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Scanning Electron Microscopy of the Cortex of the Ciliate *Stentor coeruleus*. A View from the Inside*

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SYNOPSIS. A microdissection procedure was developed which permits the viewing of the inside surface of the cortex of *Stentor coeruleus* with scanning electron microscopy. Parallel bands of myonemes cover the entire inner surface of the cortex. The myonemes of the stalk region are ribbon-shaped and lack cross connections. The myonemes of the anterior cortex are flattened against the surface and are interconnected by an extensive system of cross branches. The inner surface of the frontal field is covered with a regularly cross-branched myoneme system which follows the curved pattern of frontal field kinety. The observed branching patterns and shapes of the myonemes support the hypothesis that they cause contraction of the cell. The membranellar root system was examined. Each membranellar root makes a 90° counterclockwise twist along its vertical axis (viewed from the inside) as it descends into the cell. The outer edge of each root fuses with the inner edge of the adjacent one, forming a continuous fiber sheet linking the roots together.

Index Key Words: *Stentor coeruleus*; cortex; contractile fibers; myonemes; membranellar roots; scanning electron microscopy.

THE cortex of the contractile ciliate *Stentor* contains two fiber systems, the km fibers and the myonemes, or M-bands. The km fibers are composed of stacks of microtubules with each fiber running the length of the cell. The myonemes lie directly beneath the km fibers and are composed of bundles of microfilaments.

Several transmission electron microscopy (TEM) studies of *Stentor* have indicated functions and probable mechanisms of operation for these fiber systems. The myonemes appear to be the "muscle" responsible for contraction (1, 5, 6, 13, 15). The km fibers constitute an active extension system (5, 12, 13). Detailed ultrastructural studies of these systems have been conducted (5, 9, 10, 13, 17, 18).

TEM studies have certain limitations when dealing with the 3-dimensional form of a structure. Serial sections must be analyzed in order to reconstruct the shape and relative positioning of the objects being studied. This method is sometimes difficult to do and can result in faulty interpretations. Although the ultrastructure of *Stentor*'s fiber systems has been investigated, the overall form and connectivity of these systems is not known in detail. Conflicting reports concerning interconnections between myonemes have appeared (5, 18). The reported connectivity of the membranellar roots is also contradictory (4, 18).

Scanning electron microscopy (SEM), in contrast to TEM, can reveal the overall form of a structure. Guttman (11) used SEM to view the internal organelles of *Euglena* that had been broken open. Paulin and Bussey (17) pictured the regeneration of the membranelles on the surface of *Stentor* with SEM. A similar technique, viewing the cortex from the inside rather than from the outside might clarify the form and connectivity of the fiber systems and other internal structures that remain unclear following TEM studies.

To this end, a method of microdissection was developed which allowed the viewing of the inside surface of the cortex of *Stentor coeruleus* with SEM. Myonemes and the km fibers can be visualized with this method, as can membranellar roots, vacuoles and macronuclei. This technique has been useful in studying the structure and function of these organelles.

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MATERIALS AND METHODS

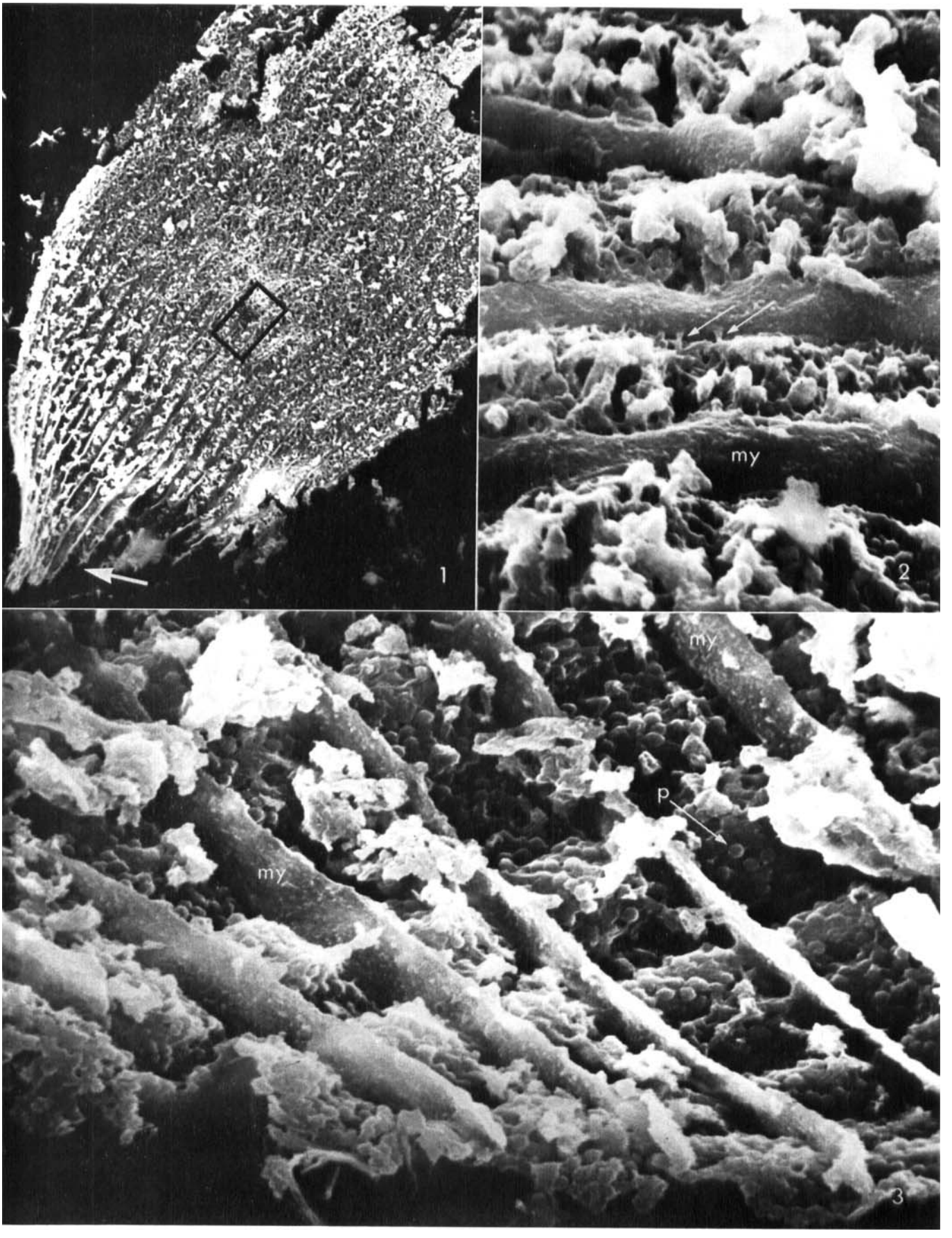
Stentor coeruleus were obtained from Connecticut Valley Biological Supply (Southampton, Mass.). They were cultured at room temperature in either filtered pond water or "M" solution [0.1 mM KCl; 0.1 mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM $NaHCO_3$; 1 mM Trisma, pH 7.4 (14)], the latter proving more reliable. They were subcultured every few weeks and were fed on mixed protozoa and heat-killed wheat grains.

In some experiments, the organisms were slow-bleached prior to fixation and freeze-drying to remove pigment granules from their cortex (7, 23). In these cases, they were kept for up to a week in "M" solution to which 1 mM caffeine was added. The caffeine solution was changed every day or two in order to facilitate bleaching.

Methods of fixation and dehydration were similar to those employed by Paulin and Bussey (17) and Small and Marszalek (21). *Stentor* were fixed for three minutes in Párducz's fixative [6 parts, 2% (w/v) OsO_4; 1 part saturated HgCl (16)]. All *Stentor* contracted into spheres when added to the fixative and were fixed in that form. The protozoa *Stentor* were then pipetted through four changes of distilled water. Drops of 10-20 *Stentor* were frozen by dropping them onto a foil boat floating on liquid nitrogen. They were then freeze-dried at −20 C, a process which was completed within 2-3 hr.

Freeze-dried *Stentor* were transferred with an eyelash onto the surface of a SEM specimen block coated with a very thin layer of quick-setting epoxy. Microdissection was accomplished with a glass needle drawn to a fine point with a microelectrode puller. The needle was oriented slightly downward and held in a Leitz micromanipulator whose horizontal movements were controlled by a joy stick. The specimen block was placed on the rotary stage of a compound microscope. A fiber optics bundle positioned above the specimen block provided illumination. The tip of the dissection needle was placed in the focal plane of the microscope. Horizontal movements of the needle were controlled by the joy stick. Vertical movements of the specimen were accomplished by raising or lowering the microscope stage.

Patches of cortex were cut out and separated from specimens by jabbing and prying them with the dissecting needle. Freed patches were picked up with the needle and transferred to a 2nd epoxy-coated specimen block. The patches of cortex were glued to the 2nd specimen block with their insides facing upwards. Loose particles of cytoplasm were easily removed by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sweeping motions of the needle, literally scraping the surface clean.

All specimens were coated, by evaporation, with a thin layer of gold (21). They were viewed with a JSM-U3 microscope operating at 25 kV.

OBSERVATIONS

The Myonemes

Figure 1 shows the overall appearance of a patch of cortex which has been scraped clean and viewed from the inside. Most prominent in the picture is a fiber system which runs in parallel bands down the entire length of the patch. The system has been identified from its shape and dimensions as the myoneme system, as will be demonstrated below. It is most prominent in the posterior end of the cortex (the stalk region), but each myoneme can be easily traced adorally toward the frontal field to the anterior edge of the patch.

The cortex was thoroughly scraped to clear the surface of cytoplasmic material. Small patches of material, however, usually remained on the surface and can be seen as white blotches in Fig. 1. Mitochondria, which are known to be associated with the cortex (5, 18), might make up part of this cytoplasmic material, although no positive identification has been made.

The myoneme system was almost entirely intact in all patches of cortex viewed. An effort was made to break off segments of myonemes in order to view the km fibers beneath them. The cortex was scraped to the point where it was torn apart. A portion of a myoneme, however, could rarely be detached from the underlying cortex (Fig. 7).

The posterior portions of the myonemes are distinctly ribbon-shaped, standing out clearly from the underlying surface (i.e., projecting into the cell interior) (Figs. 2, 3, 7). They are approximately 0.5 μm thick when viewed edge on from overhead (Fig. 9). The width of these myonemes measured from lateral views (Figs. 2, 3) varied from 2-3 μm. These dimensions are in agreement with measurements made from published TEMs of contracted myonemes: 2.5 × 0.6 μm (Fig. 12 of ref. 5); 2.3 × 0.5 μm (Fig. 1 of ref. 6). No cross branches between adjacent myonemes have ever been seen in the posterior region of *Stentor*.

Myonemes in the anterior cortex contrast sharply with the posterior portions of the system. These myonemes do not rise from the surface, but rather lie flat against it (Fig. 4). In addition, the anterior myonemes are connected together by an extensive system of cross branches. The primary, longitudinally oriented myonemes are sometimes interrupted by donut-shaped portions which join with converging connectives (Fig. 4). The cross branches are cylindrical, while the primary myonemes are more flattened in appearance.

The inside surface of the frontal field of *Stentor* is covered with an extensively branched myoneme system (Fig. 5). The myonemes follow a curved path whose overall pattern closely resembles the pattern of frontal field stripes and kineties seen with the light microscope (22). The spacing between myonemes is similar to the spacing between kineties measured in contracted cells. Thus, there is probably a 1:1 correspondence between myonemes and kineties in the frontal field, as is the case for the lateral surface of *Stentor* (5, 18).

Figure 6 shows a portion of the frontal field pictured in

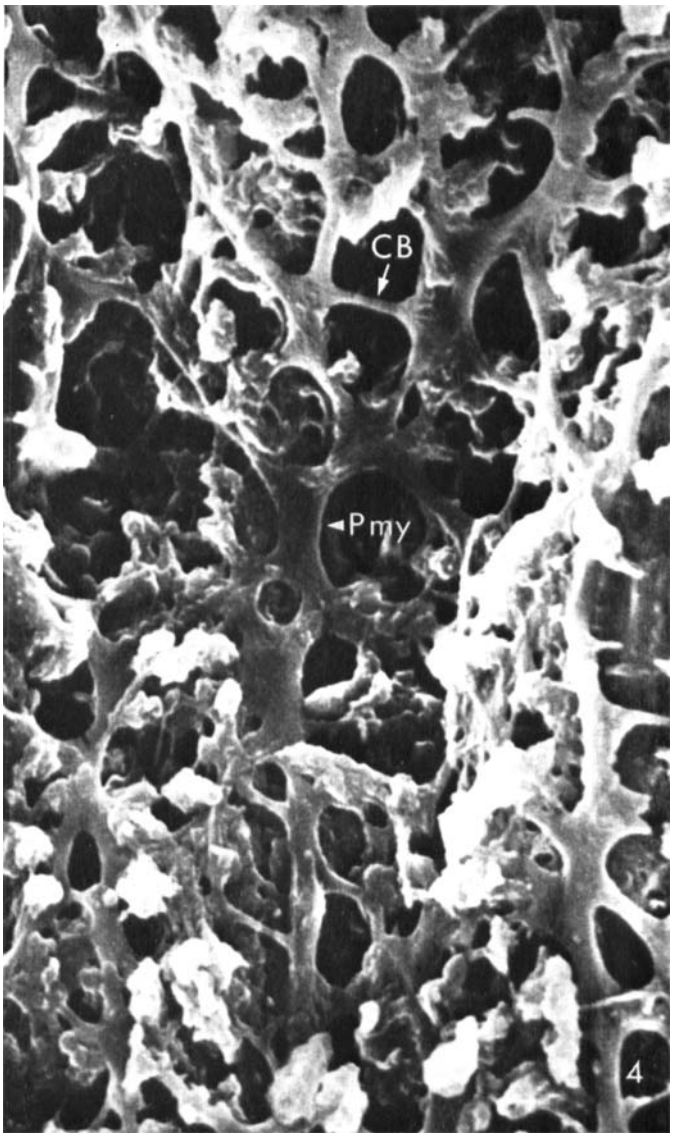

Fig. 4. Anterior region of the myoneme system. The anterior end is at the top of the figure. Primary myonemes (Pmy) and their cross branches (CB) are visible. × 6,100.

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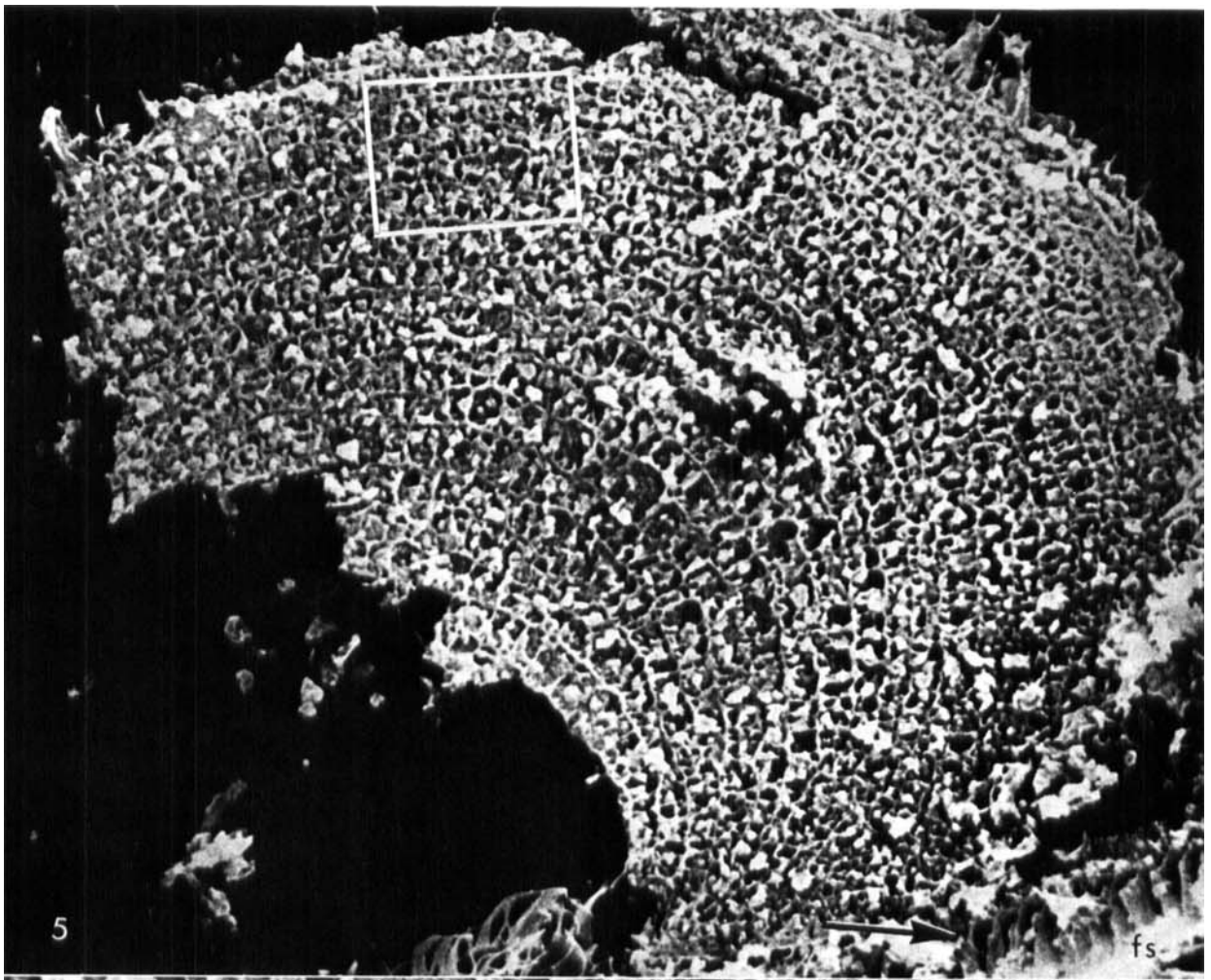
All figures are scanning electron micrographs of *Stentor coeruleus*.

Fig. 1. A large patch of the lateral cortex, viewed from the inside. The anterior end is toward the upper right. This piece of cortex was originally curved around the spherically shaped cell but was glued flat for viewing purposes. The myonemes are seen as parallel lines to the lower left and can be traced anteriorly the length of the patch. The arrow indicates the area shown in Figs. 2, 3. The enclosed region is enlarged in Fig. 4. × 560.

Fig. 2. Three posterior myonemes viewed laterally. The anterior end is toward the left. Arrows indicate infrequently seen secondary connections between the myonemes (my) and the inner surface of the cortex. × 5,000.

Fig. 3. Posterior myonemes (my) viewed looking anteriorly and to the right. The cortex is completely covered with pigment granules (p). × 5,000.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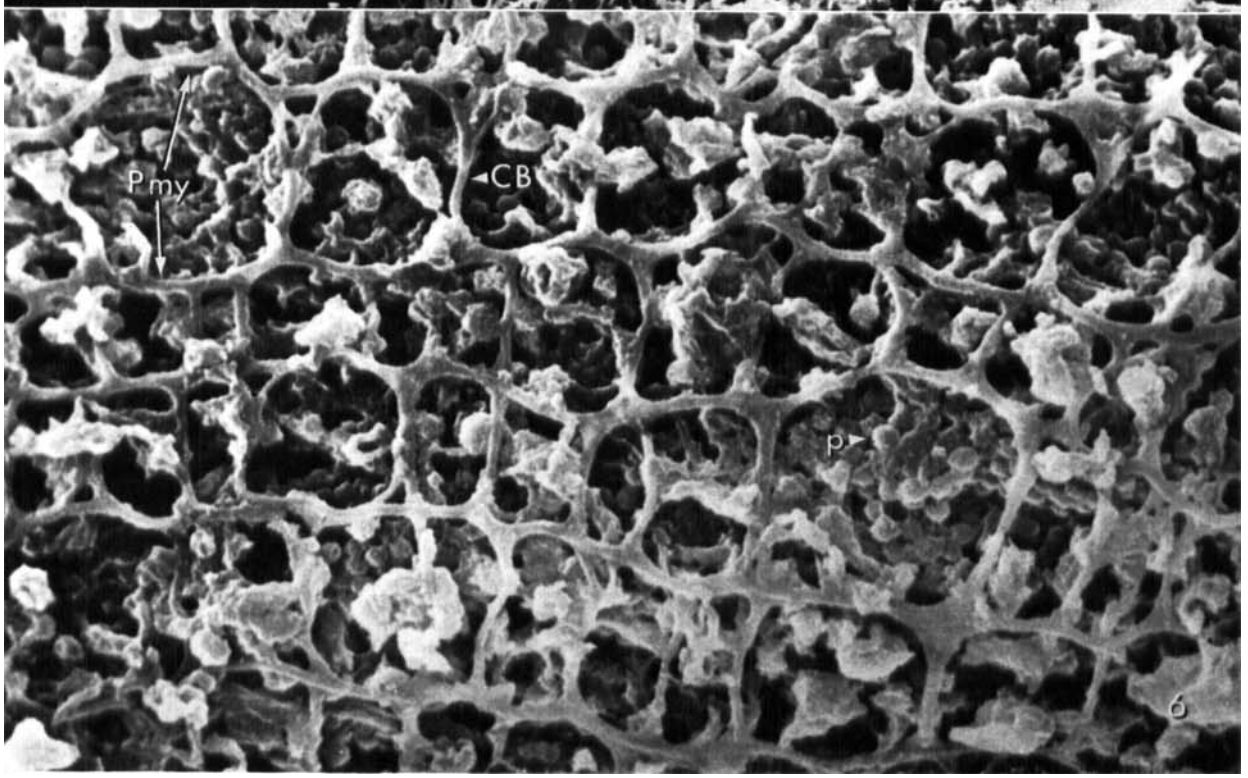
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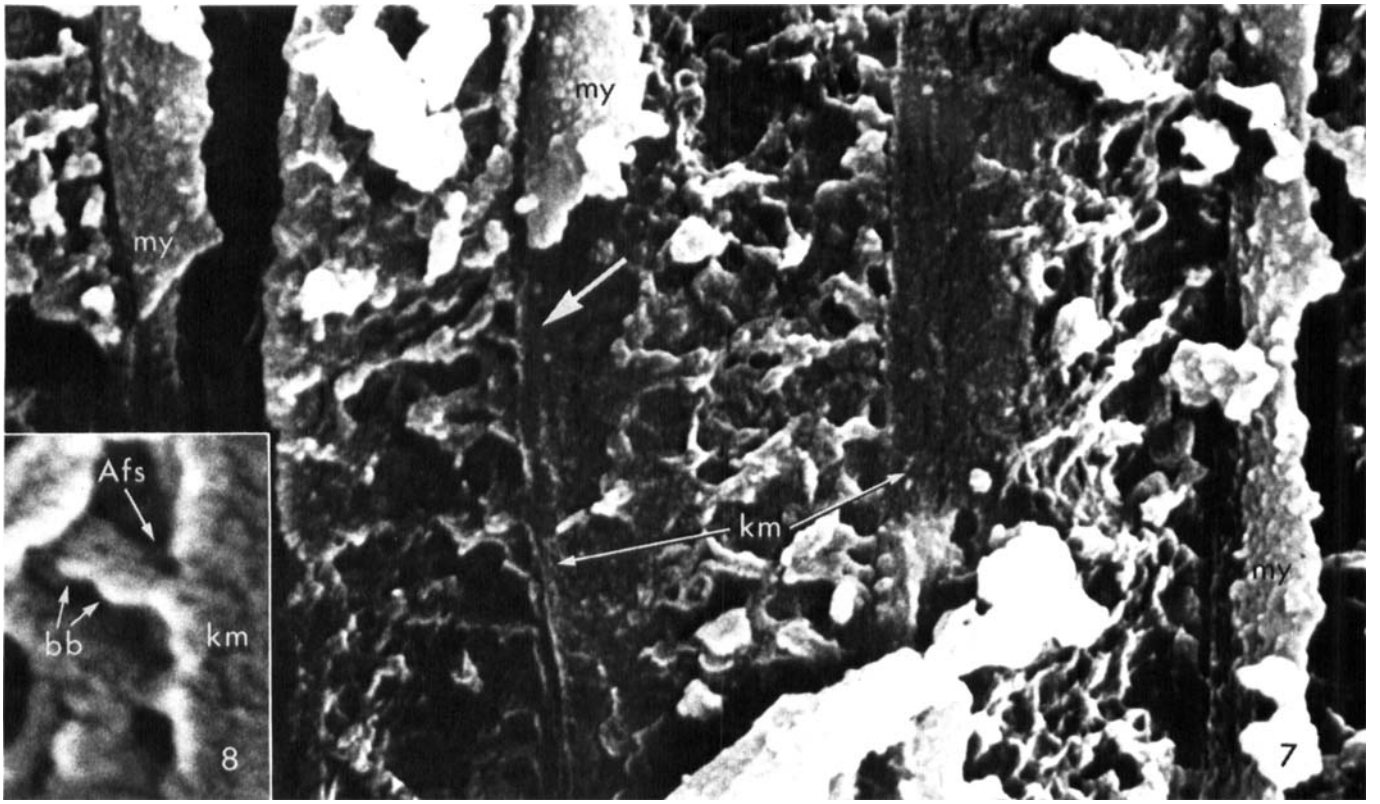

Fig. 7. Km fibers of the posterior cortex. The specimen was pretreated with 1 mM caffeine for 4 days. The anterior end is at the top. The outlines of 2 km fibers (km) (having a smooth appearance in contrast to the surrounding cortex) are seen lying directly below broken-off myonemes (my). A portion of a detached myoneme is seen in the lower center. Arrow indicates region shown in Fig. 8. × 7,100.

Fig. 8. A higher magnification view of the cortex seen in Fig. 7. A pair of basal bodies (bb) is connected to a km fiber (km) by a short, downward slanting structure. Afs, anterior fiber sheet. × 27,200.

Fig. 5. The primary myonemes, i.e., those running parallel to the kineties (oriented horizontally in Fig. 6) can be easily identified. Cross branches connecting adjacent myonemes are as numerous as the primary ones. These branches have the same cross sectional shape (round) and dimensions (0.35 μm in diameter) as the primaries and usually intersect them at right angles. Thus, an equal amount of myoneme material is oriented in two orthogonal directions, roughly circumferential and radial, beneath the circular frontal field.

The appearance of the surface of the myonemes differs in various regions of the cortex. Myoneme surfaces are always well defined, but the posterior myonemes are covered with far more bumps than are those found in the anterior or frontal field regions, giving them a textured appearance. The bumps might correspond to the small dense staining bodies associated with the myoneme membrane seen in TEM studies (5, 13). Alternatively, they might represent broken-off attachments between the myoneme membrane and the surrounding endoplasmic reticulum (2, 13).

Connections between the myonemes and the underlying cortical surface have occasionally been seen in SEM photographs in both anterior and posterior regions of the cortex. Figure 2 pictures thin strands of material projecting from a posterior myoneme towards the surface. Most connections presumably lie directly beneath the myonemes and out of view.

Large wrinkles in the surface, oriented perpendicular to the myonemes have frequently been seen in the posterior region of *Stentor* (Fig. 3). These wrinkles correspond to similar folds, seen as overlapping regions of the pigmented pellicle, in living, super-contracted cells (Fig. 4 in Ref. 5).

Km Fibers

Sections of myonemes were broken off during the dissection procedure in order to view the km fibers lying beneath them. Visualization of the fibers and their connections to basal bodies was facilitated by using cells whose cortical pigment granules had been removed with caffeine bleaching, leaving a smooth, inner cortical surface (7, 23).

Transmission studies have shown that the km fibers are composed of stacks of microtubular ribbons (5, 13). Each ribbon arises from a pair of basal bodies and projects diagonally downward and to the right in the plane of the surface (as

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Fig. 5. A large portion of the frontal field viewed from the inside. The broken-off gullet region would lie to the left. The dark area in the center was damaged during the dissection procedure. Enclosed area is shown in Fig. 6. The arrow to the lower right indicates the membranellar roots seen in Fig. 13. fs, fiber sheet (see text). × 1,200.

Fig. 6. A region of the frontal field cortex. The gullet end is toward the left. The cortex is covered with pigment granules (p). Pmy, primary myonemes; CB, cross branches. × 5,250.

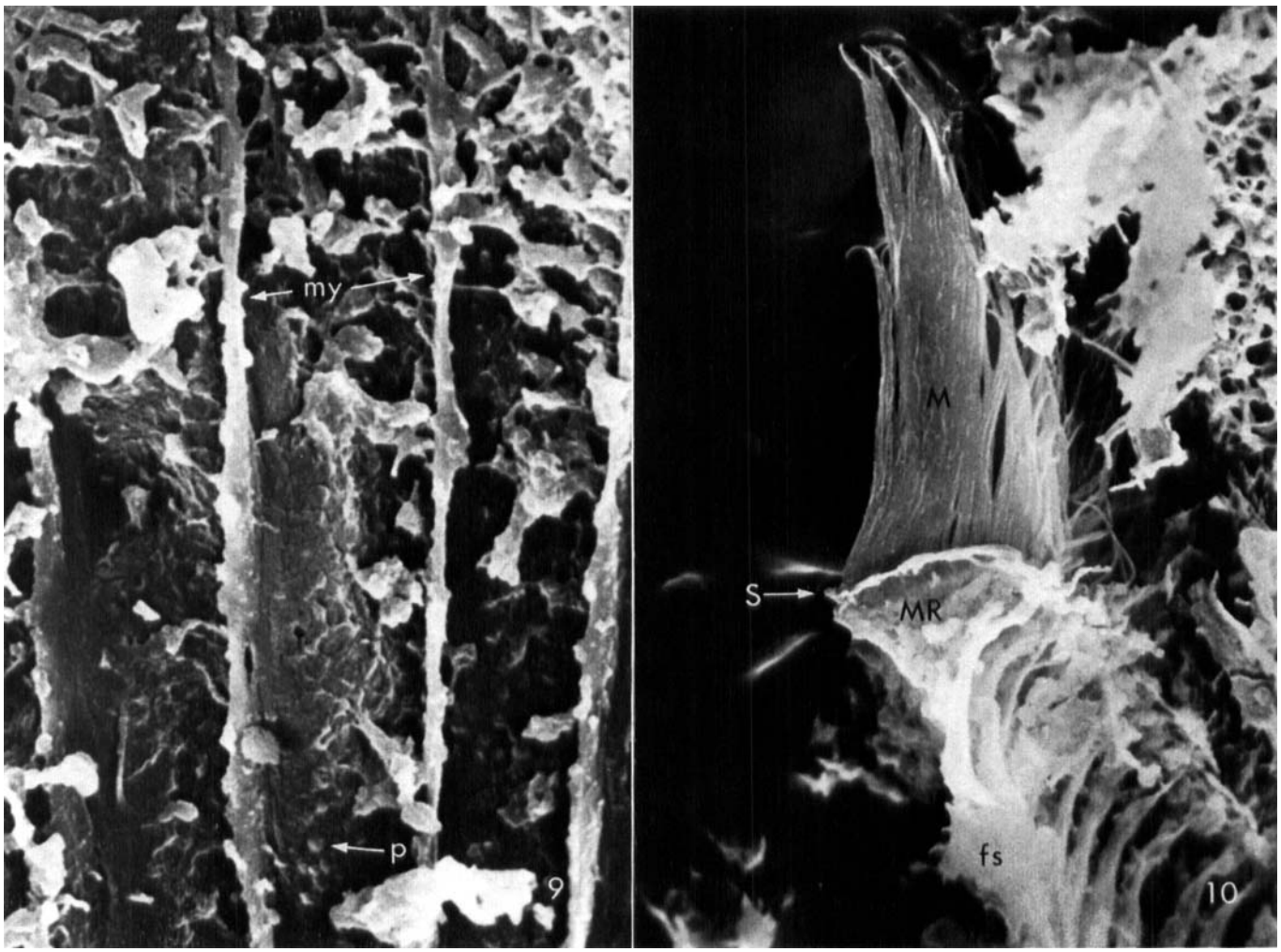


Fig. **9**. The posterior region of the cortex of a *Stentor* bleached for 4 days in 1mM caffeine. Very few pigment granules (p) are associated with the surface. my, myoneme. × 3,800.

Fig. **10**. A membranelle and its root at the end of a broken-off portion of the membranellar band. The view is from the interior of the cell looking anteriorly and toward the gullet end of the membranellar band. The membranelle (M) is separated from its root (MR) by a thin extension of the frontal field surface (S). The roots are connected together by a basal fiber sheet (fs). The structure at the upper right is the underside of a dislocated portion of the frontal field. × 2,900.

viewed from the inside of an upright cell). It combines with microtubular ribbons arising anterior to itself, forming a longitudinally oriented fiber.

Two km fibers lying beneath broken-off myonemes are shown in Fig. 7. A higher magnification view of one of these km fibers (Fig. 8) reveals 2 circular projections connected to the fiber by a short diagonal structure. These paired projections are basal bodies, as shown by their center-to-center spacing and orientation with respect to the km fiber. This agrees with measurements made from TEM micrographs (5, 10, 13). The center-to-center spacing between basal bodies in Fig. 8 measures ~ 0.24 μm as compared with 0.25 μm measured from Grain (plate IIe of Ref. 10). A secondary connection identified as the anterior fiber sheet by Bannister & Tatchell (5), is also seen. Frequently the km fibers were found to be flush against the surrounding cortex and difficult to distinguish.

Pigment Granules

Pigment granules, 0.5-0.7 μm in diameter, pebble the inside surface of all regions of the cortex (Figs. 3, 6, 13). The size and distribution of the granules seen in this study are in agreement with previous observations (7, 17, 22). The granules appear to be held to the surface by an overlying membrane.

Most cortically bound pigment granules disappear when *Stentor* is slow-bleached with caffeine. A view of the surface of a cell treated with 1 mM caffeine for 4 days (Fig. 9) reveals only a few granules associated with the surface. This confirms the results of Blumberg et al. (7) who have shown that the pigment granules of caffeine-bleached *Stentor* migrate from the surface toward the inside of the cell.

The Membranellar Root System

The frontal field of *Stentor* is surrounded by a band of membranelles, or compound cilia (22). Transmission studies have been shown that each membranelle is composed of ~60 cilia arranged in 3 rows of 20 (2 rows of 20 in the gullet region) (18). Ten to 12 nemadesmal microtubules (the root fibrils of Randall and Jackson) descend into the cell interior from each basal body of 2 of the rows of cilia of each membranelle

(17, 18). Each root is composed of a matrix of these nemadesmal microtubules.

Scanning micrographs show that the membranellar root system is a complex structure composed of fibers and fibril sheets. Figure 10 shows a low-magnification view of a complete membranelle and its root. The root is seen to narrow as it projects into the cell. The posterior end of the root, however, does not assume a round, fiber-like form as reported by Randall and Jackson (18). Figure 11 reveals that the root undergoes a 90° counterclockwise twist along its vertical axis (when viewed from the inside) as it descends into the cell. This quarter-turn brings each root's outside edge in direct contact with the inside edge of the neighboring root. In this manner, a continuous fiber sheet connecting all roots is formed.

Two observations suggest that the nemadesmal microtubules comprising the membranellar roots remain vertically oriented in the basal fiber sheet. (a) High magnification views of the fiber sheet (Fig. 12) reveal a surface covered with vertically directed striations. These striations probably represent the outline of an orderly array of microtubules. (b) The fiber sheet was easily damaged during the dissection procedure, and tears in the sheet were frequently observed (Fig. 12). These breaks always occurred vertically, along the lines of surface striations.

Adjacent membranellar roots are also connected together by a series of branching fibers ~0.25 μm in diameter, lying directly beneath the frontal field surface. These fibers form a complex series of connections with the anterior end of each root (Fig. 13). They were observed by Randall & Jackson (18) and might represent the connectives between the membranellar roots and the myoneme system seen by Bannister & Tatchell (3).

DISCUSSION

The Myoneme System

Several lines of evidence indicate that the myoneme system causes contraction in *Stentor*. Bannister & Tatchell (5) demonstrated that the myonemes are intrinsically contractile by examining the convoluted myonemes which form following cell contraction. These myonemes straighten (i.e., contract) following stimulation of the cell. A high speed cinematic study of convoluted myonemes (15) showed that following stimulation, the myoneme contraction precedes contraction of the entire cell.

Ultrastructural studies of myonemes also indicate that they constitute the contractile organelle. Huang & Pitelka (13) demonstrated a transformation of the myoneme microfilaments, from 4 nm in diameter in extended cells to 12 nm in contracted ones. Bannister & Tatchell (6) reported an aggregation of the microfilaments in contracted myonemes. Both these studies indicate that there is a molecular rearrangement of the microfilaments during contraction, although they do not in themselves resolve whether contraction is based on a contracting or a sliding filament model (1, 24).

Stentor is not uniformly contractile over its entire surface (22). The stalk region, which can extend to more than a mm in length, will contract into the posterior tip of a cell only 200 μm in diameter. The anterior end of *Stentor*, in contrast, undergoes very little extension or contraction. The frontal field is moderately contractile, but being circular in shape, contracts uniformly in all directions instead of along one axis.

The scanning micrographs in this study have shown that the structure of the myoneme system varies significantly in different regions of *Stentor*. If the myonemes represent *Stentor*'s contractile system, then their form and connectivity should correlate with, and help to account for, the cell's differential contractility.

The observed branching patterns of the myonemes are consistent with their proposed contractile function. The posterior myonemes are totally lacking in cross branches. This configuration is well suited to participation in the large amounts of extension and contraction which occur only in a longitudinal direction in the stalk region. The anterior cortex, on the other hand, undergoes little change in size. The extensively cross-linked myonemes in this region surround the nearly spherically-shaped anterior end of the cell with a web of contractile material. This contractile web should exert a uniform tension over the entire anterior surface of the cell and might serve to counter the sudden increase in internal pressure caused by stalk contraction.

Similarly, the frontal field myonemes appear well suited for mediating the uniform contraction observed in the circular frontal field. Here, cross branches are highly organized, intersecting the primary myonemes at right angles. Approximately the same amount of myoneme material is oriented in 2 orthogonal directions, one circumferential and the other radial. This results in a system that should contract uniformly in all directions.

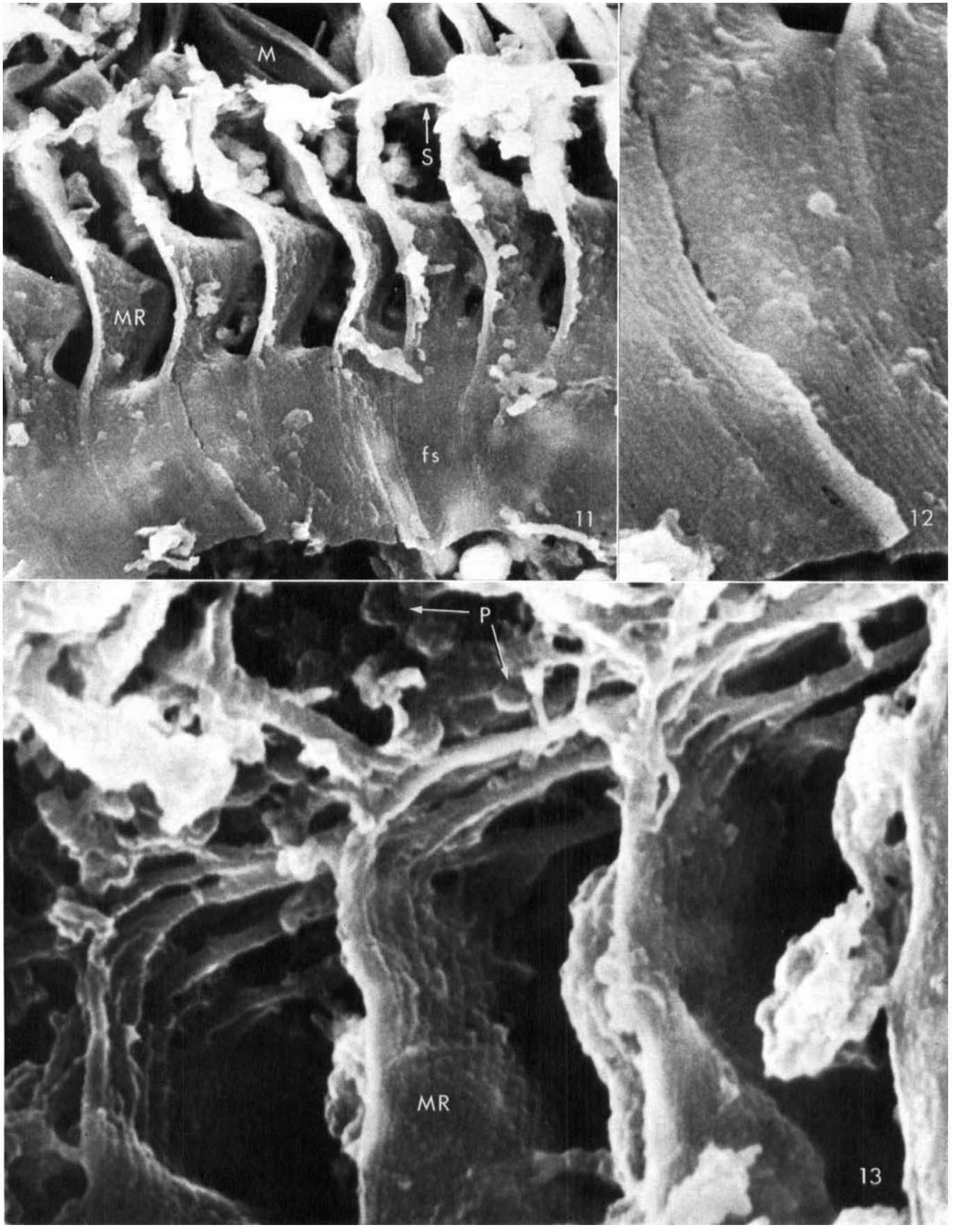
The observed cross-sectional shapes of the myonemes suggest that they were in a state of tension while being fixed. The ribbon-on-edge-shaped form of the posterior myonemes probably arises because (a) the microfilamentous material of the myonemes is held together by a bounding membrane (2, 5, 18); (b) the myonemes are connected to the overlying cortex over a narrow width (0.5 μm) (5, 13); (c) the myonemes lie along a concave surface (see Fig. 3). Given these conditions, a myoneme will assume a ribbon-shaped form if its microfilaments are in a state of tension. Furthermore, posterior myonemes were never observed to pull away from the cortex, suggesting that connections between the myonemes and the cortex occur at frequent intervals. This is in agreement with TEM studies (5, 12) which note possible myoneme connections to basal body pairs which are spaced at approximately 0.5 μm intervals in contracted cells.

The primary anterior myonemes, although they too lie along a concave surface, are flattened against the cell cortex rather than projected into the cell interior. The flattened form of these myonemes is undoubtedly due to the lateral tension exerted by their cross branches. In the frontal field, the similarity in appearance of the primary myonemes and their cross branches suggests that the 2 are in approximately the same state of tension.

The Membranellar Root System

The ultrastructure of the membranellar root system was first described in detail by Randall & Jackson (18). They reported that as the roots descended into the cell interior, the root fibrils (nemadesmal microtubules) collected into rounded bundles which branched and turned horizontally, forming a basal fiber joining the roots together. This scheme was cited by Tartar in his review (22). Bannister & Tatchell, contradicting the generally accepted opinion, reported that a flat fiber sheet of nemadesmal microtubules, rather than a rounded basal fiber, joined the roots (4). Their report extended the earlier light microscopic observations of Dierks (8) who described the membranellar roots as rectangular sheets which twisted as they descended into the cell.

The scanning electron micrographs in this study clearly show that the membranellar roots are connected together by a flat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fiber sheet (Fig. 11) as reported by Bannister & Tatchell. This sheet is formed as each of the flat membranellar roots makes a quarter-turn twist along its vertical axis as it descends. The outer edge of each root fuses with the inner edge of the adjacent root, forming a continuous sheet which encircles the anterior end of the cell. Micrographs showing vertically oriented striations and tears in the fiber sheet indicate that the nemadesmal microtubules remain vertically oriented as they descend through the twisting roots and into the fiber sheet.

The original transmission micrographs of Randall & Jackson (18), as well as those of Grain (10) and Paulin & Bussey (17) are consistent with the existence of a basal fiber sheet. These photographs show curving nemadesmal microtubules and fiber-like connections between roots and were originally interpreted as demonstrating the existence of a basal fiber. However, these same photographs would have resulted from obliquely cut sections through the fiber sheet and twisting roots. A sheet of microtubules would appear as a narrow fiber bundle in a non-vertically cut section of the sheet.

The function of the connectives between membranellar roots remains unknown. One or more of these structures could play a role in the conduction of the metachronal wave of ciliary activity which travels down the membranellar band (19, 20). It is intriguing to note that *via* the nemadesmal microtubules, there is a 1-1 mapping of each cilium in every membranelle (excepting those cilia lacking nemadesmata) onto the basal fiber sheet. Each cilium in every membranelle would be stimulated sequentially (forming a metachronal wave) if a propagated impulse were to travel as a front across the length of the fiber sheet and secondarily up each excited nemadesmal microtubule to its corresponding basal body. Because membranellar roots twist counterclockwise as they descend, the projection of the outside edge of each membranelle lies closer to the gullet end of the fiber sheet than does the projection of the inside edge. The metachronal wave travels away from the gullet end of the membranellar band (19, 22). Thus, this model of metachronal conduction predicts that during membranellar beating, the outside edge of each membranelle is stimulated and begins its active stroke prior to the stimulation of its inside edge. Future experiments must determine the validity of this model.

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Fig. 11. The membranellar root system as viewed from the cell interior. The gullet end of the membranellar band lies to the right. Membranellar roots (MR) twist along their vertical axes, forming a continuous basal fiber sheet (fs). The outline of the frontal field surface (S) and the lower part of the membranelles (M) are visible. × 4,300.

Fig. 12. A higher magnification view of the fiber sheet seen in Fig. 11. Vertically oriented striations and a tear in the fiber sheet are visible. × 12,500.

Fig. 13. The anterior portion of 3 membranellar roots, as viewed from the cell interior looking anteriorly and to the left. The gullet end of the membranellar band lies to the right. A branching fiber system, lying along the underside of the frontal field surface connects the roots (MR) together. p, pigment granules. × 18,000.