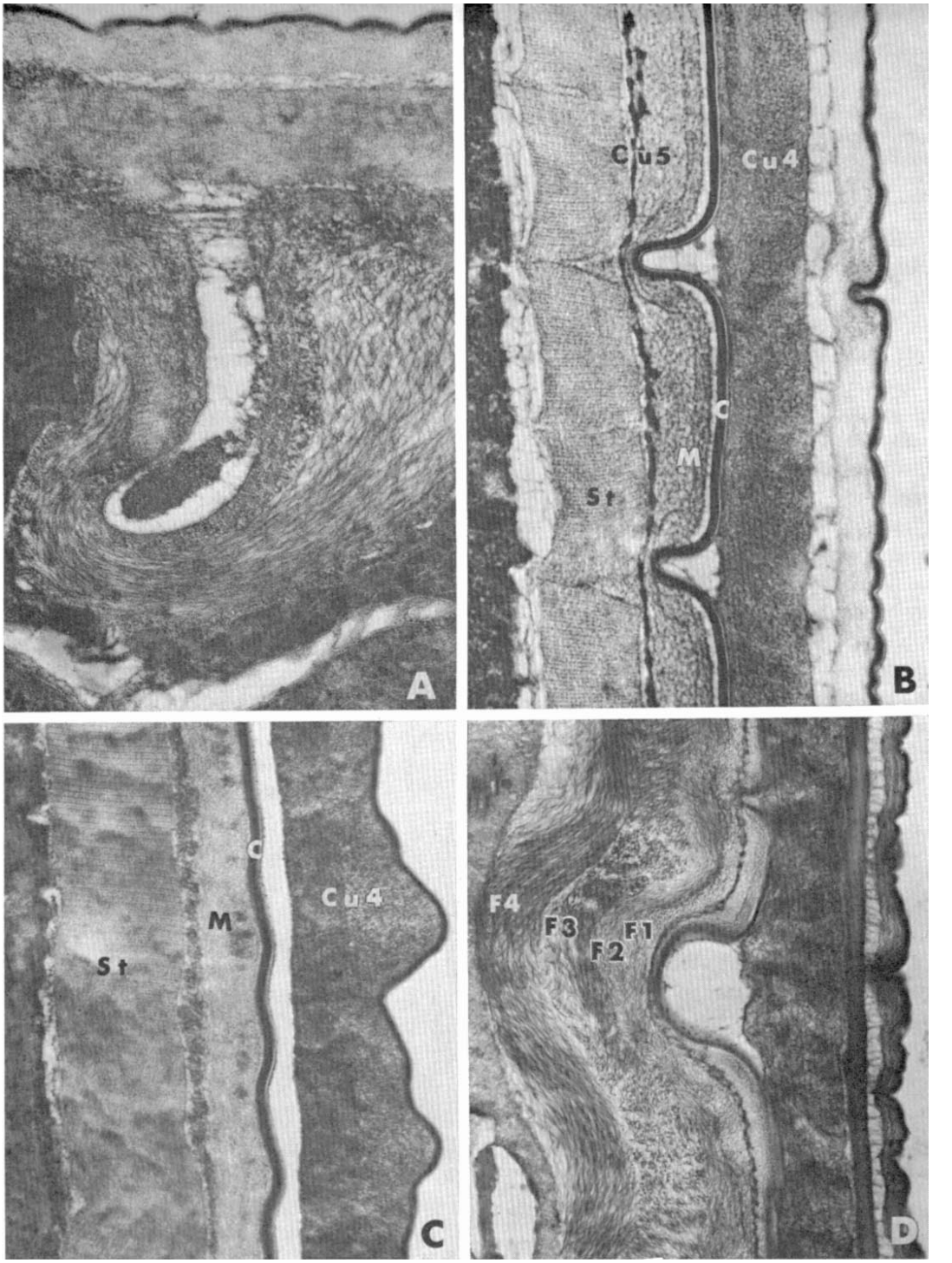

Figure 14.