IDENTIFICATION OF ACTIN *IN SITU* AT THE ECTOPLASM-ENDOPLASM INTERFACE OF *NITELLA*

Microfilament-Chloroplast Association

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ABSTRACT

Using a glycerination procedure designed to avoid excessive plasmolysis or disruption of the ectoplasm, microfilaments in bundles at the ectoplasm-endoplasm interface of *Nitella* internode cell segments were found to bind rabbit heavy meromyosin (HMM) *in situ*. All HMM arrowheads in a bundle seem to have the same polarity and many lie in register as judged from the electron micrographs; the arrowhead periodicity is approximately 380 Å. The decorated microfilaments are thus similar to those seen in negatively stained cytoplasmic suspensions of internode cells. In glycerinated material, as well as in suspensions, the microfilaments are closely associated with chloroplasts. The microfilaments lie adjacent to or are attached to the chloroplast envelope. The results provide further evidence that the microfilaments thought to play a role in cytoplasmic streaming in vivo in *Nitella* consist of actin and suggest that they may be anchored to the chloroplasts.

Actin-myosin interactions are now thought to be responsible for a variety of motility phenomena in nonmuscle cells (25). In the green plants, although actin and myosin have not been characterized biochemically, bundles of 50–70-Å microfilaments, similar to actin-containing microfilaments of other cells, seem to be associated with cytoplasmic streaming (8, 11, 12, 13, 14, 19, 20, 22). Classic studies with the multicellular green algae *Nitella* and *Chara* have shown that these microfilaments are present at the site of motive force generation for streaming, the interface between the stationary ectoplasm and mobile endoplasm (7, 9, 11, 13, 14, 17, 19, 23). A recent report, however, suggests that filaments responsible for streaming are located in the endoplasm of these cells (1).

In a recent study it has been shown that bundles of 50-Å microfilaments are present in cytoplasmic suspensions of *Nitella*, that the microfilaments are similar in appearance to muscle actin, and that they reversibly bind rabbit muscle heavy meromyosin (HMM) in arrowhead arrays nearly identical to those produced on F-actin (21). Williamson has subsequently shown HMM binding to microfilaments obtained from *Chara* (32). Although these results strongly indicate that actin is present in suspensions, they do not absolutely demonstrate that it corresponds to the microfilaments present at the ectoplasm-endoplasm interface in vivo. Of interest is the fact that the microfilaments in suspensions often are associated with chloroplasts. It is well known that files of chloroplasts are normally located in the ectoplasm in *Nitella* internode cells (e.g., 6, 11, 12), and thus the

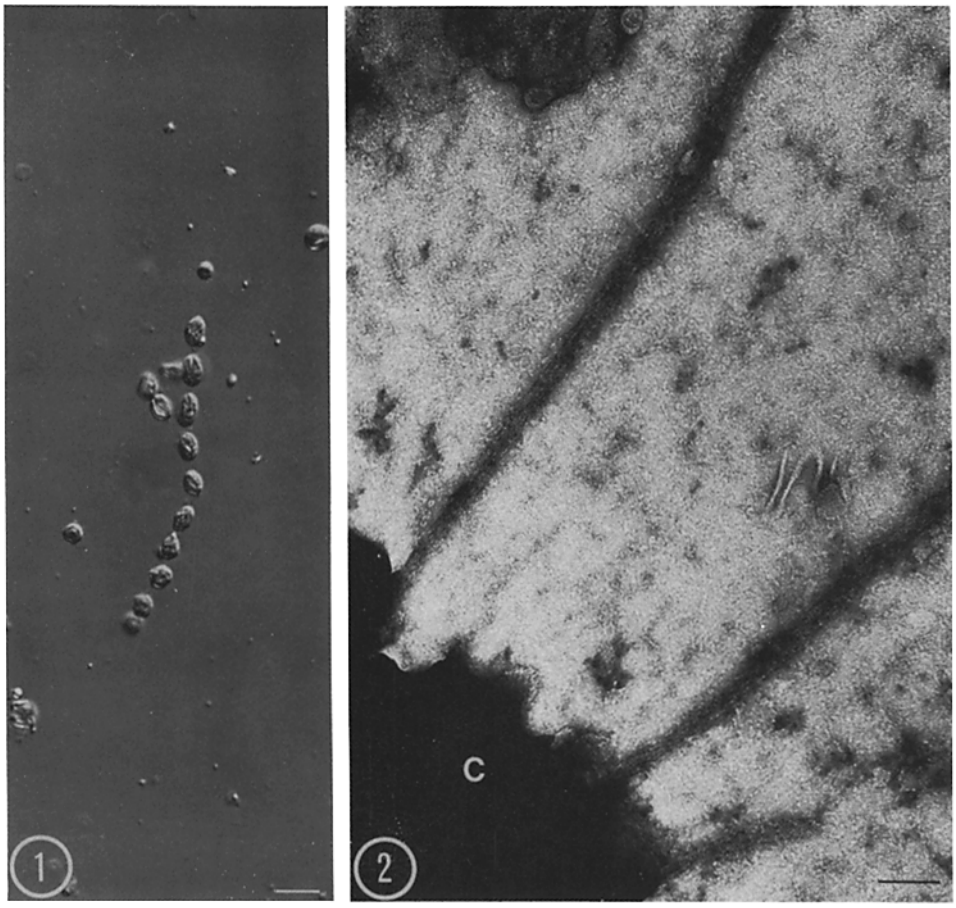


FIGURE 1 A Nomarski micrograph of a chain of chloroplasts in a cytoplasmic suspension obtained from *Nitella* internode cells. The fibers connecting the chloroplasts are not visible in this micrograph, but can be seen with careful focusing of the microscope. × 1,100. Markers on all light micrographs equal 5 μm, on all electron micrographs, 0.1 μm.

FIGURE 2 A portion of a single negatively stained chloroplast (*C*), with several bundles of undecorated microfilaments radiating from its surface, on a coated grid containing cytoplasmic suspension treated with HMM followed by 1 mM ATP. × 80,000.

association of these organelles with microfilaments in suspensions would indicate the location of the latter at the site in vivo.

Considerable attention has been devoted recently to possible microfilament-membrane attachment sites in nonmuscle cells (3, 8, 18, 24, 28, 30, 31). Such sites are of importance in studying the polarity of microfilaments and how they are anchored in the cell. Therefore, the association of microfilaments and chloroplasts in *Nitella* is of great interest. In the present study, we present further evidence for this association and demonstrate the HMM decoration of interface microfilaments *in situ* in internodes, a glycerination procedure designed to avoid excessive plasmolysis and disruption of the internode cytoplasm.

MATERIALS AND METHODS

Cultures of *Nitella flexilis*, started from samples provided by Professor Paul B. Green, were maintained in soil medium under fluorescent and incandescent light. Cytoplasmic suspensions of *Nitella* were prepared by gently pressing excised internodes in a salt solution containing 0.1 M KCl, 5 mM $MgCl_2$, and 5 mM potassium phosphate buffer, pH 7.0, until the solution turned slightly green. Samples of the suspension were

either viewed with Reichert Nomarski optics or negatively stained with uranyl acetate on Formvar-carbon-coated grids.

In experiments on HMM decoration *in situ*, internode cells were cut at both ends in either distilled water or salt solution. The open excised cells were then passed stepwise through a glycerol plus salt solution series at 0°-4°C (5% glycerol, 0.5 h; 10% glycerol, 1 h; 25% glycerol, 2 h; 50% glycerol, overnight.) The cells were deglycerinated in 25% (2 h) and 5% (5 h) glycerol plus salts, before being incubated for 24 h at 0°-4°C in HMM solution (rabbit back striated muscle HMM; 5.6 mg protein/ml in 5% glycerol plus salts). The internodes were either rinsed in salt solution or treated with 10 mM ATP plus salts (pH 7.0) before being fixed in 3% glutaraldehyde plus salts for 1–2 h. After several rinses in salt solution for 1 h, the cell segments were postfixed in 2% osmium tetroxide in phosphate buffer, pH 7.0, for 2 h. They were then dehydrated in an acetone series and propylene oxide before embedment in Spurr's medium (27) between slides and cover slips coated with MS-122 fluorocarbon dry release agent (Miller-Stephenson Chemical Co., Inc., Los Angeles, Calif.). After curing, the thin plastic wafers were examined with Reichert Nomarski optics. When suitable regions were located, they were cut out, mounted, and thin-sectioned on a Reichert OM-U2 ultramicrotome. Grids containing sections were poststained with aqueous uranyl acetate and lead citrate before being viewed on a Hitachi HU-11E electron microscope at 50 kV.

Rationale for Glycerination Procedure

Glycerination of plant material must be approached with caution, since plant cells are under high turgor pressure and therefore cannot be treated with 50% glycerol without causing plasmolysis and disruption of cytoplasmic components. Moreover, the wall around plant cells might hinder penetration of the HMM. Our rationale for the glycerination procedure was based on the idea that although cutting the internodes might cause surging and substantial exfoliation of the ectoplasm, it would relieve the turgor pressure before the introduction of glycerol and prevent plasmolysis. The gradual glycerination in a graded series would also reduce plasmolysis. A significant number of unexfoliated, unplasmolyzed regions might therefore be preserved and be easily located with Nomarski optics. Cutting the ends of the internodes would also facilitate penetration of the HMM.

RESULTS

Cytoplasmic suspensions of *Nitella* prepared by pressing excised internode cells in salt solution are seen to contain free chloroplasts and chloroplasts aggregates, similar to those previously reported in

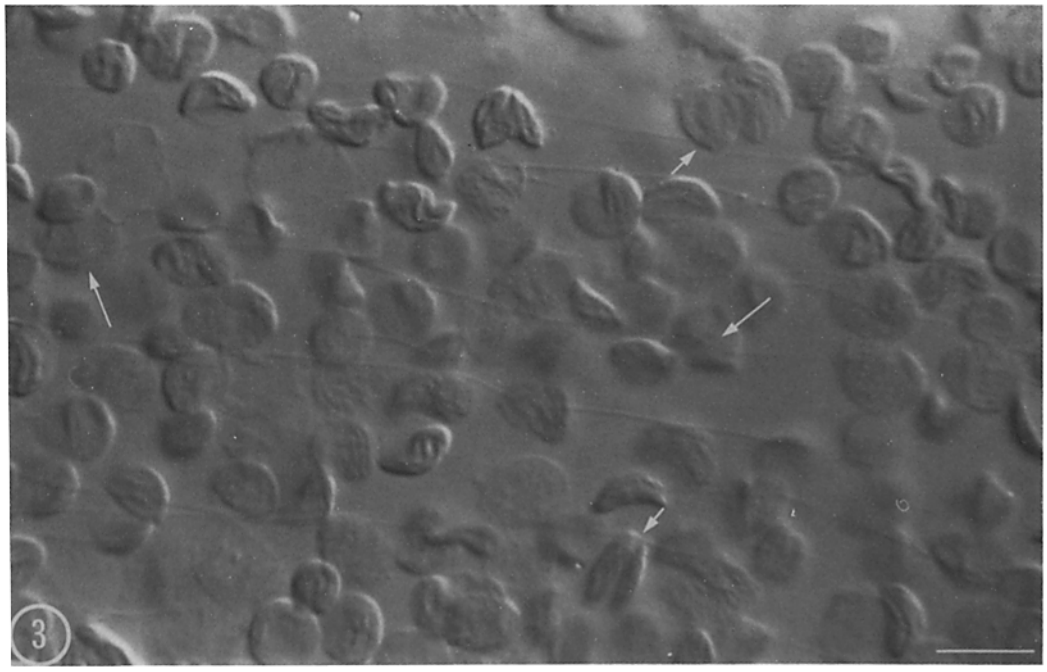


FIGURE 3 A Nomarski micrograph of a glycerinated, HMM-treated *Nitella* internode cell segment. Fibers at the ectoplasm-endoplasm interface appear to arc around (long arrows) and make contact with (short arrows) the chloroplasts. × 2,600.

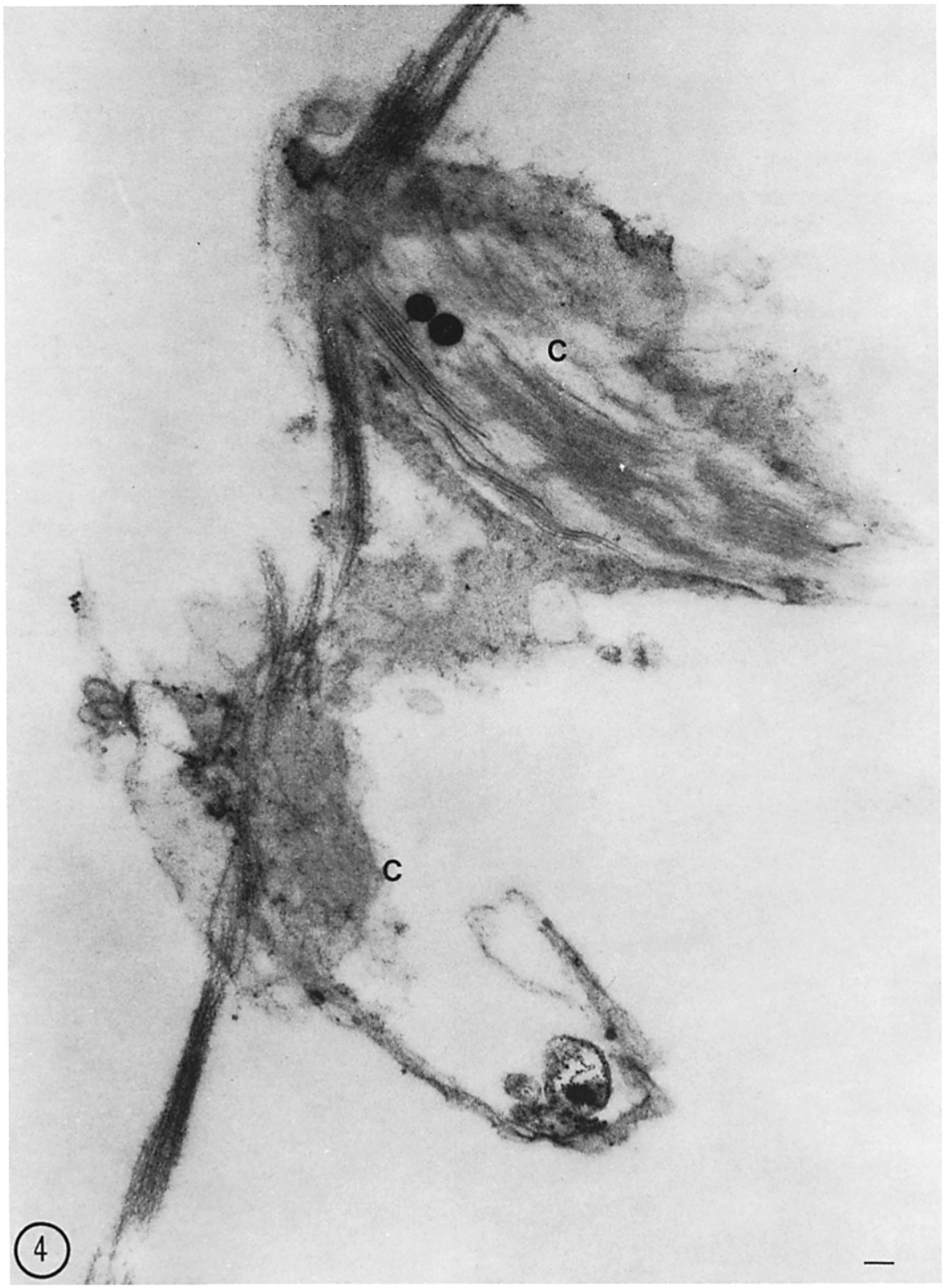
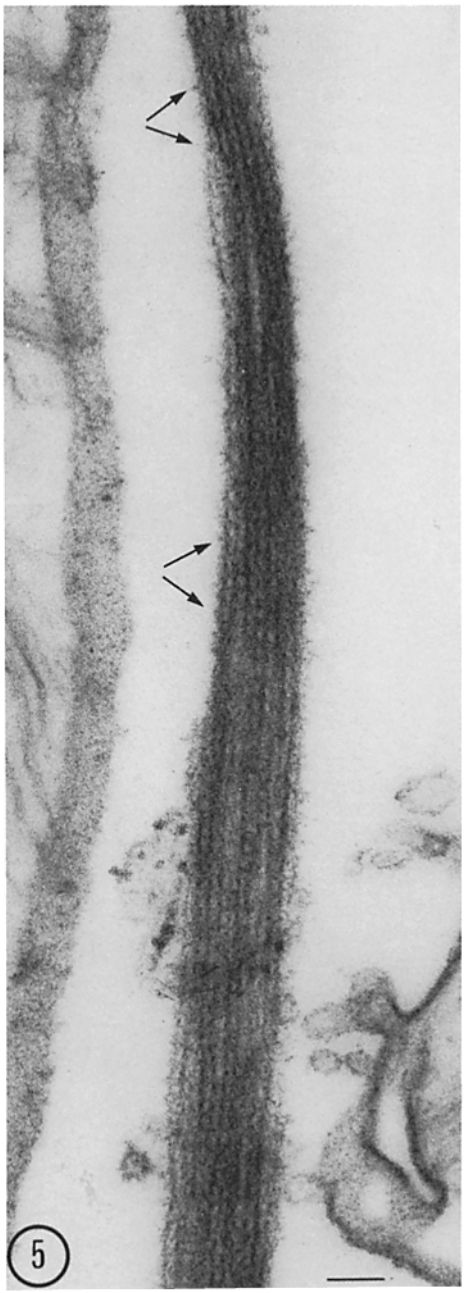
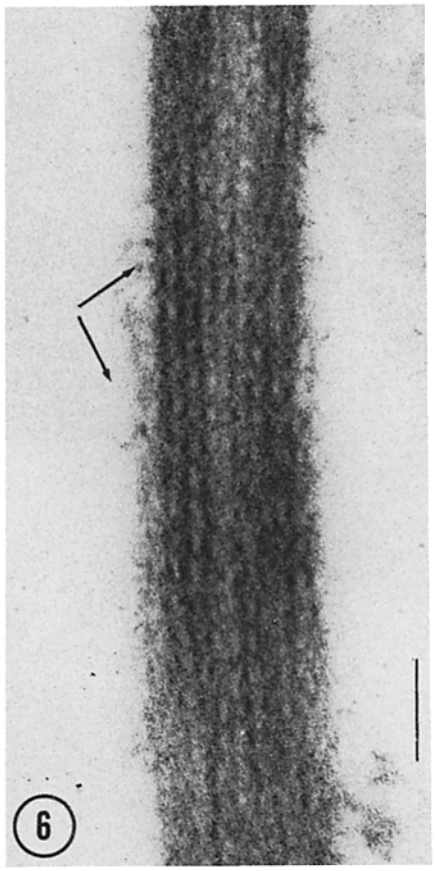


FIGURE 4 A fiber linking adjacent chloroplasts (*C*) in a thin-sectioned region of the ectoplasm similar to that shown in Fig. 3. The fiber consists of microfilaments which closely invest part of the surface of the chloroplasts. × 45,000.

isolated cytoplasmic droplets (9, 13, 14, 15), when viewed with Nomarski optics. Many of the aggregates occur as files or chains (Fig. 1) and careful focusing reveals that the chloroplasts are linked by fine fibers. After negative staining, such chains become more rare, but individual large dense structures, probably chloroplasts, are present and have bundles of actin-like microfilaments radiating from their surfaces (Fig. 2).

Observations of glycerinated, HMM-treated material embedded in thin plastic wafers with Nomarski optics reveal that although the internode segments show signs of disruption, many contain unplasmolyzed, unexfoliated regions of the





FIGURES 5,6 The microfilaments in the bundles (fibers) are decorated with HMM arrowheads, all of which point in the same direction (toward the top of the page). In certain regions, the arrowheads lie in register (arrows). Fig. 5, × 74,000; Fig. 6, × 135,000.

ectoplasmic layer. Such regions contain chloroplasts spanned by numerous fibers (Fig. 3) similar in appearance to those seen by Kamitsubo (11, 12) in living characean cells. The fibers arc around the chloroplasts and seem to make contact with them (Fig. 3, arrows). When thin sections are viewed with the electron microscope, all the fibers appear as bundles of microfilaments (Fig. 4–6). The bundles exhibit a "herringbone" pattern due to the presence of HMM arrowheads arrayed on the constituent microfilaments (Figs. 5, 8). In certain parts of the bundles, arrowheads are particularly evident (Figs. 5, 6, 8 arrows) probably due to an advantageous plane of section. Since all the discernible arrowheads in all such regions of a bundle point in the same direction, it is likely that all microfilaments in each bundle have the same polarity. The same finding was obtained with negatively stained microfilament bundles (21). In addition, the arrowheads seem to lie in register, indicating a paracrystalline packing (Figs. 5, 6, 8). The spacing or repeat of the arrowheads (380 Å) is in close agreement with the value found after negative staining (360–375 Å). The bundles are clearly associated with chloroplasts. Often bundles or their branches arc around part of the chloroplast surface (Fig. 4). In many instances the bundles bend or arc to make contact with successive chloroplasts in a file (Fig. 4, 7). Although such bending may be an artifact of the preparation procedures (compare the degree of preservation of orderly arrangement of bundles and chlorplasts in our figures with that of Kamitsubo [12]), it nevertheless shows that successive chloroplasts are mutually linked. All microfilaments and bundles in the vicinity of chloroplasts are similar in appearance, and no specialized "jacket" fibers linking chloroplasts were found (11). The microfilaments impinge upon, make contact with, or lie closely adjacent to the outer membrane of the chloroplast envelope (Figs. 4, 7–10). Usually the envelope is poorly preserved, except where the microfilament bundle makes contact (Figs. 9, 10). At such points, both membranes of the envelope remain intact. Finally, in certain micrographs, branches of bundles seem to attach to the outer membrane of the envelope, although such images may merely show the branch going out of the plane of section. (Fig. 10). Although some reports have indicated that an association exists between microfilaments and cisternae of endoplasmic reticulum in *Nitella* (2, 19), we found little evidence of this in our studies.

Attempts were made to reverse HMM binding

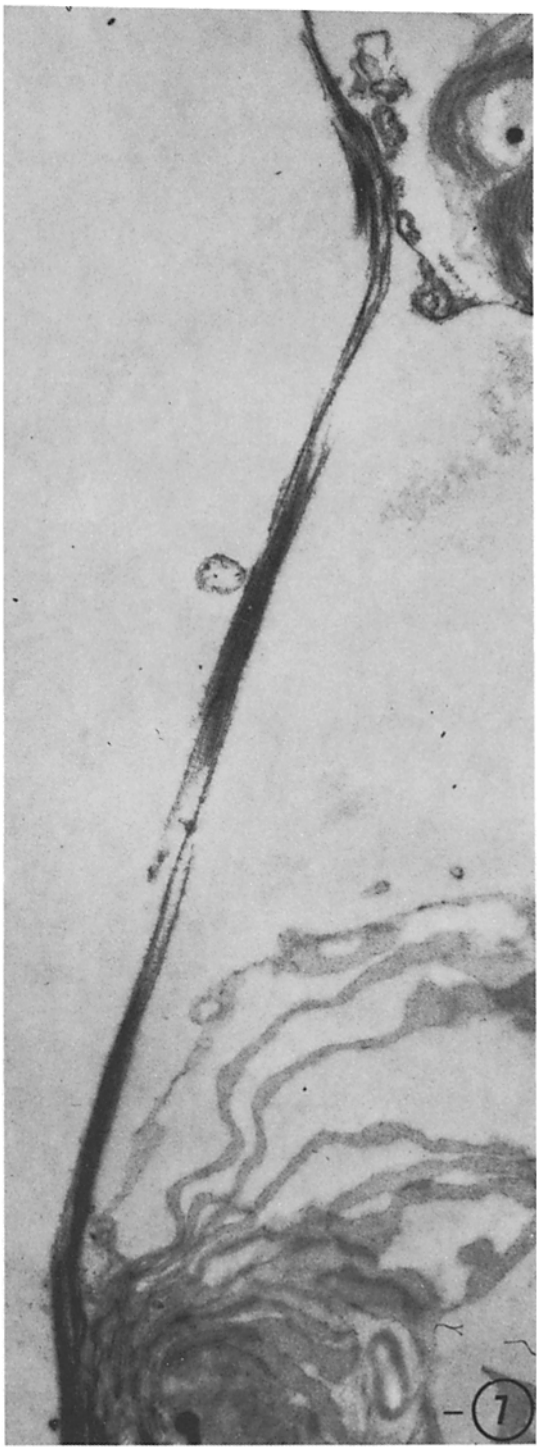


FIGURE 7 Two successive chloroplasts linked by a microfilament bundle. The bundle appears bound to the surface of the chloroplasts. × 21,000.

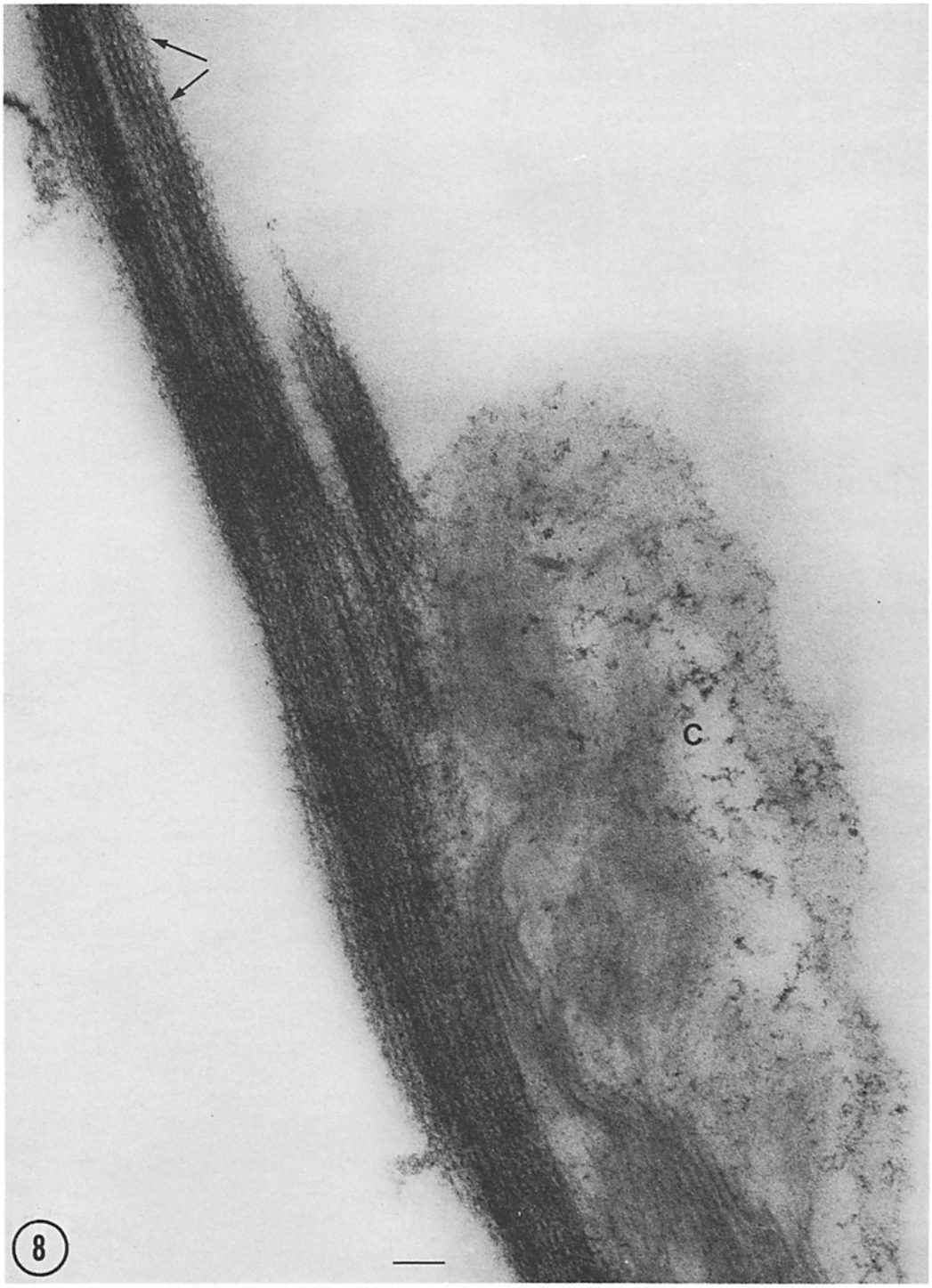
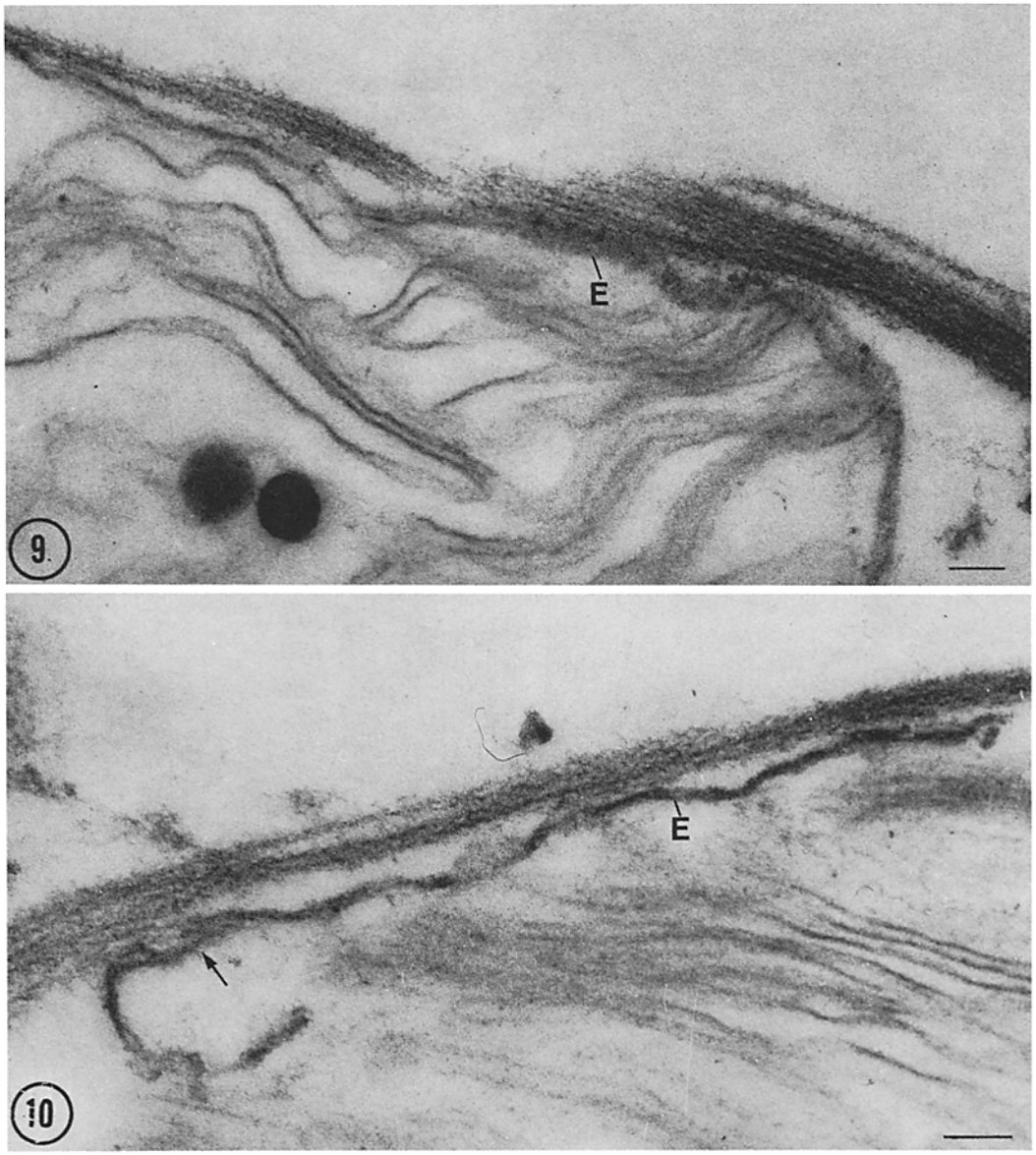


FIGURE 8 Tangential section of a chloroplast (*C*). One side of its surface is closely invested by decorated microfilaments. The registry of the arrowheads is evident in certain regions (arrows). × 74,000.



FIGURES 9,10 The membranes of the chloroplast envelope (*E*) are preferentially preserved adjacent to areas of contact with the microfilament bundles. In Fig. 9 the microfilaments lie closely adjacent to the envelope, and in Fig. 10 some of them seem to attach to the envelope (arrow). Fig. 9 × 70,000. Fig. 10 × 90,000.

in situ by treating internode segments with ATP in salt solution before fixation. However, microfilament bundles are not preserved during fixation after such treatment. Thus the presence of bound HMM seems markedly to enhance the preservation of microfilaments in *Nitella*, as it does in other nonmuscle cells (4, 5).

DISCUSSION

The diverse data reported here establish that HMM-binding microfilaments organized as bundles are located at the ectoplasm-endoplasm interface in *Nitella* internodes and are associated with chloroplasts. The bundles are probably identical to the fibers or microfilament bundles previously

shown to be at the site (11–14, 19, 23) and to those already identified in cytoplasmic suspensions (21).

The characteristics of the *in situ* decorated microfilaments closely match those of filaments seen after negative staining. For example, the arrowhead repeats are similar (360–380 Å). In addition, all arrowheads on all microfilaments in a given bundle and its branches appear to point in the same direction. Moreover, in many instances the arrowheads on adjacent microfilaments lie in register, indicating a paracrystalline packing. This is also evident in the negatively stained preparations.

Previous investigations (6, 7, 11, 12, 19, 23), including the elegant studies of Kamiya and Jarosch (references 9, 15; reviewed in 10, 13, 14, 17) have indicated that microfilament bundles at the ectoplasm-endoplasm interface of characean internode cells are associated with the chloroplasts. For example, it has been found that the plastids in a file oscillate or vibrate together as if they are under tension or mutually linked (6, 11). We observed that chains of chloroplasts are present in crude cytoplasmic suspensions of *Nitella* internodes. The chloroplasts, which were undoubtedly exfoliated during the preparation procedure, seem to be linked by fibers. After negative staining, individual chloroplasts are found and appear to be associated with microfilaments. Our results with glycerinated internode segments establish that a relationship between the microfilaments and the chloroplast envelope exists.

Although the exact nature of the connections between the microfilaments and the envelope membranes is still unknown, our observations indicate that attachments to the chloroplasts envelope may indeed occur. Attempts have now been made to visualize microfilament-membrane attachment sites in a variety of nonmuscle cells (3, 8, 18, 24, 28, 30, 31). Such sites are usually thought to be on the plasmalemma. The results from *Nitella* indicate that the microfilaments in this species, which run parallel to the plasmalemma but at a large distance from it, are instead anchored to the chloroplasts. Whether the chloroplast envelope-microfilament association consists of cross bridges, actual insertion points, or some sort of adhesive or nonspecific linkage is still under investigation, and future experiments will be in part directed toward clarifying this question. Although we have not as yet identified any constant polarity (direction of HMM arrowheads) on microfilament bundles or their branches with reference to possible insertion points in the chloroplast envelope, our efforts will be directed to this problem as well. Experiments are now in progress on negatively stained preparations of internode cell segments to determine the polarity of bundles with reference to the direction of cytoplasmic streaming, and a preliminary report suggests that the arrowheads are oriented opposite to the direction of cytoplasmic flow in *Nitella* (16).

These results and those on the paracrystalline packing and uniform polarity of microfilaments in bundles reinforce our interest in how rotational streaming is produced in *Nitella*. The significance of the paracrystalline packing in the regulation of streaming is unknown; however, paracrystals can be formed in vitro from purified muscle actin or actin plus tropomyosin-troponin (e.g., reference 26), and microfilaments appear to be aggregated in orderly arrays in vivo in a few other species. Microfilaments seen in transverse section in higher plants cells are hexagonally packed (see Fig. 18, reference 8). Tilney (29) has observed close-packed actin microfilaments in register in the discharged acrosomal process of *Limulus*. Extension of the acrosomal process is accompanied and possibly produced by a change in packing of the microfilaments, and protein(s) (29), which seems to be associated with the microfilaments may be involved in the regulation of the packing state. Some similar process, as proposed by Jarosch (10), may occur in *Nitella*, although most evidence still favors a shear-generating interaction between actin and myosin, similar to that which occurs in muscle.

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